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# STABILIZATION OF GP120 OUTER DOMAIN FOR HIV-1-1 VACCINE IMMUNOGEN DESIGN

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STABILIZATION OF GP120 OUTER DOMAIN FOR HIV-1-1 VACCINE

IMMUNOGEN DESIGN

by

Duoyi Hu

A THESIS

Presented to the Faculty of

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Major: Veterinary Science

Under the Supervision of Professor Shi-hua Xiang

Lincoln, Nebraska

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# STABILIZATION OF GP120 OUTER DOMAIN FOR HIV-1 VACCINE

## IMMUNOGEN DESIGN

Duoyi Hu, M.S.

University of Nebraska, 2016

Advisor: Shi-hua Xiang

The sole envelope glycoprotein spike on the surface of Human Immunodeficiency Virus (HIV-1) is composed of two subunits: gp120 and gp41. The gp120 consists of three domains: the inner domain, outer domain and bridging sheet, to which the viral primary receptor CD4 binds and induces a conformational change in gp120 to expose the co-receptor binding site. Previous studies have found that the outer domain of gp120 is relatively more stable than the inner domain and bridging sheet. When superimposed, the co-crystal structures of CD4-gp120 complex and VRC01-gp120 complex revealed that the CD4-binding site (CD4-BS) antibody VRC01 actually mainly bound to the outer domain of the gp120. The outer domain (designed OD1) of gp120 has been considered as an immunogen candidate but to be ineffective, suggesting that structural-based modifications to the outer domain are required for designing an effective vaccine immunogen. Previous studies in our laboratory have introduced two disulfide bonds to stabilize the outer domain structure and named it OD2, which significantly improved the immunogenicity when compared to the wild-type outer domain (OD1). In this project, based on the OD2 set of experiment, an additional important mutation from serine to tryptophan at position 375 (S375W) in

the CD4-binding cavity was introduced to further stabilize the outer domain, which was named OD3. The OD3 immunogen was expressed and purified in *E. coli*. Subsequently, the purified OD3 immunogen was characterized *in vitro* and its immunogenicity was tested in guinea pigs. In comparison to OD1 and OD2, the results demonstrate the antibody titers and neutralizing activity were clearly higher in OD3. It is suggested that the S/375W mutation stabilized the outer domain and further improved the outer domain (OD) immunogenicity. In conclusion, this research has used a structure-based approach to design HIV-1-1 vaccine immunogens based on the gp120 outer domain (OD).

*Keywords:* Human Immunodeficiency Virus (HIV-1); Acquired Immunodeficiency Syndrome (AIDS); Structural-based Vaccine Design; HIV-1 envelope; Outer Domain of gp120; Stabilization; S375W mutation.

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**Abbreviations:**

HIV-1: Human Immunodeficiency Virus

AIDS: Acquired Immunodeficiency Syndrome

OD: Outer Domain of gp120

bNAbs: Broadly Neutralizing Antibodies

Nabs: Neutralizing Antibodies

Mabs: Monoclonal Antibodies

CTL: Cytotoxic T Lymphocyte

MHC: Major Histocompatibility Complex

DCs: Dendritic Cells

IFN- $\alpha/\beta/\gamma$ : Interferon-alpha/beta/gamma

ART: Anti-Retroviral Therapy

ELISA: Enzyme-Linked Immunosorbent Assay

CD: Circular Dichroism

## Chapter 1: Literature review

Acquired immune deficiency syndrome, known as AIDS, is an immunodeficiency disease caused by human immunodeficiency virus type 1, known as HIV-1-1. Since the virus was discovered in 1981(1-4), more than 65 million people have been infected with this virus, and more than 39 million people have died of AIDS-related diseases (5). An astonishing fact is that the number of deaths caused by AIDS exceeds the total number of death resulted from all the wars in the 20th century. Although scientists try to elongate HIV-1 patients' life to a higher extent, we currently still have no cure, and have no effective vaccine to prevent the virus infection. While researchers all over the world have united together to tackle this problem, there are still a lot of obstacles on the way to the success. The year 2014 was sad for us: MH17, an aircraft taking more than 100 HIV-1 field experts crashed by a missile attack, and no one survived this disaster (6). While people in the whole world were criticizing the missile attack, scientists in HIV-1 related field were grieving over the deaths of their colleagues. Yet we are, and will still be working on this issue: public awareness has been raised dramatically in developing countries; vaccine development is becoming promising; antiviral therapeutic treatments are also becoming more effective. In the foreseeable future, human beings will finally overcome this disease.

## **Acquired Immune Deficiency Syndrome (AIDS)**

AIDS is caused by HIV-1-1 infection. HIV-1-1 specifically attacks human immune cells such as CD4+ T cells, dendritic cells and macrophages (7). The infected lymphocytes are recognized as toxic pathogens by human cytotoxic CD8+ lymphocytes, and are killed by them. When CD4+ T cells and other vital human lymphocytes decline to a certain critical level, cell-mediated immunity is gradually destroyed, and people infected with HIV-1 eventually die from the breakdown of their immune system. Without any treatment, this whole process may take 9-12 years (8).

## **Human Immunodeficiency Virus (HIV-1)**

### **Facts and Statistics**

AIDS-related diseases have led to a total number of 39 million death since 1981, the first reported case. In 2015, there were 37 million people living with HIV-1-1, and Sub-Saharan Africa accounts for 70% of HIV-1-1-infected population. Although we have witnessed an increase of incidence in two years, the death rate and HIV-1-1-infected growth rate has dropped down significantly. New HIV-1-1 infections have fallen by 35% since 2000, and new HIV-1-1 infections among children have declined by 58% since 2000. By the end of 2015, AIDS-related deaths have fallen by 42% since the peak in 2004. By the year 2015, 5.8 million people were accessing antiretroviral therapy and 16 million of people had received antiretroviral therapy (ART) (5). With ART, people

living with HIV-1-1 are able to have their lives extended to 30 or more years, making their future promising (9).

## **HIV-1 Evolution and Genetic diversity**

HIV-1 belongs to the genus lentivirus, which is a part of the family Retroviridae (10)(11). HIV-1 is an enveloped, positive, single-stranded RNA virus. It was first reported as syndrome caused by a new virus in 1981. It was then named HIV-1 in 1982, and was finally isolated and characterized in 1983 (4). There are two types of HIV-1, classified as HIV-1-1 and HIV-1-2. HIV-1-2 originates from a simian immunodeficiency virus (SIV) that infects the Sooty Mangabey. Compared to HIV-1-1, HIV-1-2 is less infectious, less virulent, and thus is confined primarily to West Africa (12). HIV-1-1 originates from an SIV which infects common chimpanzees and gorillas. HIV-1-1 is divided into 4 groups, one major group named M group, and three minor groups named group N, O and P. Each group is believed to represent an independent transmission of SIV into humans (13). The M group accounts for 90% of HIV-1-1 infections all over the world and is further divided into 11 clades, or subtypes. They are named alphabetically from A to K. Among the subtypes, subtype A is most commonly seen in West Africa; subtype B is widely spread in Europe, United States, Australia, Thailand and Japan; and subtype C is the dominant from southern and eastern Africa, India, and parts of China. Other subtypes are less transmissible and confined to smaller areas (14).

## **HIV-1 Transmission**

The three major transmission pathways of HIV-1 are sexual contact, direct exposure to infected blood or body fluids, and mother to child transmission during pregnancy or breastfeeding (15). It is confirmed that other routes of transmission such as exposures to feces, sweat, tears, or saliva (16). Among these three transmission pathways, sexual contact (both heterosexual and homosexual contact) is responsible for the most frequent transmission. Generally, HIV-1 transmission via heterosexual contact dominates among all the transmission pathways worldwide, however, the incidence of homosexual transmission is arising in developed countries (17). In Africa, the majority of transmissions are through mother to child, and male to female sexual contact. However, homosexual contact results in higher percentage of HIV-1-1 infection in some European countries and the United States, which accounts for 64% of new cases of infection(18). Oral sexual contact also transmit HIV-1-1 through blood from minor wounds, although the risk of this is relatively low (19). The risk of transmission may increase when there are other sexually transmitted infections (20) or ulcers (18). When genital ulcers are exposed to infected blood or body fluids, the result is a five-fold increase of HIV-1-1 infection (18). The most effective protection in sexual pathway is the use of condoms during any sexual intercourse, which can reduce the risk of transmission by 73%. (21).

Blood and body fluid transmissions account for the second most frequent mode of HIV-1-1 transmission. This is particularly true in less-developed counties. By sharing



needles and needle stick injuries, blood and blood product transfusions, or medical injections with contaminated and unsterilized instruments, HIV-1-1 finds its way to lead to a positive infection. In developed countries, examination of blood products before a blood transfusion is conducted is usually very seriously considered, and thus the risk of HIV-1-1 infection is negligible (about 1 in 1.5 million in the United States in 2008) (22). While in less developed countries, HIV-1-1 screening and blood donor selection are not conducted perfectly, so up to 15% of HIV-1-1 infections in those countries actually comes from contaminated blood and blood product transfusions (23)(24)

Mother-to-child is the third most frequent mode of HIV-1-1 transmission. This transmission pathway causes 90% of cases in children infected with HIV-1-1 (25). However, with appropriate treatment during pregnancy, the risk of mother-to-child infection can be reduced to about 1% (25). A piece of good news is that during the year 2014, there were 220,000 cases of children who became newly infected with HIV-1-1, decreasing from 520,000 in 2000 (5).

## **HIV-1-1 structure and genome**

As shown in **Figure 1**, HIV-1-1 is positive, single-stranded RNA retrovirus. Two copies of RNA strands are packaged in the viral capsid and enveloped by matrix protein and lipid membrane. HIV-1-1 is roughly spherical (26). Its diameter is about 120 nm, which is 60 times smaller than a human red blood cell (27). Viral RNAs carry all the genetic

information and are the nucleotides materials responsible for encoding nine structural and non-structural proteins. As shown in **Figure 2**, the whole genome contains 9749 base pairs, and nine open reading frames which are protected by two long terminal regions on both the 3' and 5' sites. Among the nine open reading frames, there are three large precursor proteins, named Gag, Pol and Env (28). These proteins are then post transcriptionally cleaved into smaller structural and non-structural proteins. The viral RNAs are tightly bound and protected by the nucleocapsid protein p7, and the late assembly protein p6. Viral RNA-P7-P6 complex is carried by the capsid made up of 2000 copies of p24 (29) (30), and further covered by the matrix protein composed of p17. All of these proteins are post transcriptionally cleaved from the Gag precursor protein. The three most important non-structural proteins: integrase, reverse transcriptase, and viral protease, are encoded in the Pol open reading frame. Regulatory proteins such as Rev and Tat, and other auxiliary accessory proteins, such as Vpr, Vif, Vpu, and Nef are encoded in smaller open reading frames (31) . Outside the matrix is the lipid membrane layer. It is formed from human lymphocytes by taking the advantage of their lipid bilayer. HIV-1-1 envelope glycoproteins gp120 and gp41 are cleaved from their gp160 precursor encoded from Env open reading frame. These two envelope protein subunits play a vital role in viral entry, and thus are the key to inhibitor design and vaccine development (32, 33).

## **HIV-1-1 Viral Life Cycle**

HIV-1-1 lives in human lymphocytes. HIV-1-1 becomes very fragile when it is exposed in the environment outside of the body (34, 35), which reduces the amount of infectious virus by 90%-99% in just several hours. However, when HIV-1-1 is exposed to a human lymphocyte, this virus finds its way into it and makes the human lymphocyte a reproductive reservoir. The CD4 molecule binds to gp120, followed by a significant conformational change, which leads to the exposure of chemokine receptor on gp120. On the surface of the cell, the chemokine receptor, either CCR5 or CXCR4, then binds to gp120, which allows gp41 to penetrate the cell membrane, and thus fuels virus-cell membrane fusion (34, 35).

Successful viral fusion results in delivery of viral RNA and non-structural proteins into the cell. These important enzymes, including reverse transcriptase (RT), integrase, protease, etc., serve in reverse-transcription, cDNA replication, and host genome integration. Reverse transcriptase transcribes the single strand viral RNA into a complementary DNA carrying viral genetic information. The cDNA and its complement form a double-stranded viral DNA and the viral protease and integrase insert viral DNA into human host chromosome (34, 35) . During replication and transcription, the extremely error-prone reverse transcriptase generates cDNA with a large number of mutations, which can result in immune evasion and drug resistance.

Other unique features about the reverse transcriptase are its ribonuclease activity and DNA-dependent DNA polymerase activity (36, 37).

NF- $\kappa$ B is one of the cellular transcription factors that controls virus production after the viral genome has been successfully integrated into the host chromosome (38). When cytotoxic CD8<sup>+</sup> T-cells become activated, NF- $\kappa$ B is upregulated and allows the host genome to actively produce viral particles. Viral non-structural and structural proteins are produced along with host chromosome translation. During viral replication, the integrated viral DNA is transcribed back into viral RNA, which then undergoes RNA splicing and becomes viral mature mRNAs (39). Along with the translation, some viral regulatory enzymes, including Tat, allows better viral production. Another enzyme, Rev, can localize itself to the nucleus and bind to viral mRNA, making the viral mRNA possible to transport out of the nucleus. With Tat and Rev, viral mRNAs leave the nucleus (38), and these mRNAs either function as transcription and translation materials, to produce viral non-structural or structural proteins, or function as new viral genome copies. Viral mRNAs, structural proteins and non-structural proteins, are then packaged and assembled in the Golgi apparatus, and leave the host cell when mature. During maturation, the Env polyprotein gp160 is cleaved by furin protein in the Golgi apparatus. The cleaved gp160 becomes two subunits, gp120 and gp41, which are non-covalently linked and form a heterodimer. Three gp120/gp41 heterodimers form a homotrimer anchored by gp41 subunits on the surface of HIV-1 envelope. Virions are finally become mature and are then able to infect neighboring cells (40, 41).

## **Immune Response**

### **Signs and Symptoms**

When having unprotected sex with an HIV-1-1 infected individual, or having direct exposure to HIV-1-1 infected blood or body fluid samples, people are at risk of getting infected with HIV-1-1. Initial symptoms may be inconspicuous since large numbers of individuals only develop influenza-like symptoms (42)(43), and some others have no noticeable symptoms (44)(45). Most commonly reported initial HIV-1-1 symptoms are fever, lymphadenopathy, rash, headache, and throat/mouth/genital inflammation (46). Some opportunistic infections like gastrointestinal symptoms, and neurological symptoms may occur (47). However, all symptoms are not severe, and usually disappear without any treatment in one to two weeks (48). Considering the fact that these symptoms are usually not specific, and are easy to “be cured”, HIV-1-1 infections are often not being recognized or diagnosed. Typically, people who had unprotected sex with an individual with HIV-1-1 before developing with such symptoms, are suggested to have a HIV-1-1 antibody test 1-3 months after suspecting that they have been exposed to HIV-1-1.

In most cases, an HIV-1-1-infected individual first develops the non-specific Flu-like symptoms described above, then the immune system starts to eliminate the virus but leaves some latent virus harboring in CD4+ T cells, Macrophages or Dendritic cells (49). A patient will live normal life until HIV-1-1 progresses and AIDS or AIDS-related

symptoms arise after several years. Without any treatment, this clinical latency may last three to 20 years, with an average of eight years (50)(51)(52). During the latency, patients may first experience asymptotically, or just have a low-grade fever for a short period of time, but at a later latency stage, CD4+ T cells are lost at a higher rate and individuals will eventually progress to AIDS (53). Interestingly, there are some exceptions: about 5% of HIV-1-1 infected individuals actually retain a high level of CD4+ T cells for more than five years without any treatment (54)(55), these people are known as “long-term non-progressors” (55); another group is known as “elite suppressors” whose viral load is undetectable even without any treatment.

AIDS refers to HIV-1-1 post latency symptoms and AIDS associated diseases, which is clinically defined by the number of CD4+ T cells declined to a certain critical level (usually, below 200 CD4+ T cells per microliter) (56). Without any antiretroviral treatment, individuals infected with HIV-1-1 develop these symptoms within 10 years, and swiftly progress to death due to the depletion of the vast majority of CD4+ T-cells and infectious virus domination in plasma. Opportunistic infectious disease develops along with this stage and can be caused by almost any pathogens including bacteria, viruses, fungi and parasites (57). HIV-1-1 patients at a later clinical state are also very vulnerable to cancers. Kaposi’s sarcoma occurs in 10 to 20% of cases of HIV-1-1 infected patients, which becomes the most common cancer accompanying AIDS (58). Lymphoma, leading to death in 16% of people living with HIV-1-1, is the second most common cancer (59). Human papillomavirus (HPV) and Hepatitis C virus (HCV) are

reported to co-infect with HIV-1-1 and these two viruses also lead to virus-induced cancers.

## **Innate Immunity**

Innate immunity refers to the non-specific in-born immunity. Innate immunity plays a very important role in early HIV-1-1 defense. The innate immunity is also the sub-immune system that bridges humoral and cell mediated adaptive immune response at a later stage. Moreover, white blood cells serve in innate immunity and in the case of HIV-1-1 infection, play a unique role in HIV-1-1 latency, which is a major barrier of HIV-1-1 eradication.

During the first stage of HIV-1-1 infection (also referred to as acute HIV-1-1 infection), HIV-1-1 viral particles can be recognized by phagocytes such as macrophages and dendritic cells (60, 61). HIV-1-1 envelope proteins, viral mRNAs, and other viral structural or non-structural proteins, are termed as viral pathogen-associated molecular patterns (PAMPs) (61). These viral PAMPs are recognized by pathogen-recognition receptors (PRRs) of infected cells which trigger a series of signaling cascades. These signaling cascades result in an intracellular antiviral immune response such as a production or upregulation of antiviral molecules including interferons, interleukins, cytokines and chemokines (60). These molecules and the phagocytes are also involved in initiation of the adaptive immune system(62). At the first infection stage, HIV-1-1 is recognized by a mode of PRR termed as Toll-like receptors (TLRs) (63). Among

these TLRs, plasmacytoid dendritic cells (pDCs) utilize TLR7 and TLR9 to recognize single-stranded RNA (ssRNA) and double-stranded DNAs (dsDNAs) (63), triggering signaling cascades to control viral replication and reproduction.

Viral replication usually indicates a successful viral infection, however, in the case of pDCs, TLR7 and TLR9 recognition usually does not require HIV-1-1 reproductive infection (63). There therefore must be other PRRs recognition pathways being activated in those infected cells. It has been demonstrated that the infected cells use RIG-I-like receptors (RLR) and other PRRs such as DAI, IFI16, and AIM2 to recognize viral DNAs. Biological interplay between viral DNAs and PRRs leads to transcriptional activation of pro-inflammatory cytokines, chemokines, and type I interferons (I-IFNs) (64, 65). For example, upon TLR7 recognition, I-IFNs are induced, and these IFNs bind to IFN receptors on the surface of the infected and surrounding cells, which further triggers other signaling cascades that activates the transcription and translation of many IFN-stimulated genes (ISGs). ISGs are important in blocking viral replications. One of the most important ISGs is IFI16 (66-68). As mentioned above, IFI16 serves a role as PRR. IFI16 is both a nuclear and a cytosolic protein. This protein binds to cDNA under the assistance of a hin domain (67). After HIV-1-1 has successfully entered and replicated in the reservoir, the viral mRNAs are reversely transcribed into cDNA. IFI16 recognizes and captures viral DNA through hin domain, destroying HIV-1-1 replications (69). A knockdown of IFI16 study shows that the target host cells that undergo HIV-1-1 infection in the absence of IFI16, have a significant higher level of



HIV-1-1 permissiveness and viral replication(68). Thus IFI16 are confirmed to be both an HIV-1-1 PRR and a restriction factor.

RLRs are a family of DExD/H box RNA helicases that function as cytoplasmic sensors of PAMPs. Among these RLRs, RIG-I functions in HIV-1-1-infected cells by binding to HIV-1-1 mRNAs (70), which leads to activation of pro-inflammatory innate immunity response to target cells. Importantly, RIG-I also contributes to crosstalk with TLRs and other PRRs to modulate the adaptive immune response (71). Another sensor that recognizes HIV-1-1 PAMPs in HIV-1-1-infected cells is the cyclic GMP-AMP synthase (cGAS) (69), which recognizes and binds to HIV-1-1 reverse transcriptase in its early replication cycle. cGAS contains an amino-terminal DNA binding domains and a nucleotidyltransferase domain, which allows viral DNA binding and produces cyclic GMP-AMP (cGAMP) (68, 72). cGAMP is a cellular factor that works along with cGAS to induce ISGs. cGAMP also functions as a second signaling factor and binds to an adaptor protein called STING. The cGAMP-STING complex activates a signaling cofactor TBK1, and interferon regulatory factors IRF3 and IRF7 (72), which finally stimulates antiviral effector genes. Transcriptional factors such as IRF3, IRF7 and NF- $\kappa$ B, also drive the production of viral restriction factors (APOBEC, TRIM5a, SAMHD1, and tetherin) (61, 73) limiting viral replication. Each of these factors has been heavily studied. Recent studies found other restriction factors including SLFN11, IFITM and MX2 (74-77). Interestingly, these factors are not highly expressed in resting cells only until they are induced by IFN. SLFN11 is an RNA helicase-like protein that suppresses

HIV-1-1 replication at a later stage, so it is also associated with control of HIV-1-1 chronic infections. IFITM is transmembrane protein that inhibits HIV-1-1 entry (77). MX2 is GTPase that suppresses HIV-1-1 replication by stopping HIV-1-1 reverse transcribed DNA integration into the host chromosome.

During the first few hours of HIV-1-1 infection, viruses successfully obtain opportunities to tear into the mucosal epithelium and undergo further interactions with conventional DCs (cDCs) and Monocytes (60). The HIV-1-1-bearing stromal cDCs also have CD4 molecule and CCR5 coreceptor on the cell membrane for HIV-1-1 infection. Activation of cDCs leads virus degradation by the secretion of restriction factors such as APOBEC3G and SAMHD1, to restrict HIV-1-1 replication (78). Also, IFNs and Interleukins such as IL-12, IL-15 and IL-18 induced by the activated cDCs are able to either inhibit viral replication or recruit the NK cells to kill the infected cDCs (79). However, HIV-1-1 uptake by the C-type lectin DC-SIGN on the surface of the DC cell membrane blocks DC maturation thus resulting in DC dysfunction (80). DC-SIGN binds to HIV-1-1 envelope glycoproteins to internalize virus by endocytosis. Binding of DC-SIGN with HIV-1-1 envelope glycoproteins also facilitates HIV-1-1 transmission to CD4<sup>+</sup> helper T cells. During the activation of cDCs, the plasmacytoid DCs are also activated. pDCs recognition of HIV-1-1 is achieved by TLR7 and TLR9 recognition. Unlike cDCs, the recognition usually does not require productive HIV-1-1 infection. Both cDCs and pDCs interacts with CD4<sup>+</sup> helper T cells and transmit virions via the virological synapse cell (81, 82). Based on the new understanding of the

viral transmission pathway, new ideas of blocking HIV-1-1 infection of CD4+ helper cells could be reconsidered, and this understanding also bring us more insights into HIV-1-1 antiretroviral drug design as well as vaccine development.

## **Acquired Immunity**

Currently, many studies have shown a strong link between the activation of innate immunity pathways and the adaptive immunity response (83, 84). Both the severity and the duration of primary HIV-1-1 infection are associated with the speed of CD4+ T cells lost in chronic infection, and they also positively correlate with the number of deaths in the AIDS phase. So, the events during very early HIV-1-1 infection directly have consequences for the disease. How does the immune activation indicate HIV-1-1 infection? It has been well recognized that HIV-1-1 pathogenicity is characterized by both severe immunodeficiency and significant immune activation (85, 86). There is growing evidence showing that lymphocytes obtained from HIV-1-1-infected patients are found mostly in an activated stage (87). During the acquired immunity activation, phenotypic activation markers on the surface of peripheral T cells and B cells are expressed at a decently higher level regulated by IFNs and other regulatory factors induced in the innate immunity phase, shown by an increased level of inflammatory cytokines in plasma. A sooty mangabey monkey model study (88) suggested a positive correlation between immune activation and immune deficiency. In the absence of dramatic immune activation, sooty mangabeys infected with SIV showed no manifests of infection but detected high level of SIV replication in plasma (89).

## **Cell Mediated Immunity and Viral Immune Evasion**

The acquired immunity against HIV-1-1 begins with the uptake of virus by CD4<sup>+</sup> T helper cells. During initial infections, usually a few hours post HIV-1-1 uptake by either pDCs or cDCs (90-92), the internalized HIV-1-1 virions are transmitted from DCs to CD4<sup>+</sup> T cells via virological synapse. HIV-1-1 recognizes and binds to CD4<sup>+</sup> T cells through the interplay between viral envelope protein gp120 and cellular membrane receptor and coreceptor CD4 and CCR5 or CXCR4, followed by CD8<sup>+</sup> cytotoxic T cells killing infected CD4<sup>+</sup> T cells (93). During the first week, HIV-1-1 rests in HIV-1-1-bearing T lymphocytes and amplifies within the cells in draining lymph nodes. HIV-1-1-bearing DCs present HIV-1-1 antigens to both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells via either MHC class II AND MHC class I (94). This activation of T lymphocytes in draining lymph nodes results in the clonal expansion of HIV-1-1-specific CD8<sup>+</sup> T cells and HIV-1-1-specific B cells. After activation, HIV-1-1-specific antibodies generated from B cell follicle start to neutralize viral particles and CD8<sup>+</sup> T cells begin to eliminate HIV-1-1 productively infected CD4<sup>+</sup> T cells.

Typically, during virus progression, the activated adaptive immunity eliminates both viruses and virus-infected cells. However, the outcome of HIV-1-1 infection is that after about 10 years of HIV-1-1 latency (95), infected individuals finally end their lives in immunodeficiency resulted AIDS. So, what are the reasons make our immune system abnormal? And how does the virus escape from immune detection and establish viral

latency? Unlike other viruses which target on non-lymphocytes, our working immune system clears HIV-1-1-infected CD4<sup>+</sup> lymphocytes. This can be achieved by CD8<sup>+</sup> cytotoxic T cells, and NK cells, cytotoxic killing from which eventually results in inadequate CD4<sup>+</sup> T cells in plasma. Following initial HIV-1-1 infections, CD8<sup>+</sup> cytotoxic T cells do help control virus proliferation. The infected cells present viral peptide antigens by HLAs (human leukocyte antigens) to CD8<sup>+</sup> cytotoxic T cells, resulting in apoptosis of infected cells either by perforin generating holes in the target cells, by granzymes, or by Fas ligand-Fas (CD95) induced cell death (91). CD8<sup>+</sup> cytotoxic T cells also regulate the delivery of antiviral soluble factors to infected cells, such as cytokines and IFN-gamma, which promote nearby cells becoming less vulnerable to viral infection (96)(97)(98). Despite these strategies utilized by CD8<sup>+</sup> T cells work to some extent eliminated infected cells, the viral defense story is just a beginning, for HIV-1-1 is a master of escaping from cytotoxic lymphocytes. Under the selective pressure from CD8<sup>+</sup> cytotoxic T cells, HIV-1-1 swiftly acquires mutations against cytotoxic T cell killing. Strong evidence has been found suggesting two viral protein, Nef and Env are responsible for viral immune evasion (99, 100). The Nef protein is necessary for high viral loads in AIDS progression, but the most important and well characterized function of Nef, is its role in antigen presentation during immune evasion. An example is that during an adoptive transfer of cytotoxic T lymphocytes to a HIV-1-1-infected patient, the transferred cytotoxic T lymphocytes swiftly and effectively selected for viral variants contains a deletion mutation in Nef protein (101, 102). Another method of immune evasion for HIV-1-1 is that a mutation at the second

residue of the Env epitope reduces binding affinity of peptide to MHC during the cytotoxic T lymphocyte immune response domination. This result indicates that Env mutations can be another immune evasion strategy used by HIV-1-1 during cytotoxic T lymphocytes antiviral selection. Other viral proteins that play roles in immune evasion include Gag and Pol. These viral proteins allow the virus to replicate without recognition by the host (103, 104).

To summarize, strategies of HIV-1-1 escaping cytotoxic T lymphocyte-mediated immunity include: If infected CD4<sup>+</sup> T cells are successfully eradicated by CTL killing, the resulting substantial loss of CD4<sup>+</sup> T cells will directly influence T helper cell function, and this finally undermines CD8<sup>+</sup> T cells immune response to clear infection. On the other hand, HIV-1-1 evolves to obtain antigenic variability through the error-prone-reverse transcriptase resulting in mutations. These viral mutations can result in the dysfunction of cytotoxic T lymphocytes in viral recognition, viral replication inhibition, or viral proliferation. Eventually, the repeated T cell activation ends in T cell exhaustion and leads to chronic HIV-1-1 infection.

### **Humoral Immunity and Viral Immune Evasion**

During early HIV-1-1 infection, typically 1-4 weeks post infection, CD4<sup>+</sup> T cells activated by DCs presenting antigens via MHC II differentiate into T follicular helper cells and migrate into the B cell follicle (105, 106)(107). HIV-1-1-specific B cell and early antibody responses are established in the interplay of follicular DCs, follicular B

cells and follicular helper T cells (108). In the early immune activation stage, pro-inflammatory cytokines mediated from innate immune response events lead to the increase of turnover and polyclonal activation of B cells (109). This however will results in either increased B cell apoptosis, follicular hyperplasia, or mature B cell activation. A successful B cell maturation and memory B cell differentiation guided by HIV-1-1 infection establishes the foundation for HIV-1-1-specific antibody production. However, neutralizing antibodies against autologous HIV-1-1 strains develop slowly, usually no earlier than 12 weeks post initial infection (110), and what is worse, even though about 20% of HIV-1-1 patients develop neutralizing antibodies against HIV-1-1, a majority of these antibodies are of only moderate degree of neutralization and aroused after years of infection (106, 111). It is thus important to look at B cells at their early response to HIV-1-1 infection to understand why a neutralizing antibody response cannot be acquired in acute infection as is the case in other viral infections.

The first detectable B cell response is found on day 8 post infection, whereas the first detectable antibody response appears on day 13 post infection. These early induced antibodies are however non-neutralizing antibodies against either gp41 or gp120 (111-115). It is still unknown why these early phase antibodies are non-neutralizing. One possible reason is that denatured or non-functional Env forms may be dominant in the antigen pool (116, 117). The first neutralizing antibodies that induce escape mutants develop 12 weeks post infection (118-120). Although studies have characterized a number of neutralizing antibodies, these neutralizing antibodies majorly bind to the

highly conserved regions of gp120 or gp41. For example, the monoclonal antibodies b12 and VRC01, neutralize virus by specific binding to the CD4-binding site on gp120. Another example would be the monoclonal antibodies that recognize the membrane-proximal external region of gp41, such as 2F5, Z13 and 4E10 (120). Observations based on the neutralizing antibodies binding locus reveal that the HIV-1-1 variable loops under highly-shielded glycans create an antigenic barrier for antibodies neutralizing either HIV-1-1 envelope core domains, or HIV-1-1 envelope variable loops.

Reasons for the broad-specificity, poor neutralizing, and rarely induced antibodies are complex. First, as mentioned above, HIV-1-1 escapes antibody recognition by the error-prone reverse transcriptase-resulted antigenic mutations. Under antibody selection, more than 95% of gp120 nucleotide changes resulted in an amino acid change (121). Second, during HIV-1-1 progressive infection, follicular B cells are damaged by virus-induced and pro-cytokine induced apoptosis. The loss of follicular B cells has a direct influence on antibody production, resulting either in a poor antibody response or hypergammaglobulinemia. This damage further contributes to substantial loss of germinal centers (about 50%) (122) and gradually progresses to HIV-1-1 chronic infection. Third, during the chronic and advanced infection, CD4+ T cell lymphopenia stimulates the secretion of IL-7, which increases the number of immature transitional B cells. The consequence of expansion of immature B cells promotes a declined B cell response to HIV-1-1 antigens. In addition, Nef mediated class-switch recombination, during the expansion of immature B cells and naive mature B cells, results in the paucity



of HIV-1-specific IgA at mucosal sites (122). At a later AIDS stage, inadequate CD4<sup>+</sup> T helper cells are not functional enough to transit the decreased number of resting memory B cells or splenic marginal zone B cells into memory B cells, which ends up with memory B cell exhaustion. Taken together, the antigenic variability of HIV-1 envelope proteins, the vast damage in both germinal centers and follicular B cells, the loss of CD4<sup>+</sup> T helper cells and the exhausted memory B cells are eventually not able to produce high affinity broadly neutralizing antibodies against HIV-1 progression.

## **HIV-1-1 Viral Latency**

HIV-1-1 can infect both activated and non-activated, resting CD4<sup>+</sup> T cells. Infection of activated CD4<sup>+</sup> T cells recruits CD8<sup>+</sup> T cell cytotoxic killing and results in a substantial depletion of CD4<sup>+</sup> T cells. Whereas infection of resting CD4<sup>+</sup> T cells usually leads to viral latency. Viral latency is therefore a major challenge for virus eradication, even under the treatment of highly active antiretroviral therapy (HAART) (123). In a normal T cell immunity model, naive CD4<sup>+</sup> T cells are activated by antigens presented by antigen presenting cells (APCs). Following activation, CD4<sup>+</sup> T cells start clonal proliferation, during which, a majority of T cells undergo apoptosis and some effector T cells differentiate into memory T cells. Other naive or memory T cells stay at the G0 phase of the cell cycle. However, during HIV-1-1 infection, viral genomes are reverse transcribed and replicated into double-stranded cDNAs (dscDNAs) (124). The dscDNAs are relatively unstable and exist in the cytosol. This is called a reversible pre-integration latent state. However, when ATP is abundant in the cytosol, pre-integration

state dscDNAs are permanently integrated into the host genome. The provirus after successful integration remains quiescent before another cycle of immune activation. These latent HIV-1-1 provirus bearing CD4+ T cells can be differentiated into memory CD4+ T cells (125), and the nightmare for the host starts from here. As the antigens fade, most effector T cells die by apoptosis, but a small portion of T cells remains as memory T cells, and carry a permanent HIV-1-1 genome in their chromosome (126, 127). Some of these memory CD4+ T cells transit into a resting state that have an extended lifespan and lack the markers for T cell activation. These virus-bearing resting CD4+ T cells are thus not recognized by immune system and are less likely to be activated during HIV-1-1 progression (128, 129). Viral latency is therefore established.

## **Anti-HIV-1-1 Therapy Development against AIDS**

### **Antiretroviral Therapy**

There is no cure for AIDS at the moment. Neither antiretroviral therapy (ART) nor a protective vaccine can eliminate the virus or cure the disease. However, with ART or HAART, HIV-1-1 infected individuals are able to live with HIV-1-1 for a longer time and have their life extended to a close-to-normal life span. Also, ART does great job in preventing mother-to-child HIV-1-1 transmission when treatment is given at a very early stage to both child and mother. In the absence of any treatment, mother-to-child transmission ranges from 15% to 45%, and ART brings the number down to below 5% (130). This ART also provides optimal efficacy of reducing HIV-1-1 transmission during sexual contact, reducing viremia and transmission by more than 96% (131)(132).

Before given any ART, infected individuals are advised to undergo some tests to examine the patient's medical history, to determine the best choices of medicine, or to avoid potential side effects. These tests includes, but not confined to, complete blood count (including CD4+ T cell count), urinalysis, liver and kidney function tests, infectious disease or sexually transmitted diseases tests, and a coreceptor tropism assay. Typically, ART consists of several classes of HIV-1-1 medicines, and each individual may take a regimen of different medicines in combination. The anti-HIV-1-1 medicine classes are: non-nucleoside reverse transcriptase inhibitors (NNRTIs), which prevents HIV-1-1 reverse transcriptase converting viral RNA to cDNA; nucleoside reverse transcriptase inhibitors (NRTIs), which also prevents reverse transcription; protease inhibitors, which inhibit viral-cleavage of the host genome; fusion inhibitors (e.g. T20), which blocks virus-cell membrane fusion; CCR5 antagonists (e.g. Maraviroc), which inhibits viral entry through coreceptor blocking; and integrase strand transfer inhibitors (INSTIs) (133). There are however side effects that come with these medical treatments. Physicians prescribing ART medicines always try to find a right combination of medicines without eliciting too many side effects. However, these drugs may lead to some short-term problems such as diarrhea, fatigue or headaches etc., or long-term side effects such as lipodystrophy, insulin resistance, and lactic acidosis etc. The short-term side effects usually last and fade from days to a month, but people suffering from the long-term side effects should consider seeing their doctors periodically (134). Drug resistance is another problem. HIV-1-1 mutates during their replications, and the

mutations selected by antiretroviral drugs will gradually decrease antiretroviral drugs efficacy.

## **Immunotherapy**

Immunotherapy is another way of treating HIV-1-1-infected individuals. Immunotherapy refers to passively transferring HIV-1-1-specific neutralizing antibodies to HIV-1-1 infected patients. However, pre-clinical and clinical trials of these therapies all have failed to neutralize viruses (135-137). Therefore immunotherapy against HIV-1-1 had been abandoned due to its ineffectiveness for many years. However, recent studies taking advantage of novel strategies such as cell-based immunotherapy successfully suppressed HIV-1-1 in infected humans. On June, 2015, Caskey M et al. proved that HIV-1-1 viremia can be suppressed in HIV-1-1 infected humans by passive transfer of a monoclonal antibody named 3BNC117 (138). Based on the single-cell-based antibody cloning methods, a new generation of potent broadly neutralizing antibodies have been uncovered (139-142), making it possible for researchers to produce large amount of broadly neutralizing antibodies (bNAbs). These bNAbs have shown promising protection and viremia suppression in humanized mice and nonhuman primates (143-145). In addition, a very recent study carried out by Nancy Haigwood in 2016 (146), showed that by passively transferring human bNAbs as a cocktail of PGT121 and VRC07-523 to infant macaques, SHIV-1 infection can be halted with no viral emergence even after CD8<sup>+</sup> T cell depletion. This results suggest that this human bNAb cocktail immunotherapy can eliminate both viral foci and viral

reservoirs at very early stage, making this immunotherapy promising to completely eradicate HIV-1-1 and stop HIV-1-1 infection in human infants.

## **Vaccine Development against AIDS**

The typhoid vaccine against typhoid bacterium was commercially available after a 105 year endeavor since pathogen had been discovered. A pertussis vaccine was available 89 years after the first reported case. It took us 47 years to develop a vaccine against polio and 42 years to develop a vaccine against measles. It therefore seems quite understandable that over 35 years of HIV-1-1 vaccine research has not provided us with an effective HIV-1-1 vaccine. We must however realize that the time course for those released vaccines was reducing, and it only took us 16 years to develop a vaccine against the hepatitis B virus. It is predictable that at this age an effective vaccine for HIV-1-1 is coming in the foreseeable future.

Traditional vaccine strategies such as subunit, killed or live attenuated vaccines are not protective against HIV-1 infection. These vaccines either induce non-bNAbs or NAbs but strain specific, so that the low bNAb content is inadequate to have antibody protection against HIV-1-1 infection. The first generation HIV-1-1 vaccine, typically is represented by the AIDSVAX gp120 subunit vaccine, stimulates antibodies towards HIV-1-1 envelope glycoproteins, but proved to be non-neutralizing, and thus failed to protect vaccinated individuals from HIV-1 infection (147). The second generation, named the STEP vaccine, uses adenovirus serotype 5 virus as backbone vector, to

carry HIV-1-1 Gag, Pol and Nef genes, expressing these HIV-1 viral proteins. This design was predicted to stimulate CD8+ T cell mediated immunity against HIV-1, but again showed no effective protection (148, 149). After the above two failed trials, a third generation vaccine was created (or we should call it a third efficacy trial, because it utilizes a combination of two vaccines described above). This trial, known as RV144 (150) was conducted in Thailand. This vaccine strategy is described as using a canary pox viral vector carrying HIV-1-1 envelope gene gp120, Gag, and Pol to prime the immune response, followed by the AIDSVAX gp120 subunit vaccine to boost. The outcome of this trial is not promising: the vaccine efficacy is only 31.2% in their modified intention-to-treat study involved 16,395 subjects. In addition, there are no significant changes in the degree of viremia or the number of CD4+ T cells. This limited protection although not promising, offers insight for further research.

What lessons shall we learn from these vaccine trial failures? First, we understand that an effective vaccine should have the potential to stimulate both CD8+ T cell and B cell response to multiple epitopes, especially to those highly conserved regions. In addition, an effective vaccine that allows T cell response to the common epitopes of different variants of the founder virus could inhibit viral immune evasion. Second, the fact that bNAbs induced by these conventional vaccines are usually low in number, poor in quality, and elicited slow in time reveals that a more reasonable strategy would be to create a vaccine that exposes as many conserved epitopes as possible, and also stably preserves the native form without denaturing *in vivo*. In addition, more advanced

adjuvants and therapies that can protect bNAbs-producing B cells or rescue the damaged or exhausted CD8<sup>+</sup> T cells and bNAbs-producing B cells may be necessary to work synergistically with vaccines. Third, it will be very wise to understand the HIV-1-1 envelope structures both in a native form, and in a complex form with potent bNAbs binding. Understanding the structures of the HIV-1-1 virion as well as the envelope glycoproteins allows us to investigate the epitopes that are targeted by bNAbs, or to find out how the conserved regions are shielded by the variable loops and glycans.

There is a remarkable endeavor in recent HIV-1-1 vaccine studies. In 2013, a study carried in China designed a novel adjuvant for HIV-1 vaccine delivery (151). Fullerenol is self-assembled into virus-sized nanoparticles, and they use this dual-functional nano-adjuvant to deliver a HIV-1-1 DNA vaccine. Surprisingly, this adjuvant not only shows promising adjuvant activity, but also decreases the antigen dosage and immunization frequency while maintaining decent immunity levels. What is more, the nano-adjuvant promotes effector memory CD8<sup>+</sup> T cells in immune response, which has great significance in earlier immunity against HIV-1 infection. In November, 2015, a new method investigating how HIV-1-1 vaccine induce humoral immunity, termed “systems serology”, reported by Chung AW et al. (152), provides us more ideas to understand the multi-dimensional relational comparisons in humoral immune response against HIV-1-1 vaccine. Furthermore, researchers from Duke University designed a HIV-1 Env using the novel 3D-printing technique (153). A ligand-free HIV-1-1-Env trimer was fixed in its native conformation, and epitopes were analyzed. These antigenicity-

guided structural designs are promising for a new generation of vaccine antigens. In February, 2016, one research team lead by Harvard Medical School and Beth Israel Deaconess Medical Center, recombined an HIV-1-1 clade A Env gene into adenovirus serotypes 26 and 35 (154). Assessment of the safety and immunogenicity of these two adenovirus-vectored-HIV-1 vaccines showed that both vaccines elicited significant immune responses without noticeable side effects in monkey model tests. This study provides us ideas about designing a desirable HIV-1-1 vaccine delivery system, but how long the protection lasts and how well the vaccine protects human after HIV-1 infection remains unknown. These recent HIV-1 vaccine studies, either focus on the adjuvant development, on understanding the humoral response rationale beyond vaccination, on choosing a safe and effective antigen delivery vector, or on a structural basis to design immunogens with reasonable antigenicity, are very good examples representing the promising directions of HIV-1 vaccine development.



## Chapter 2: Introduction and Objectives

The structure-based design of HIV-1 vaccine emphasizes the rationale on the structural relationship between antigens and antibodies. This method is substantially rational and sophisticated in designing immunogens to elicit potent neutralizing antibodies. Traditional HIV-1-1 vaccine designs do not pay much attention to the structural instability or the potential for antigen denaturing after *in vivo* antigen delivery, which is likely one of the reasons why potent bNAbs cannot be elicited. It is in this context that studies focused on structural-based design of HIV-1-1 vaccines have been started recently (146, 155).

The envelope glycoprotein of HIV-1-1 is expressed as the gp160 precursor, which is then proteolytically cleaved into gp120 and gp41 during maturation in the Golgi apparatus (35, 155-158). The two glycoprotein subunits gp120 and gp41 are the sole spikes on the surface of HIV-1 virion, so a large number of vaccine designs are based on these glycoproteins. Naturally, these two glycoproteins are non-covalently linked and are formed as heterodimers, and eventually function as trimers of gp120/gp41 dimers. Despite the fact that viral envelope protein complexes are well exposed on the surface, generation of HIV-1-1-specific neutralizing antibodies are shown to be narrow in breadth. As been discussed above, possible reasons may include viral mutation resulting in immune evasion. However, recent studies focusing on the HIV-1-1 envelope trimer structures reveal that another HIV-1-1 immune evasion mechanism

may be associated with the rearrangement of the envelope trimer structure during a pre-fusion to post-fusion transition (159-161). During recent years, the Cryo-EM technique has brought the structure of a soluble HIV-1-1 envelope trimer to our horizons (159-161). This trimer structure was represented in a cleaved state without gp41 MPER region and cytoplasmic tail. Later, the crystal structure of this cleaved trimer captured by antibodies PGT122 and 35O22 was also established. These findings make blueprints for mapping the prevalence and location of effective HIV-1-1 immune recognition.

Over the past 20 years, substantial HIV-1-1 envelope subunits structures have been obtained at atomic or near atomic levels. Some of these important findings include the structure of the gp120 core and its interaction with CD4 molecule (162), and the structure of post-fusion gp41 (163-165). In the context of the trimer, gp120 subunits shield most of the gp41 subunit and becomes the most exposed subunit, so that gp120, compared to gp41, has been more commonly studied (166)(167)(168)(169)(170). The first generation of candidate immunogens was designed based on monomeric gp120 (146, 164, 165, 171-173). The peptide sequence of gp120 is made up of five conserved regions and five variable regions (174-177), the five conserved regions together represent the gp120 core. The gp120 core is shielded by highly glycosylated variable regions, protecting the core from being neutralized.

Monoclonal antibodies (Mabs) against HIV-1-1 envelope proteins are primarily generated from naturally infected human beings or other animals. Among these Mabs,

a large number are antibodies that have no neutralizing ability. However, there are some potent Mabs which are able to neutralize several primary HIV-1-1 isolates (165, 178, 179). These neutralizing Mabs include 2F5, 2G12 and IgG1b12. Other Mabs particularly interact with the variable loops and are classified as V1/V2/V3 antibodies, but they are however only able to neutralize certain epitopes, so their value in vaccine development is less promising (178-188). The last group of antibodies directly targets the CD4 binding site, called CD4-BS antibodies. These antibodies also provide limited neutralizing effect, but they are able to neutralize laboratory-adapted isolates such as MN27 and HXBc2 (189, 190) (191). X-ray studies looking at the crystal structures of the HIV-1-1 gp120 (191)(192) core region demonstrated that the variable regions in two isolates HXBc2 and YU2 share very little accordance in terms of neutralizing antibody responses and coreceptor binding. However, the X-ray 3D crystal structures of these two isolates are nearly identical (191, 193, 194).

**Figure 3** represents the crystal structure of the gp120 core, which is composed of three domains: the inner domain, outer domain and bridging sheet (198-201). Moreover, a red circle at the junction of three domains indicates the location of the CD4 binding site. Recent studies looking at the binding of the structure of gp120 to VRC01, suggests that its binding site on gp120 seems to be, to some extent, identical to the CD4 binding site, as shown in **Figure 4**. However, a closer look at their binding sites revealed that VRC01 majorly binds to the outer domain of gp120, whereas the CD4 binding site consists of the junction of the three domains of gp120. These studies provide us with more details

about the outer domain of gp120. Compared to the inner domain and bridging sheet, the gp120 outer domain is relatively more stable, and is also highly conserved. One idea is that by making the outer domain protein as an immunogen, VRC01-like antibodies could possibly be induced. A recent study in our lab created two disulfide bonds to stabilize the C-N terminal, as well as the  $\beta$ 21/22 hairpin in the CD4-bound conformation of the gp120 outer domain (195). Immunogenicity of that modified OD has been evaluated and showed that OD2 (stabilized with additional two disulfide bonds from OD1) compared to OD1, was able to induce and circulate a higher amount of neutralizing antibodies in guinea pigs (196). Based on that study, we have introduced an additional S375W mutation to the CD4-bound conformation of outer domain, and named it OD3, to further investigate its potential of inducing neutralizing antibodies compared to OD1 and OD2. An X-ray crystal study of the gp120-CD4 binding complex reveals how the structural conformation of gp120 differs from the unbound state, and a thermodynamic study (195) of S375W gp120 binding to sCD4 reveals that there is less of an enthalpic and entropic change exhibited upon CD4 binding, which confirms the S375W-triggered CD4-bound conformation. The S375W-induced gp120 CD4-bound conformation appears to be a valuable conformation for further studies in vaccine design. We hope to make the gp120 outer domain a more stable immunogen and bring insights for further HIV-1-1 vaccine development.

## Chapter 3: Materials and Methods

### General Experimental Design

**Figure 5** illustrates the general experimental design in this study. The objective of the study is to evaluate the immunogenicity of our designed OD3 immunogen compared to OD1 and OD2. Primers were designed to generate the S375W mutation by site-directed mutagenesis, and the new gene segment was cloned into the expression vector pET28b. Plasmids were transformed into *E.coli* BL21 (DE3) for protein expression and purification. Purified samples were validated by SDS-PAGE, to evaluate immunogens' purity, followed by Western-blot with antibodies targeting different epitopes of the gp120 outer domain. Circular dichroism was conducted to confirm the secondary structure of the immunogens and to ensure their folding status. Verified immunogens were sterilized using the Minisart® 0.20 µm non-pyrogenic single-use filter for guinea pig immunization. A 63-day immunization protocol was followed. Guinea pig serum was collected at different time points for immunogenicity studies. HIV-1-1 pseudotyped viruses were generated by transfection of viral envelope plasmid, a CMV packaging plasmid and a luciferase plasmid into 293T cells. These pseudotyped viruses were utilized in the viral neutralizing assay.

### Virus Strains

Viruses used for the neutralization assay were pseudotyped HIV-1-1 viruses produced by transfection of 293T cells with three plasmids: a plasmid carrying luciferase

expressing gene (pHIV-1-luc), a plasmid packaging HIV-1 Gag and Pol gene (pCMV Gag-Pol) and plasmids expressing the HIV-1-1 envelope proteins of isolate variants (pSVIIIenv) (197, 198). Transfection of these three plasmid produces luciferase-expressing HIV-1-1 isolates in 293T cells after three days of incubation.

## **Molecular Modeling and Designing of OD3**

The glycoprotein-antibody complex, HIV-1-1 subtype B strain gp120 and the VRC01 monoclonal antibody complex were used as structural models to study and predict the gp120 structure of the HIV-1-1 subtype C strain 1084i. Based on a paper published from our lab (199), we designed the OD1 and OD2 immunogens using the software Discovery Studio Client 3.5 (Accelrys, San Diego, CA). OD1 is the wildtype gp120 outer domain and OD2 refers to the disulfide bond induced gp120 outer domain. OD3, used in this study, was modified from the OD2 protein with a serine to tryptophan mutation at location 375. This S375W mutation was first assessed in a full length gp120 in 2002 (200). The S375W mutation resulted a tryptophan indole ring occupying the Phe 43 cavity, which favors a gp120 conformation close to the CD4-bound state, which was better recognized by either CD4 or CD4-induced antibodies, and less favorable to CD4-binding-site antibodies. Considering the fact that S375 is located in the outer domain of gp120, we introduced the S375W mutation and designed OD3 to test its immunogenicity. All structural-based modification designs were performed on the platform of Discovery Studio Client. We numbered the protein sequences of OD1, OD2,

and OD3 based on the reference of the envelope sequence of the HIV-1-1 prototypic HXBc2 strain (201).

## **Cloning and Expression of OD3**

The gene design of OD3 was based on our previous work on OD1 and OD2, and all the genes were designed using the HIV-1-1 subtype C strain 1084i (201). OD1 and OD2 were synthesized by GenScript®. Basically, the wildtype OD1 has 214 amino acids, from 246 to 460 of gp120, and the design of OD2 was modified from OD1 to introduce two disulfide bonds (4 cysteine substitutions were introduced at the amino acid locations 257, 370, 425, and 472). In the design of OD3, a serine at the amino acid location 375 was mutated to tryptophan (S375W) from OD2 by mutagenesis. To be specific, the forward primer (5'-CGT TGC ATC ACC ATG CAC TGG TTC AAC TGC CGT GGT-3') and the reverse primer (5'-ACC ACG GCA GTT GAA CCA GTG CAT GGT GAT GCA CAG-3') were synthesized by GeneScript®. Primers were used in site-directed mutagenesis and OD3 was cloned into the vector pET28b. All the genes were designed with a 6xHis-tag at the N-terminus for the future purpose of protein purification.

The designed OD3 gene was cloned along with OD1 and OD2 into the pET28b expression vector (Novagen), and *E.coli* BL21 (DE3) was used as the protein expression system. Sequence confirmation was conducted by Operon® to make sure the open reading frame, the designed mutation as well as the N-terminal 6x histidine

tag were correct and present. Three genes in pET28b were transformed into *E.coli* BL21 (DE3) competent cells. 60  $\mu$ L competent cells were mixed with 100ng plasmid of each gene on ice for 10 minutes in a 1.5 mL Eppendorf tube. The tubes with plasmid and cells mixture were then incubated in a 42°C water bath for 2 minutes. Tubes were then placed on ice following heat shock for 5 minutes. 0.5 mL of LB Broth (Lennox) media with no antibiotics was added into each tube containing the OD1, OD2, and OD3 plasmid transformed cells, and were then incubated in the 37 °C shaker at 200 rpm for 20 minutes. 50  $\mu$ L of cell culture suspension were then taken from each tube, and were spread on LB agar plates supplemented with 50 mg/L kanamycin. After 14 hours of incubation at 37°, single colonies from each of the 3 plates were picked up and grown in 3 mL LB media with 50 mg/L kanamycin in the 37 °C shaker at 250 rpm until the optical density reached between 0.4 and 0.6 at 600 nm. The cells were induced by adding 1mM isopropyl-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and continuously grown for another 3.5 hours. All the bacterial culture was finally harvested and collected by centrifugation at 6000 G for 15 minutes. The cell pellets were washed once with STE buffer containing 100 mM NaCl, 10 mM pH 7.5 Tris, and 1 mM Ethylenedinitrilo tetraacetic acid disodium salt (EDTA). Cells were suspended in STE+ buffer (100 mM NaCl, 10 mM pH 7.5 Tris, 1 mM EDTA, 0.5% NP40, 100 mg/L lysozyme, and protease inhibitors), and incubated on ice for 20 minutes. Cells were then disrupted by sonication and were centrifuged at 14000 rpm at 4°C for 10 minutes. Both supernatant and cell debris were collected to determine whether the protein of interest was in supernatant or insoluble fraction. An SDS-PAGE test followed



by Coomassie blue staining and anti-6xHis western blot were conducted to determine the existence of our protein of interest. We found that the majority of the recombinant protein existed as inclusion bodies. The transformed strain *E.coli* BL21 (DE3) containing each OD1, OD2, OD3 plasmid were made into glycerol stocks for further experiments.

## **Immunogen Purification**

OD1, OD2 and OD3 protein expression began by the inoculation from the glycerol stock of *E.coli* BL21 (DE3) containing each OD1, OD2, OD3 plasmid into 3 mL LB media with 50 mg/L kanamycin. 3 mL bacterial culture were then added into 300 mL LB (1:100) with 50 mg/L kanamycin, then were incubated, induced with IPTG, collected, washed, and lysed using the same method mentioned above. To purify the recombinant protein as inclusion bodies, 4 subsequent washes were performed to gradually increase the purity of our protein of interest. First, they were washed with 1x PBS+0.1% SDS, then with 1x PBS+1% NP-40, then with 1x PBS+1% TritonX-100 and finally with 1x PBS+2 M urea. Short periods of sonication impulse were introduced during the washes to better diffuse the inclusion bodies. The insoluble fraction after 4 washes was finally solubilized in 3 mL solubilization buffer (8 M urea, 50 mM NaCl, 20 mM Tris (pH=7.5), and 5 mM Dithiothreitol (DTT) at 4°C for 30 minutes. The solubilized sample was then transferred into a dialysis cassette (Slide-A-Lyzer™ G2, Thermo Scientific). Our sample was then renatured by successive dialysis against the refolding buffer at 4°C (pH=7.5 20 mM Tris, 400 mM L-Arginine, 1 mM EDTA, 5%

glycerol, 0.4 mM Glutathione (GSH) and 1 mM Glutathione disulfide (GSSG) containing 4 M, 2 M, 1M, 0M urea), for 2 hours, 8 hours, 8 hours, and 24 hours respectively. The refolded protein samples then underwent an affinity purification known as immobilized metal affinity chromatography. Ni-NTA resin beads (Thermo Scientific) were used to further purify the protein. Protein purity was measured by Coomassie blue staining and Western blot analysis. Concentration of the protein samples was determined by a BCA protein kit (Pierce). Purified and renatured protein samples were then used for further characterization.

## **Immunoblotting**

Purified protein samples were loaded into a 10% SDS-denaturing gel along with negative (bovine serum albumin (BSA)) or positive (recombinant gp120) controls. The gels were run for 75 minutes at 60 volts. Nitrocellulose membrane transfer followed the SDS-PAGE (204). After 30 minutes blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS), nitrocellulose membranes were incubated for one hour at room temperature with primary antibodies. Following six washing steps (five minutes each) with PBS+0.1% Tween-20, horse radish peroxidase (HRP) conjugated secondary antibodies were incubated with membranes for 30 minutes at room temperature. Six washes were followed as explained previously. The outcome of antigen-antibody binding was detected either using SuperSignal West Dura Extended Duration Substrate kit (Pierce) or using a BioRad ChemiDoc (LiCor Inc., Lincoln, NE) (205).

## Secondary Structure Characterization

A desalting process was performed prior to the circular dichroism (CD) characterization. A desalting column (zeba<sup>TM</sup> Spin Desalting Columns, 7K MWCO, 5 mL) was used to remove L-arginine from the protein solution to avoid noisy signals. The instrument used for CD is a Jasco J-815, which is equipped with a cell compartment with a Peltier-type thermostat and a water circulator. The acquisition and data analysis are also from Jasco. Data was recorded using either a 1 mm cell-path 300  $\mu$ L quartz cuvet or a 0.1 mm 15000  $\mu$ L circular quartz cuvet at 20°C. The data was exported in XY ascii format and analyzed with the program CD-Pro located at the DichroWeb site from the University of London, for the purpose of estimation of the secondary structure of the protein. The data was analyzed in the raw machine units (millidegrees) with the same resolution as the acquired data (1 data point per nanometer). The cell path, average molecular weight and protein concentration were provided prior to the fitting, which was carried out using the various models available (CONTINLL and SELCON3 yielded the lowest RMSD values) until a satisfactory fit was obtained. The results were imported into Sigmaplot (SPSS) which was used to plot the data along with the simulated secondary structure spectrum. Our three protein samples were measured at the same concentration of 0.35  $\mu$ g/ml, solubilized in buffer containing 20mmTris-HCl and 2mm EDTA at pH=8.0, and the recording pathlength is 0.01 cm.

## **Guinea Pig Immunization**

The standard 63-day immunization protocol of Cocalico Biologicals, Inc. (CBI) for guinea pigs was followed. 16 guinea pigs were assigned evenly into 4 groups: inoculated with OD1, OD2, OD3 or adjuvant only (Complete Freund's Adjuvant or Incomplete Freund's Adjuvant). Three booster injections were carried following the first injection. Blood samples were collected from all guinea pigs on Day 0, prior to immunization as prebleed samples; on day 35 as test bleed samples after 2 boosts on day 14 and day 21; and on day 63 as exsanguination samples following the third boost on day 49. Blood samples collected (pre-bleeds, test bleeds and final bleeds) were used in antibody testing, ELISA and neutralization assays. All animal experiments were performed according to the protocol approved by the IACUC of CBI (Reamstown, PA).

## **ELISA**

Flat-bottom, with low evaporation lid 96-well plates (Falcon<sup>TM</sup>) were coated with 200 ng per well of test protein (OD1, OD2 and OD3) diluted in coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35mM NaHCO<sub>3</sub>, pH 9.6). Prior to blocking, plates were washed with phosphate buffered saline with 0.1% Tween-20 (PBS-T, Sigma-Aldrich) 3 times. Plates were blocked with 200 µL/well blocking buffer (PBS-T with 5% milk) at room temperature for 1 hour. After 3 washes with PBS-T, 100 µL/well of guinea pig sera diluted in PBS-T at different ratios was added and incubated at room temperature for 2 hours. Plates were washed 5 times prior to the addition of 100 µL/well of HRP-conjugated goat anti-guinea pig IgG secondary antibody (Jackson ImmunoResearch

Laboratories Inc.), and incubated at room temperature for 2 hours. Plates were washed 5 times, and 100  $\mu$ L/well SureBlue Reserve<sup>TM</sup> TMB substrate (KPL) was added and incubated for 1 min in the dark. 100  $\mu$ L/well of 1 M HCl was added to stop the reaction. Absorbance data was collected at 450 nm using an ELX 800 UV universal microplate reader (Bio-Tek Instruments).

## **Generation and Titration of Viral Stocks**

HIV-1-1 isolates used for neutralization assay are pseudotyped HIV-1-1 viruses produced by transfection of 293T cells with three plasmids: pHIV-1-luc, pCMV Gag-Pol, and pSVIIIenv of different isolates, as mentioned above. Transfection of these three plasmid produces luciferase-expressing HIV-1-1 isolates in 293T cells after a three day incubation period. Transfected 293T cells were grown at 37°C with 5% CO<sub>2</sub>, and maintained in DMEM medium with 584 mg/L L-Glutamine (Gibco), supplemented with 10% FBS and 1X penicillin-streptomycin. Twenty-four hours prior to transfection,  $1.5 \times 10^6$  cells were plated in 10 cm dishes. Cells were transfected by adding 6 $\mu$ g of replication-deficient HIV-1 luciferase plasmid pHIV-1-luc, 2  $\mu$ g HIV-1-1 envelope construct pSVIIIenv-Yu2/1084i/89.6/JRFL/HXBc2/ADA, and 2  $\mu$ L CMV backbone plasmid diluted in 1 mL unsupplemented DMEM. Polyethylenimine (PEI) was the transfection reagent used in this experiment. Cell supernatants were collected at 72 hours post-transfection, and passed through a 0.45  $\mu$ M syringe tip filter. The amount of virus in the supernatants were measured by reverse transcriptase (RT) assay (202).

## Virus Neutralization Assay

The neutralization assay was based on methods previously described in our published paper (199). TZM-bl cells were incubated at 37°C supplement with 5% CO<sub>2</sub>, and maintained in DMEM medium containing 4.5 g/L D-glucose and 584 mg/L L-Glutamine, supplemented with 10% FBS and 1X penicillin-streptomycin. One day prior to the assay, cells for infections were seeded at  $0.6 \times 10^4$  per well in 96-well plates, flat-bottom, black-sided plates (Grenier Bio-One). In another separate 96-well plate, heat-inactivated guinea pig serum was serially diluted to a final volume of 100  $\mu$ L of cell growth media, mixed with 2000 RT units of pseudotyped HIV-1-1 in 50  $\mu$ L cell growth media. The virus and cell mixtures were incubated for 1 hour at 37°C with 5% CO<sub>2</sub>, and during this incubation, the TZM-bl growth media was replaced with 50  $\mu$ L media containing 160  $\mu$ g/mL DEAE-Dextran (Sigma-Aldrich) (40  $\mu$ g/mL final concentration). The serum-virus mixture was added to cells and incubated at 37°C for 60 hrs. The luciferase assay system (Promega) was used to detect Tat-inducible luciferase. Luminescence was measured on a Veritas luminometer (Turner Biosystems). Percent neutralization was determined using the following equation:  $(1 - ((\text{test well} - \text{cell control well}) / (\text{virus control well} - \text{cell control well}))) \times 100$  (203).

## Chapter 4: Results and Discussions

### Structural Based Immunogen Design

The outer domain (OD) of HIV-1-1 gp120 envelope glycoprotein contains epitopes for CD4-BS antibodies due to the biological nature of its interactions with CD4 molecule. Some CD4-BS bNAbs neutralize 90% of HIV-1-1 isolates, such as VRC01. Considering the fact that the outer domain is relatively more stable than other domains of gp120, the outer domain has been considered to be a potential HIV-1-1 vaccine immunogen candidate. However, efforts to elicit bNAbs with the outer domain have not been successful. Studies of the outer domain crystal structures revealed that the when the outer domain only was represented in a truncated form, the protein structure does not remain consistent with the native form when the outer domain was presented within gp120 (204)(195) (205). To be more specific, the C and N terminal of OD1 (refers to the truncated outer domain without any structural modifications) are more widely open, and the  $\beta$ 20-21 hairpin is less closed to the outer domain core and the structure around the Phe 43 cavity does not exactly resemble the native form. Here, we based our design the OD2 structure and introduced a S375W mutation to further stable the outer domain. In summary, an OD3 immunogen design involves the following strategies: 1) keeping the outer domain core structure by removing all the variable loops; 2) introducing two disulfide bonds to stabilize the C-N termini and the  $\beta$ 20- $\beta$ 21 hairpin; 3) introducing an S375W mutation to have the indole ring from tryptophan filled into the Phe 43 cavity.

**Figure 6** represents details of superimposed structures of the OD3 compared to OD1, and **Figure 7** shows the designed structures of three proteins, and the amino acid locations of the mutations for the disulfide bonds and S375W. The outer domain designed in this way is predicted to be more favorable to CD4-BS bNAbs binding and thus is more promising to recruit potent bNAbs such as VRC01-like antibodies. The highly conserved outer domain, as mentioned previously, has more antigenic importance in eliciting effective antibodies than the variable loops. In our design, the variable loops have been completely removed so that the outer domain core is free from variable loop masking and glycan shielding. Furthermore, the S375W mutation is predicted to further stabilize the outer domain into a CD4-bound state, which is more vulnerable to CD4-BS antibody neutralizing. This taken together may explain why our OD3 design is able to induce higher level of potent neutralizing antibodies compared to OD1 and OD2, which will be further discussed.

## Generation and Validation of Immunogens

The designed OD3 gene was cloned into a bacterial vector pET28b and expressed in the *E. coli* strain BL21 (DE3). The strain BL21 is an *E. coli* K12 derivative which contains mutations in both the thioredoxin reductase and glutathione reductase genes that allows an intracellular environment for proper disulfide bond formation. OD3 was expressed along with OD1 and OD2 in this strain and was induced by IPTG for optimal recombinant protein expression. Purification of these three immunogens was achieved



by washing inclusion bodies followed by protein refolding dialysis and 6x His-tag metal affinity chromatography purification. A SDS-PAGE gel, shown in **Figure 8**, reflects the size and purity of three proteins. The clear bands around the molecular weight of 25 kDa marker suggested the recombinant proteins (weight 22.2 kDa) were detected at correct size. Proteins were concentrated by Pierce™ Protein Concentrators PES, 10K MWCO to a range from 0.2-2 µg/µl for experiments.

Purified proteins were further validated by immunoblotting, as shown in **Figure 9**. To be more specific, the CD4-BS monoclonal antibodies F105, VRC01 and b12 demonstrated binding affinity to all three proteins. In addition, polyclonal antibodies including patient sera and a commercially available anti-gp120 (abcam Inc. ab53840) all showed distinct bands responding to the immunogens. Last, a CD4 induced monoclonal antibody 17b, also showed binding activity to all three immunogens but was relatively weaker than other antibodies.

## Secondary Structure Verification

Circular dichroism (CD) is an excellent method of rapid protein secondary structure determination. Typically, protein samples with  $\alpha$ -helixes will show negative ellipticity bands at 222 nm and 208 nm and a positive band at 193 nm (206), whereas protein samples containing  $\beta$ -sheets have negative bands at 218 nm and positive bands at 195 nm (207). Proteins with disordered turns have very low ellipticity above 210 nm and negative bands near 195 nm (208). **Figure 10** shows the secondary structure analytical

results of our three outer domain immunogens (OD1, OD2 and OD3) obtained through circular dichroism. The data was analyzed by software CDSSTR and is summarized in **Table 1**. The analytical results showed that: 1) OD2 is different from OD1 with two disulfide bonds, and this difference is reflected by the increase in the OD2 positive ellipticity absorbance at 193nm and same absorbance at 195nm. This result suggested that the two disulfide bonds help the protein organize some unordered turns into well-ordered  $\alpha$ -helix-like structures, without disrupting  $\beta$ -sheets. 2) OD3 is different from OD2 with a S375W mutation, which stabilizes the outer domain to a CD4-bound state. In the **Figure 10**, an OD3 curve not only showed positive ellipticity absorbance at both 193nm and 195nm, but also showed an increase in the negative band at 208nm, 218 nm and 222 nm, suggesting more unordered peptide structures transferred to  $\alpha$ -helices, helix $\beta$ -sheets structures and ordered turns. These data provided us an idea that our S375W mutation in outer domain is very likely to stabilize the immunogen by making the outer domain secondary structure more organized.

In the total amino acid sequence, there are 26 amino acids organized in alpha helices and 70 in beta sheets, out of a total of 195. Thus the composition as far as secondary structure is predicted to be 13 % alpha helical and 36% beta sheet. This is in good agreement with the observed results for the beta sheets. Note that type 1 helix which is the most common, is usually more accurate, yielding 13-14%, in agreement with the predicted secondary structure.

## **Immunogenicity of Designed Immunogens in Guinea Pigs**

The purified, validated protein immunogen samples were then utilized in immunization studies. We ordered 16 guinea pigs and evenly divided them into 4 groups, each of those was inoculated with our three immunogens respectively, and one group was inoculated with adjuvant only as negative control. The Freund's adjuvant system (CFA/IFA) was used for this immunization test. **Figure 11** shows the basic protocol and timeline of the immunization carried out in this study. Blood samples were collected at three time points: at day 0, which is referred to as the pre-bleed serum samples used as a negative control; at day 35, which is referred to as the test bleed serum samples, and at day 63, the last day, which is referred to as the final bleed serum samples. All inoculations, boosts, and blood sample collection was conducted by Cocalico Biologicals, Inc. in Reamstown, PA. Serum samples of all guinea pigs were shipped overnight in dry ice to us and were properly stored at -80°C. These serum samples were used in immunogenicity studies including Western-blotting, ELISA and virus-serum neutralization assay.

## **Antibody Specificity Validation**

Immunogenicity of our designed immunogens was first evaluated by Western-blotting assay to see whether there were antigen specific antibodies elicited in the immunized guinea pigs. The data from these experiments is shown in the **Figure 12**. We blotted each antigen with the final bleed serum collected from all groups. All sample groups inoculated with their assigned antigens (OD1, OD2 and OD3) showed related binding

activity. And the clear bands indicated our purified antigens remained stable in the storage condition of -80°C. In addition, all groups inoculated with any antigens (except for the group immunized with adjuvant only) showed that their serum not only contains antibodies against gp120 outer domain. Instead, as we predicted, no guinea pigs elicited outer domain specific antibodies when were just inoculated with adjuvant (only some results are shown here). These results proved the inoculation of our designed immunogens successfully induced outer domain specific antibodies.

## Antibody Titer Determination

In order to further evaluate the immunogenicity of our designed antigens, typically, to compare the immunogenicity differences between the three antigens, an ELISA assay was conducted and the results compared the total outer domain-specific antibodies induced by three antigens in guinea pigs. As previously mentioned, “pre-bleed”, “test bleed” and “final bleed” refers to serum samples collected from day 0, day 35 and day 63 respectively. **Figure 13**, shows the data combining ELISA panels from the pre-bleed, test bleed, and final bleed of four groups. The three panels on the Y-axis represent the pre-bleed, test bleed and final bleed antibody titers compared in four groups, whereas the four panels on the X-axis represent the antibody titers in four groups immunized with OD1, OD2, OD3, ADJ (adjuvant only). In general, before inoculation, of all groups, no guinea pig individuals contain outer domain specific antibodies, reflected by the antibody titers of pre-bleed panels, which were all negative background. However, after antigen inoculation and two boosts followed at day 14 and day 21, test

bleed serum from day 35 showed evidence of outer domain specific antibody generation. There were no significant individual difference among all 12 guinea pigs in group one, two and three, except for one case in the OD3-immunized group, in which one guinea pig seemed to generate lower titers of outer domain specific antibodies. The difference is significant at the serum dilution ratio of 1:100 to 1:2500. However, when we looked at the final bleed panels, we found that although the individual differences are still not significant in all groups, the guinea pig in OD3-immunized group who showed a lower antibody titer at day 35 recovered with higher amounts of antibody, making the individual difference less significant at day 63 than day 35. This increase in antibody titer is supported from the third boost in day 49, reflected in the OD3 immunized test bleed and final bleed panels, in which the antibody titers (absorbance at 450nm) increased from 1.5 to 2.2. Moreover, antibody titers are slightly increased in guinea pigs at day 63 than that at day 35, although the difference in OD3 immunized group is less significant. This result suggests that the third antigen boost provided some limited help in recruiting antigen-specific antibodies. Lastly, no HIV-1-1 outer domain specific antibodies were found in all adjuvant immunized guinea pigs. It is therefore confirmed again that antibodies found in the immunized guinea pigs were specifically induced by the immunogens of the outer domains (OD1, OD2 and OD3).

Another important analysis is to evaluate the antigenic differences among these three immunogens, and the differences can be more easily assessed in **Figure 14**, which reorganizes the ELISA data in another way, represented as bar charts. From this figure

we can tell that all three antigens induced a significant amount of antibodies compared to adjuvant immunized groups. This figure however cannot detect any significant differences between the OD1 and OD2 groups, but there is a significant difference when comparing the antibody titers between OD3 and the other two groups, from which we found that at 1:100 serum dilution ratio, the antibody titers of OD3-immunized groups, reflected by absorbance at 450 nm, showed an average value of 2.87 in the test bleed panel and 3.06 in the final bleed panel. Similar results were obtained at a lower serum dilution ratio of 1:500 and 1:2500, suggesting that this assay has some value to distinguish the antigenic differences between the three antigens, reflecting a higher amount of the total antigen specific antibody titers in the OD3 immunized group.

Taken together, these results reveal that: 1) there are very small individual differences in the antibodies elicited between the four guinea pigs in the same groups; 2) there is some effect of inducing a higher amount of antigen-specific antibodies from the third boost in OD1 and OD2 group, but no significant increase in OD3 group; 3) there is no significant difference in the total antigen-specific antibodies between the OD1 and OD2 groups, but antigen specific antibody titers are significantly higher in OD3 groups. However, there is a need to further investigate the antigenicity difference of the three antigens and thus the virus-serum neutralization assay is needed to compare the differences of their antigen-specific broad neutralizing antibody titers.

## Serum Neutralization against Diverse Viral Strains

In order to evaluate the immune response to the antigens resulting in antigen-specific HIV-1-1 neutralizing antibodies, we investigated the neutralizing abilities of serum from guinea pigs in all groups. Six HIV-1-1 isolates from subtype B and C including five primary isolates YU2, ADA, 89.6, 1084i, JRFL and one lab-adapted strain HXBc2 were evaluated in this assay. **Figure 15** compares the neutralizing activity of the pre-bleed serum, test bleed serum and final bleed serum among the three antigens. Each panel line on the X-axis represents one HIV-1-1 isolate involved in the neutralizing experiment. Clearly, the figure points to a conclusion that, compared to the serum collected in day 0, all antigen-immunized guinea pigs at day 35 and day 63 generated neutralizing antibodies against all six HIV-1-1 isolates. However, the neutralizing activities differ among the three antigen-immunized groups. To be specific, for all panels, OD2 and OD3 are more capable of inducing neutralizing antibodies against all six HIV-1-1 isolates than OD1. Overall, there is no significant percentage of neutralization difference between the test bleed and final bleed serum in all antigen-immunized groups, for any virus isolates. The only exception was the isolate 89.6 neutralized by OD3 serum, in which the final bleed serum remains stronger protection at all four serum dilution ratio. Previous ELISA data showing no significant difference in the total antibody titer in all groups may explain the insignificance of neutralizing antibody titers between the test and final bleed sera in these groups. The exception of 89.6 being neutralized at a higher percentage by OD3 final bleed serum than test bleed

serum may be explained by the fact that 89.6 isolate is somehow more sensitive for serum neutralizing when the total antigen specific antibody titers are at a higher amount. The fact that overall there is no significant difference between test bleed and final bleed serum neutralizing activities suggests that during the guinea pig immune response, though the total antigen-specific antibodies were continuously recruited after the third boost, the level of broad neutralizing antigen-specific antibodies remained the same, indicating either there is an antigenic limit for these antigens to induce neutralizing antibodies, or the total neutralizing antibodies are all induced at day 35 so that there are few remaining neutralizing antibodies induced by the third boost.

To compare the biological activity of the neutralizing antibodies in guinea pig serum induced by our designed antigen OD3, to the wild type OD1 and two-disulfide-bond OD2, the original data is reorganized into **Figure 16**. Each panel on the X-axis again represents one HIV-1-1 isolate neutralization, but each of the 6 panels on the Y-axis are serum from day 0, day 35 or day 63. As negative controls, pre-bleed sera is not supposed to have neutralizing antibodies, which is clearly represented by the day 0 panels, showing that there are no HIV-1-1 neutralizing antibodies that exist before antigen inoculation. Furthermore, serum from day 35 and day 63 show all three antigens elicited neutralizing antibodies, but the neutralizing activities are different. Generally, OD1 induced less neutralizing antibodies, and the percentage of viruses neutralized by OD1-immunized serum is only slightly larger than that of adjuvant alone. Although this is not necessarily true in the cases of YU2 and JRFL, the overall performance of OD1



was not satisfactory. Serum from OD2-immunized guinea pigs showed significantly stronger protection than the OD1 group. In **Figure 16** we show that OD2-immunized sera from both day 35 and day 63 neutralized a higher percentage of viruses in all HIV-1-1 isolates than OD1. Furthermore, OD2-immunized sera from day 63 reduced 1084i and JRFL viral infection by 81% and 79% respectively, compared to the OD1 group, which reduced infection by 62% and 43% respectively. These results suggest that the disulfide bonds formation, to some extent stabilized the immunogen to become more immunogenic in terms of inducing neutralizing antibodies. Moreover, serum from the OD3-immunized group contains more neutralizing antibodies than the OD2 group. The data in **Figure 16** shows that OD3-induced neutralizing antibodies were more potent than OD2 in neutralizing five of the six HIV-1-1 isolates: 89.6, ADA, HXBc2, YU2 and 1084i. In the case of the HIV-1-1 isolate JRFL although there is no significant difference between OD2 and OD3 in antibody titers, the percentages of viral reduction were quite comparable.

Finally, data in **Figure 17** compares the neutralizing activity of OD3-immunized serum in six HIV-1-1 isolates and one non-HIV-1-1 virus strain AMLV (Amphotropic Murine Leukemia Virus). The non-HIV-1-1 AMLV served as a good negative control, reflecting that the neutralizing antibodies are HIV-1-1 specific since they did not neutralize AMLV. Among these HIV-1-1 isolates, the dual-tropic 89.6 is the most vulnerable strain to OD3-induced antibody neutralization, followed by the lab-adapted strain HXBc2. On the other hand, YU2, and ADA are relatively more challenging for

neutralization. However, all of these isolates are neutralized by OD3-immunized serum at 70% or higher at the 1:20 serum dilution ratio, suggesting OD3 to be a valuable immunogen for further characterization.

Taken together, the virus neutralization results showed that the disulfide-stabilized OD2 immunogen had better neutralization activities than the wild-type outer domain (OD1), and the S375W-stabilized OD3 had the best neutralizing activity against various HIV-1-1 isolates. The S375W mutation serves a role in better stabilizing the immunogen in a way that is favorable to elicit potent neutralizing antibodies against heterologous HIV-1-1 isolates. This example of design using structural information demonstrated that structural-based modification for immunogen stabilization is a better strategy for improving vaccine immunogen design.

## Chapter 5: Summary

Our designed OD3 was purified at an optimal purity, and validated by Western blots. The secondary structures of all three immunogens were evaluated and the designed OD3 was found to be more organized in the secondary structure. The immunogenicity of the OD3 immunogen designed with the S375W mutation and two disulfide bonds has been evaluated clearly in guinea pigs. Compared to OD1 and OD2, OD3 induces a higher level of both total antigen-specific antibodies and neutralizing antigen-specific antibodies. Our experimental results suggest that a structural-based vaccine design is a better strategy compared to conventional vaccine designs using killed or subunit particles without any structural modifications. Due to the breadth limitedness of this project, there are some imperfections in applying our designed OD3 vaccine. First, although OD3 induces both total and neutralizing antigen-specific antibodies higher than OD1 and OD2, and even though these antibodies are capable of neutralizing diverse HIV-1-1 isolates, these antibodies did not reach 100% neutralization against any HIV-1-1 isolate at the lowest serum dilution ratio. Second, although the ELISA assay showed that there is a significant amount of antigen-specific antibodies even when the serum was diluted to 1:2500, our serum lost its neutralizing activity at a serum dilution lower than 1:180. Taken together, this outer domain stabilization largely improved the outer domain's immunogenicity compared to the native wild type, however more efforts are needed to make this immunogen to become applicable. Other structural-based immunogen designs may work synergistically with our strategy. For

example, structural-based designs targeting the MPER domain of gp41 may work together with the outer domain in inducing potent broad neutralizing antibodies. Also, novel adjuvants and vaccine delivery methods may help to improve the immunogenicity of the structural-based designed vaccines and finally provide us a potent vaccine that is capable of inducing both T cell and humoral immunity to eliminate HIV-1-1.

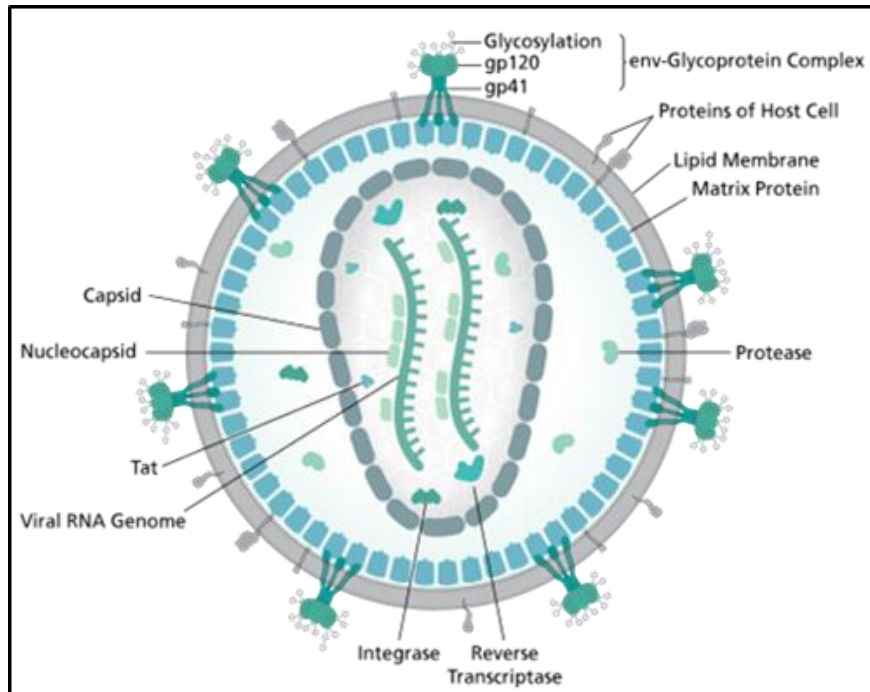
## Tables and Figures

**Table 1:** Compositions of the secondary structures in OD1, OD2 and OD3. [\[Back to](#)

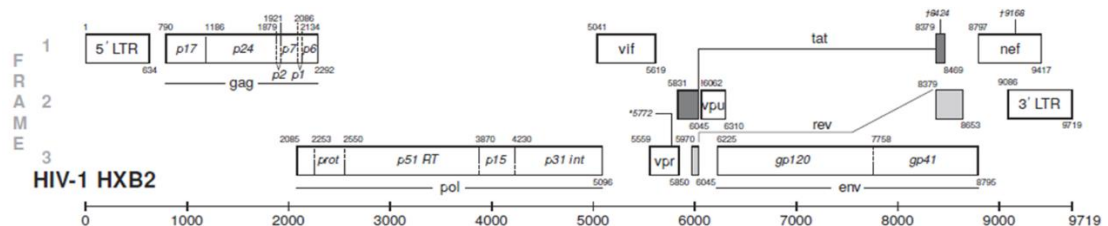
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Secondary Structures	Immunogens		
	OD1 (%)	OD2 (%)	OD3 (%)
Helix1	14	16	17
Helix2	8	9	8
Strand1	21	21	21
Strand2	14	15	17
Turns	22	23	25
Unordered	21	16	12

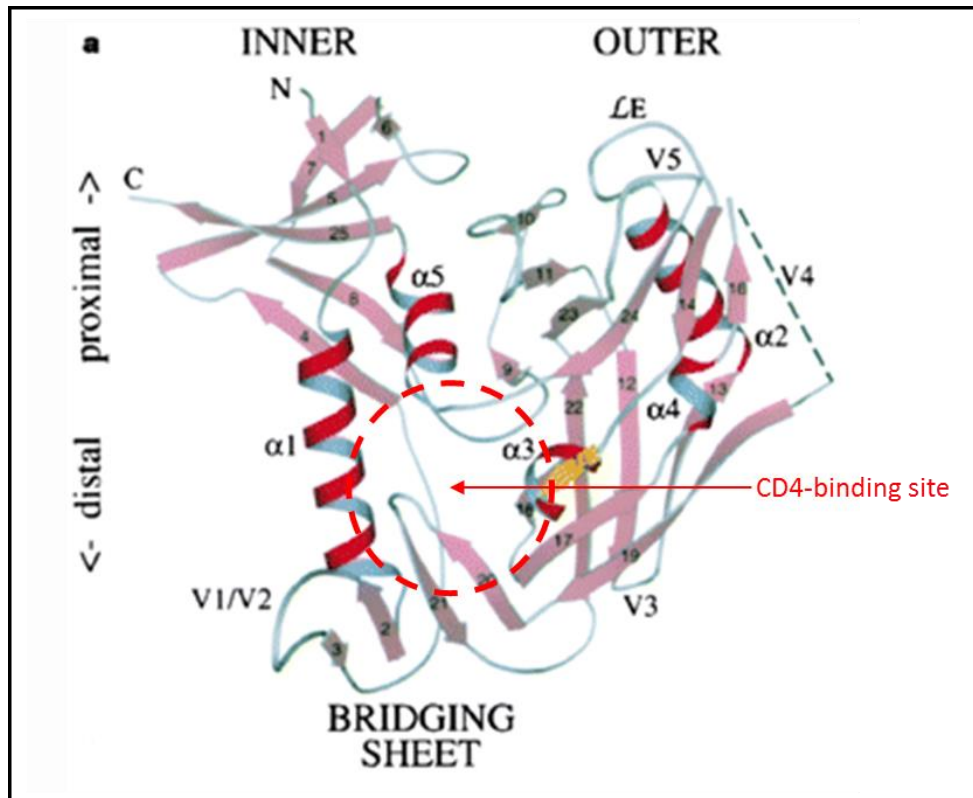
**Figure 1:** Diagram of an HIV-1 virion structure. (Adapted from the website of Wikipedia, the free encyclopedia) [\[Back to text\]](#)



**Figure 2:** Schematic presentation of HIV-1-1 genomic organization. (Adapted from HIV-1 Sequence Compendium 2015). [\[Back to text\]](#)

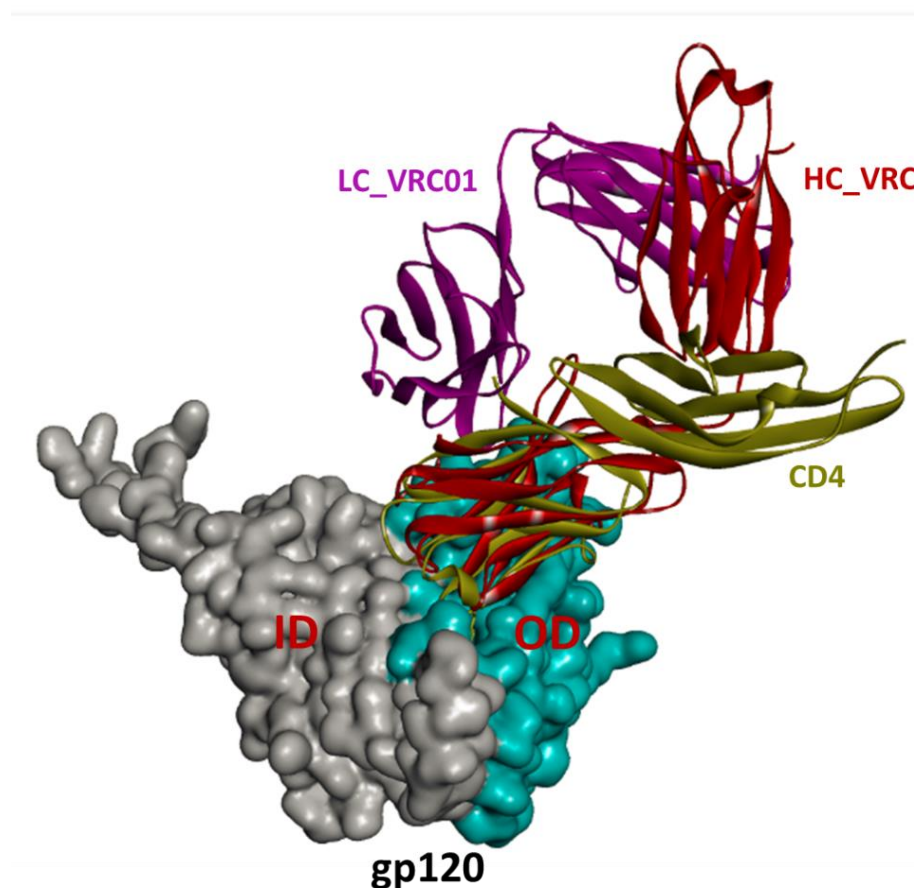


**Figure 3:** The gp120 core structure: Inner Domain (ID), Outer Domain (OD) and Bridging Sheet (BS). [\[Back to text\]](#)



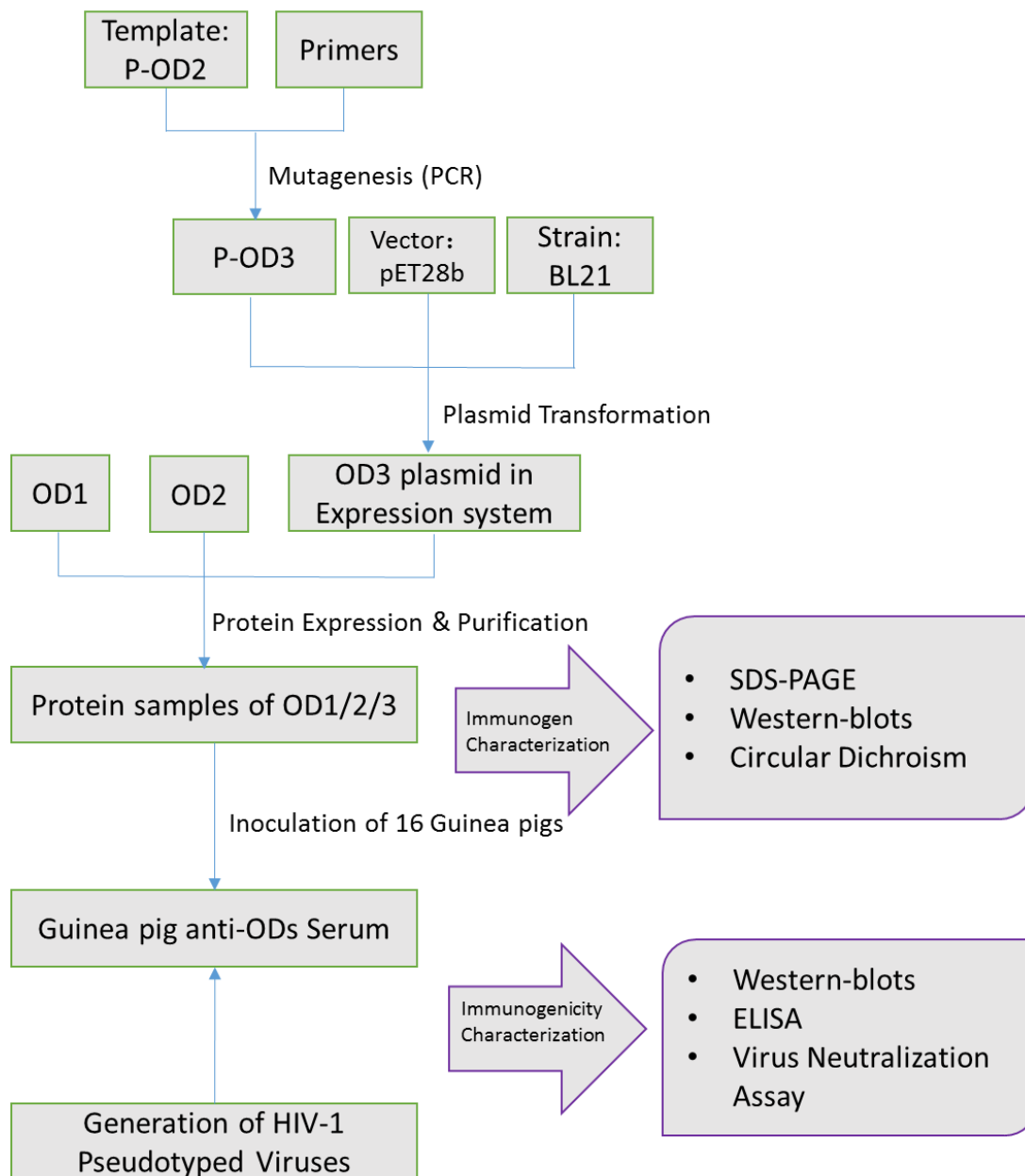
Notes: The CD4-binding site (CD4-BS) is located at the junction of three domains indicated by the red circle. The picture was adapted from Kwong et al. (Nature. 1998 Jun 18; 393(6686):648-59).

**Figure 4:** Superimposed CD4 receptor and VRC01 antibody bindings to the gp120 of HIV-1-1. [\[Back to text\]](#)



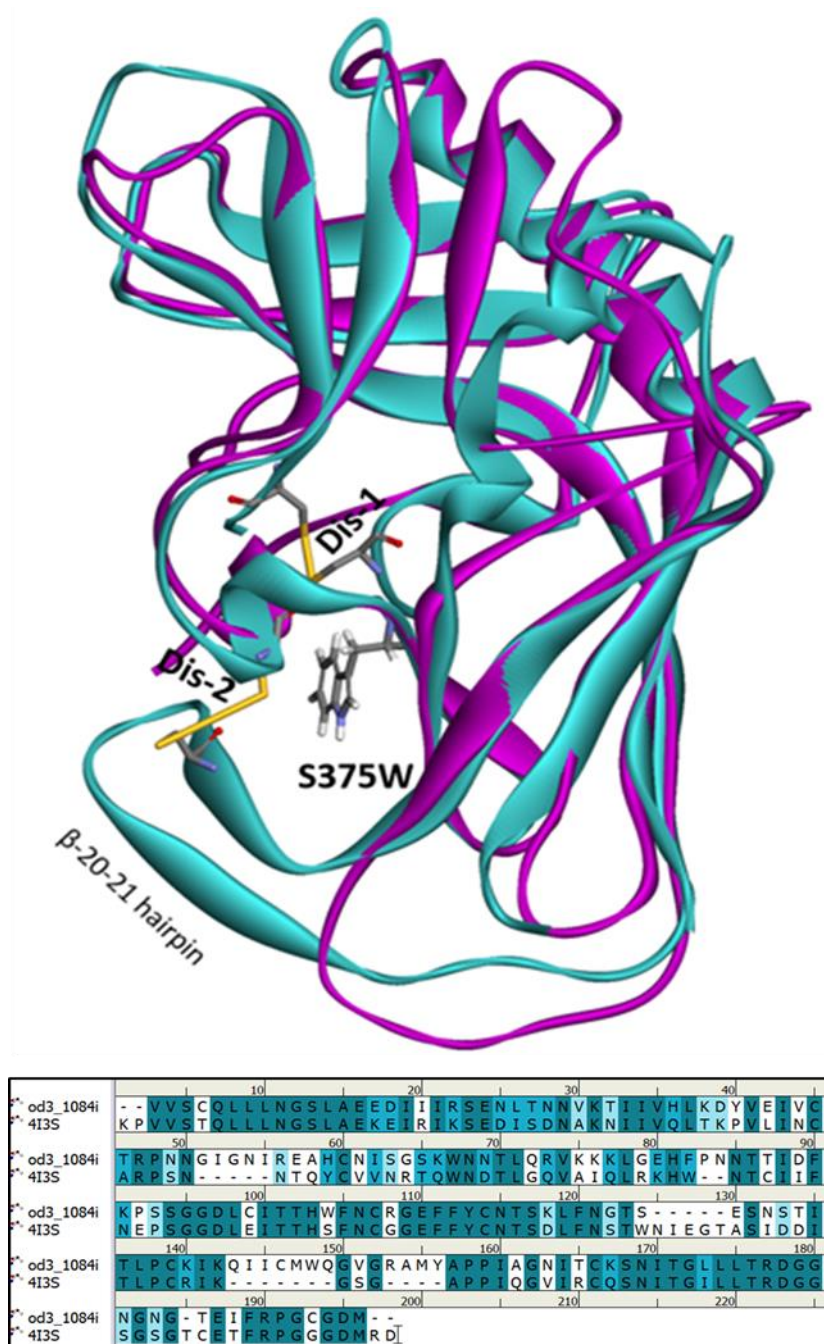
Notes: The gp120 is depicted in a surface model. The gp120 inner domain (ID) is in gray, and outer domain (OD) in cyan. The HIV-1 receptor CD4 (color yellow) and neutralizing antibody VRC01 are depicted in ribbon strips (colored red for the heavy-chain (HC), magenta for the Light-chain (LC)).

**Figure 5:** Schematic presentation of the general experimental design. P-OD2, plasmid OD2; P-OD3, plasmid\_OD3. [\[Back to text\]](#)





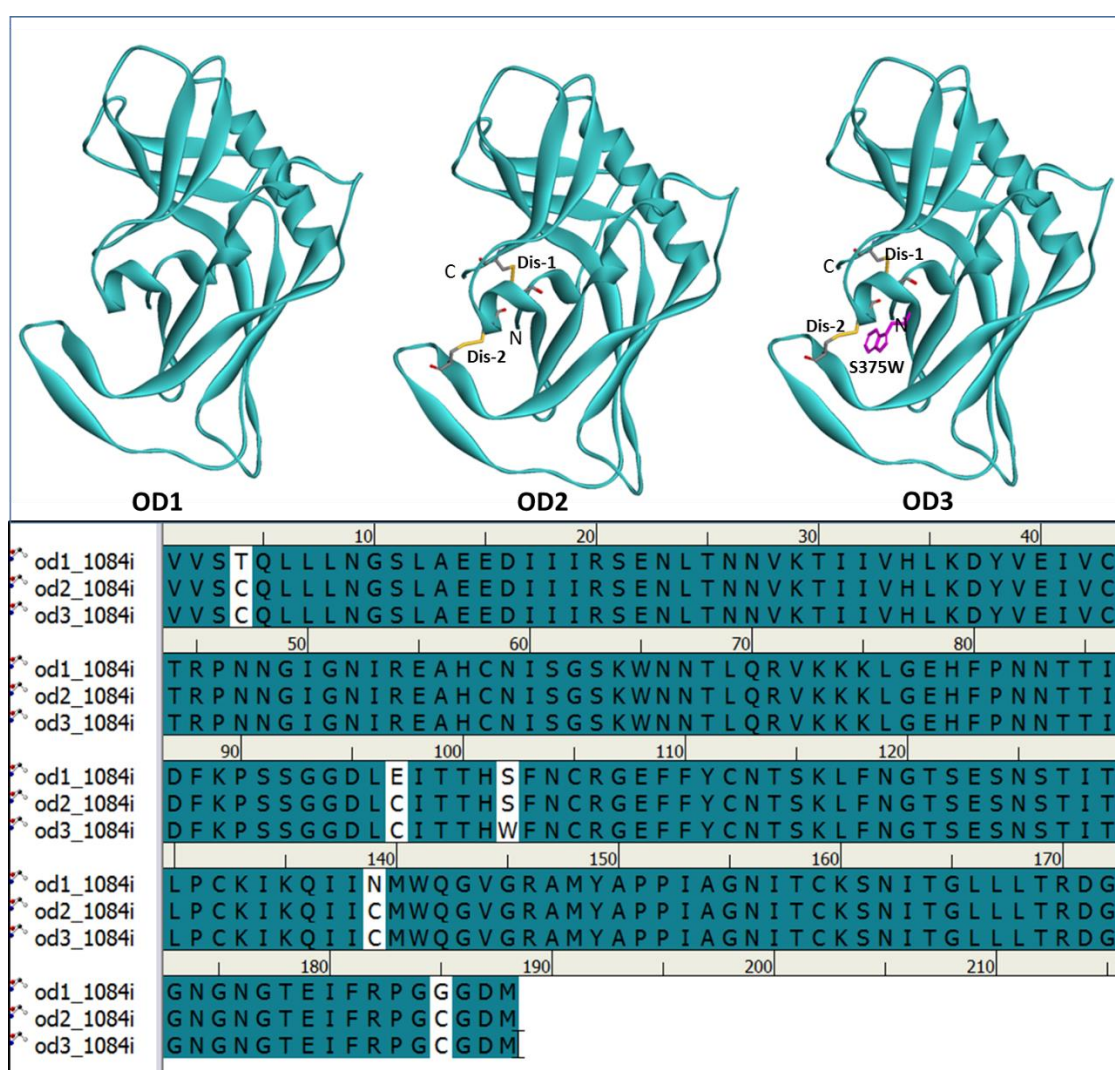
**Figure 6:** Superimposed OD3 (color cyan) and the wild-type outer domain OD1 from HIV-1-1 strain HXBc2, PDB # 4I3S, color purple) structure. [\[Back to text\]](#)



Notes: 4I3S refers to the sequence of HXBc2 outer domain. Od3\_1084i refers to the OD3 gene mutated from 1084i outer domain. Dis-1: disulfide bond-1, was created to stabilize the C-N termini, and Dis-2: disulfide bond-2, was created to stabilize the

beta 20-21 hairpin. The OD1 structure is from published data on the HXBc2 outer domain, and OD3 structure was modeled in Discovery Studio Client 3.5 based on the outer domain sequence of 1084i isolate. [Back to text]

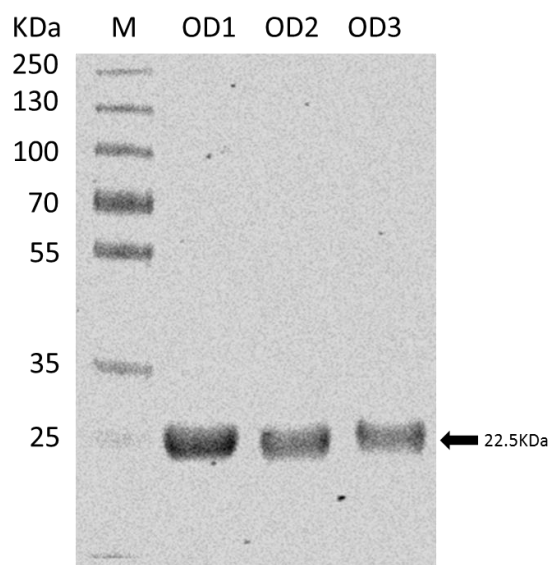
**Figure 7:** 3D modelling of the three designed immunogens and their amino acid changes. [Back to text]



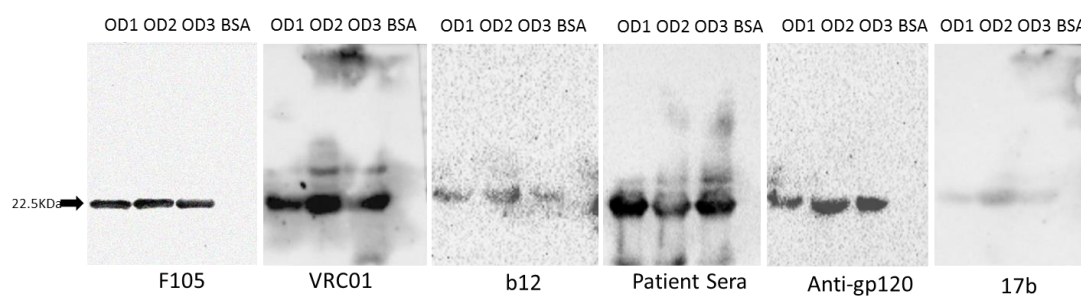
Notes: The outer domain was cut-off from the position 254 of full length gp120 molecule, which corresponds to the position 1 of the outer-domain numbering in this diagram. Based on the full length sequence of gp120 standard numbering system

(209), these mutations in this diagram are also indicated as follows: Dis 1: Dis 1: disulfide bond-1, T/C257-G/C472 (T/C4-GC185 in this table); Dis 2: disulfide bond-2, E/C370-N/C425 (E/C97-N/C139); S375W (S/W102).

**Figure 8:** Antigen purification represented by SDS-PAGE. [\[Back to text\]](#)

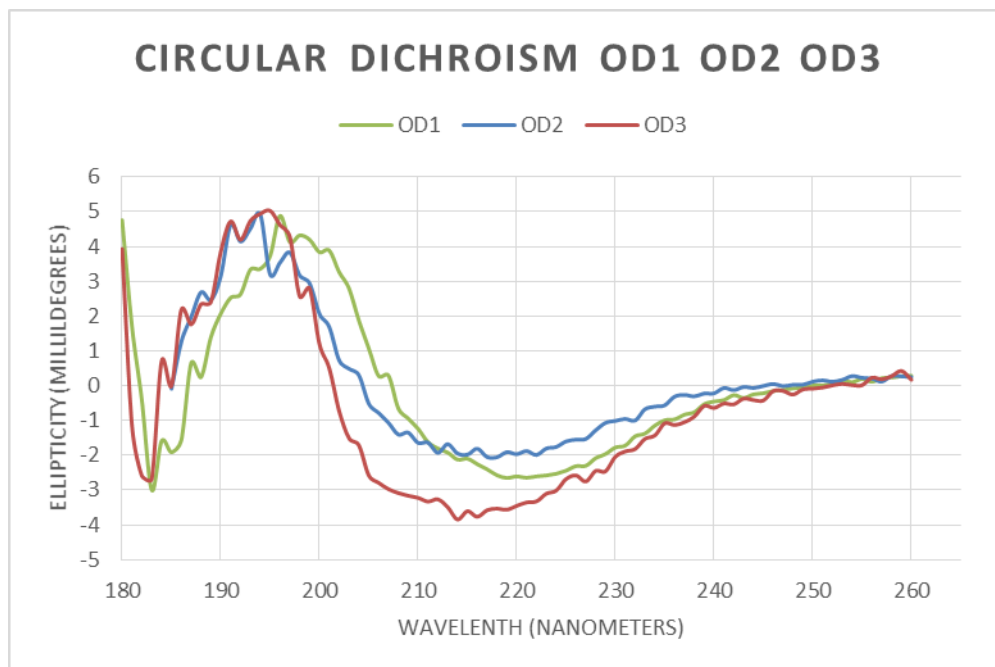


**Figure 9:** Characterization of the three immunogens by Western blot. [\[Back to text\]](#)



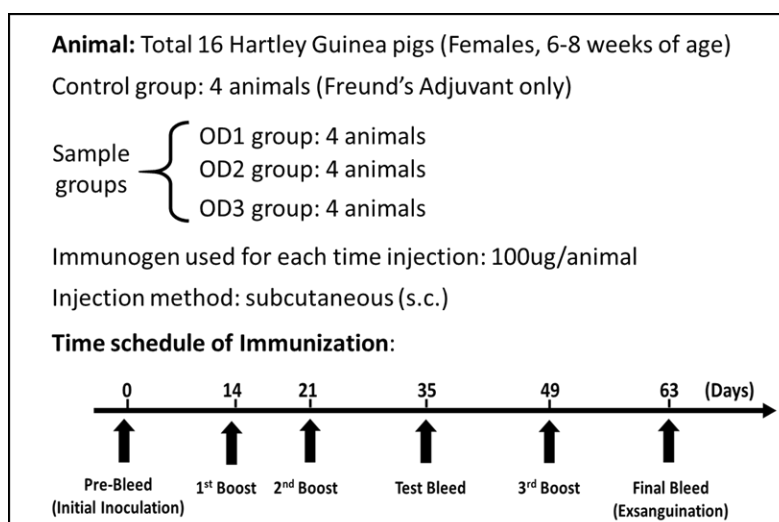
Notes: After SDS-PAGE and membrane transfer, protein samples of OD1, OD2, OD3 and BSA were probed with pooled serum from HIV-1-1 positive patients, anti-gp120 polyclonal antibody, CD4-BS monoclonal antibodies VRC01, F105, b12 or b12, and CD4-induced monoclonal antibody 17b.

**Figure 10:** Circular dichroism spectra of three immunogens with representative secondary structures. [\[Back to text\]](#)

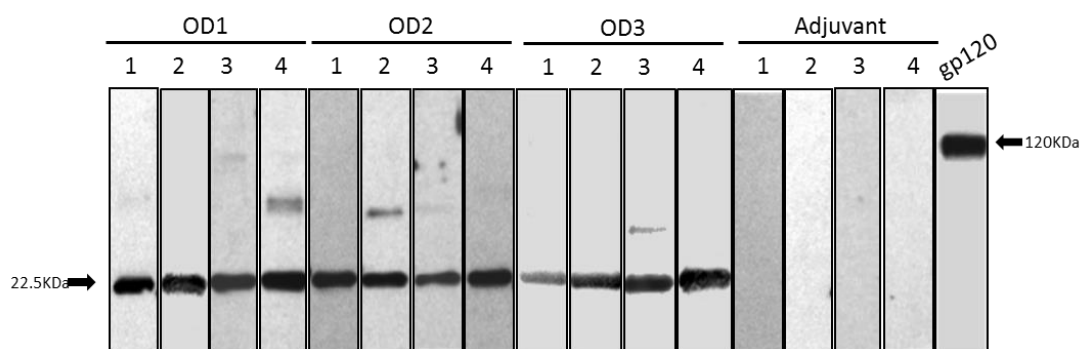


Notes: CD spectra of OD1 (black), OD2 (orange), and OD3 (green) at pH 8.0, room temperature. Spectral data analysis was performed by Dr. Javier Seravalli in the UNL protein core facility.

**Figure 11:** Guinea pigs immunization protocol. [\[Back to text\]](#)



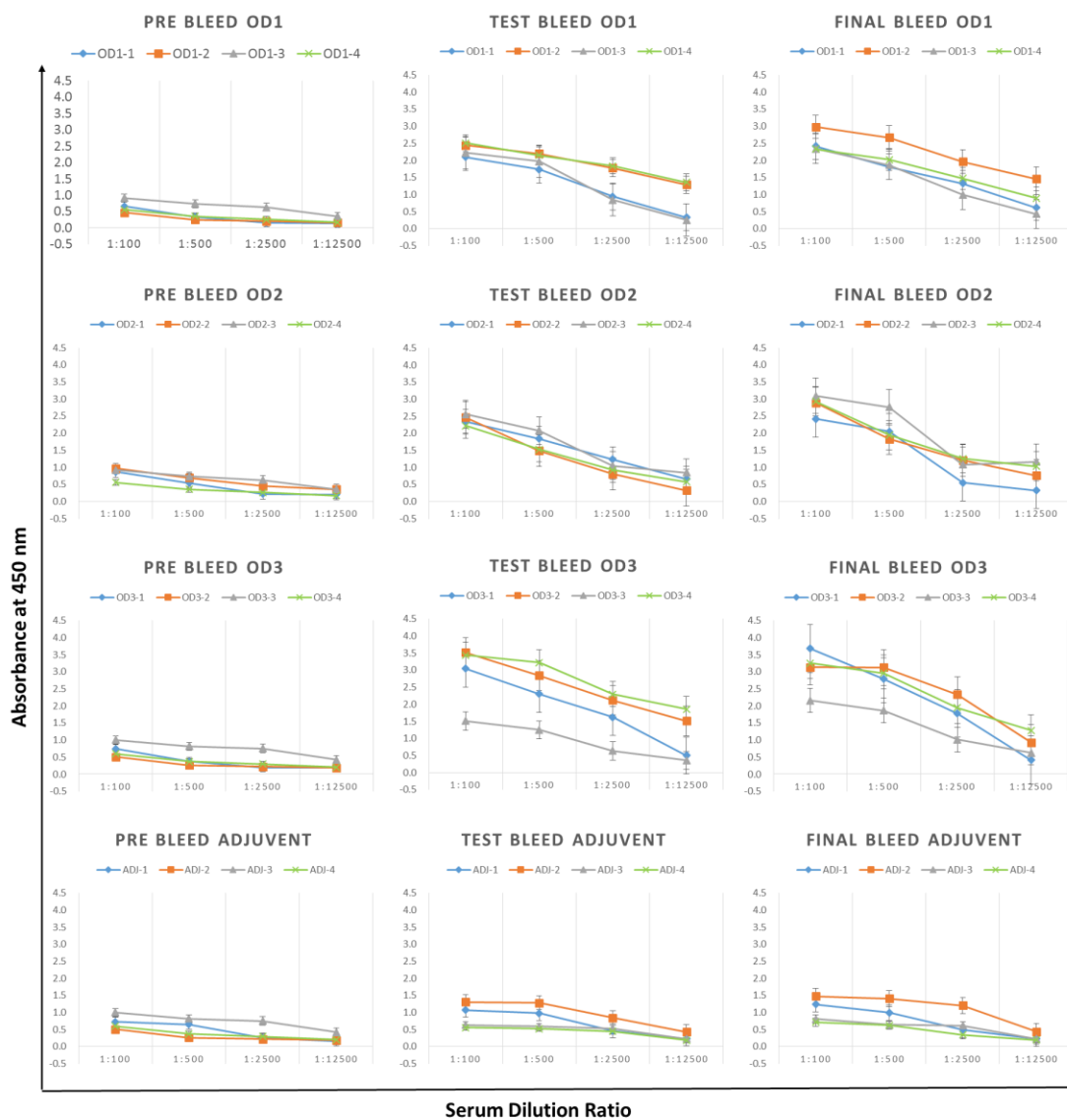
**Figure 12:** Western blot analysis of antisera from immunized guinea pigs. [\[Back to text\]](#)



Notes: Each band represents one independent Western blotting experiment. 4 serum samples from each group were used as the primary antibody against the assigned immunogens. All antigen-immunized groups showed antibody-binding activity to gp120, and here only one is shown as a positive control. All adjuvant-immunized group showed no antibody binding activity to OD1, OD2, OD3 and BSA (shows in Adjuvant 1, 2, 3, and 4 respectively).

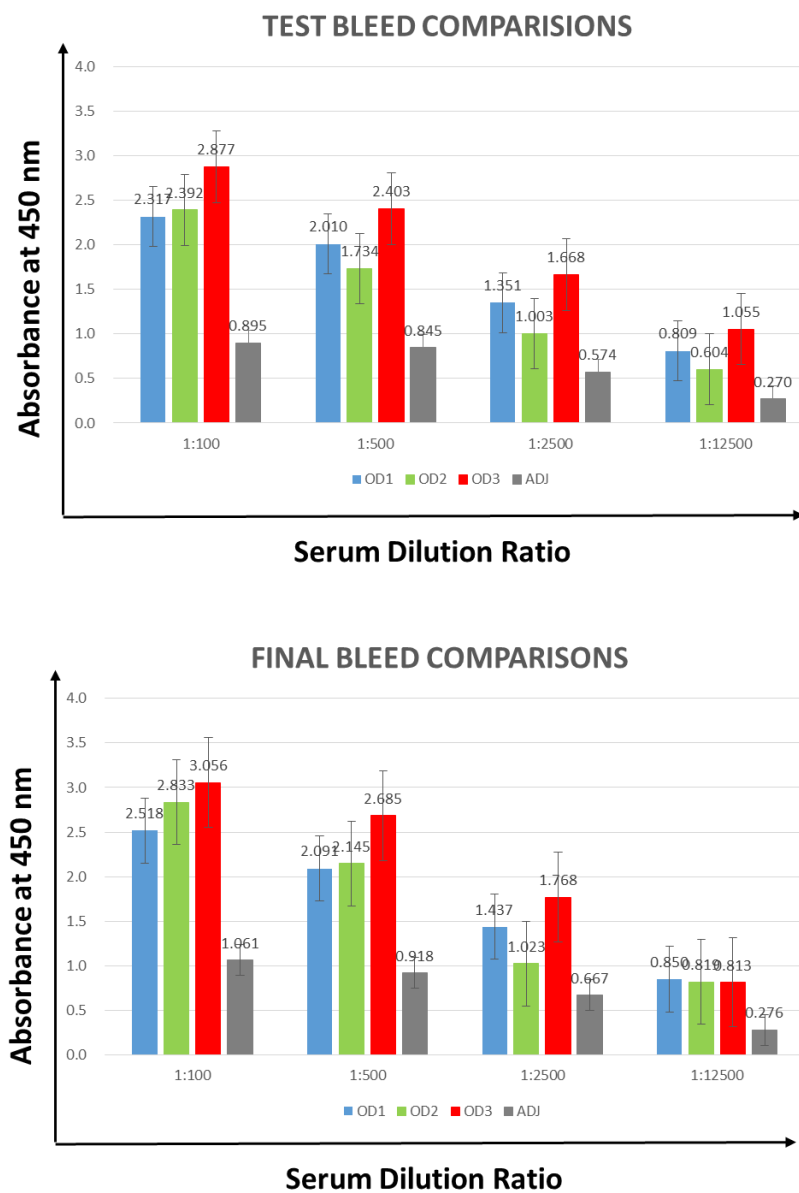


**Figure 13:** ELISA analysis of antisera from immunized guinea pigs and their individual antigenic differences. [\[Back to text\]](#)



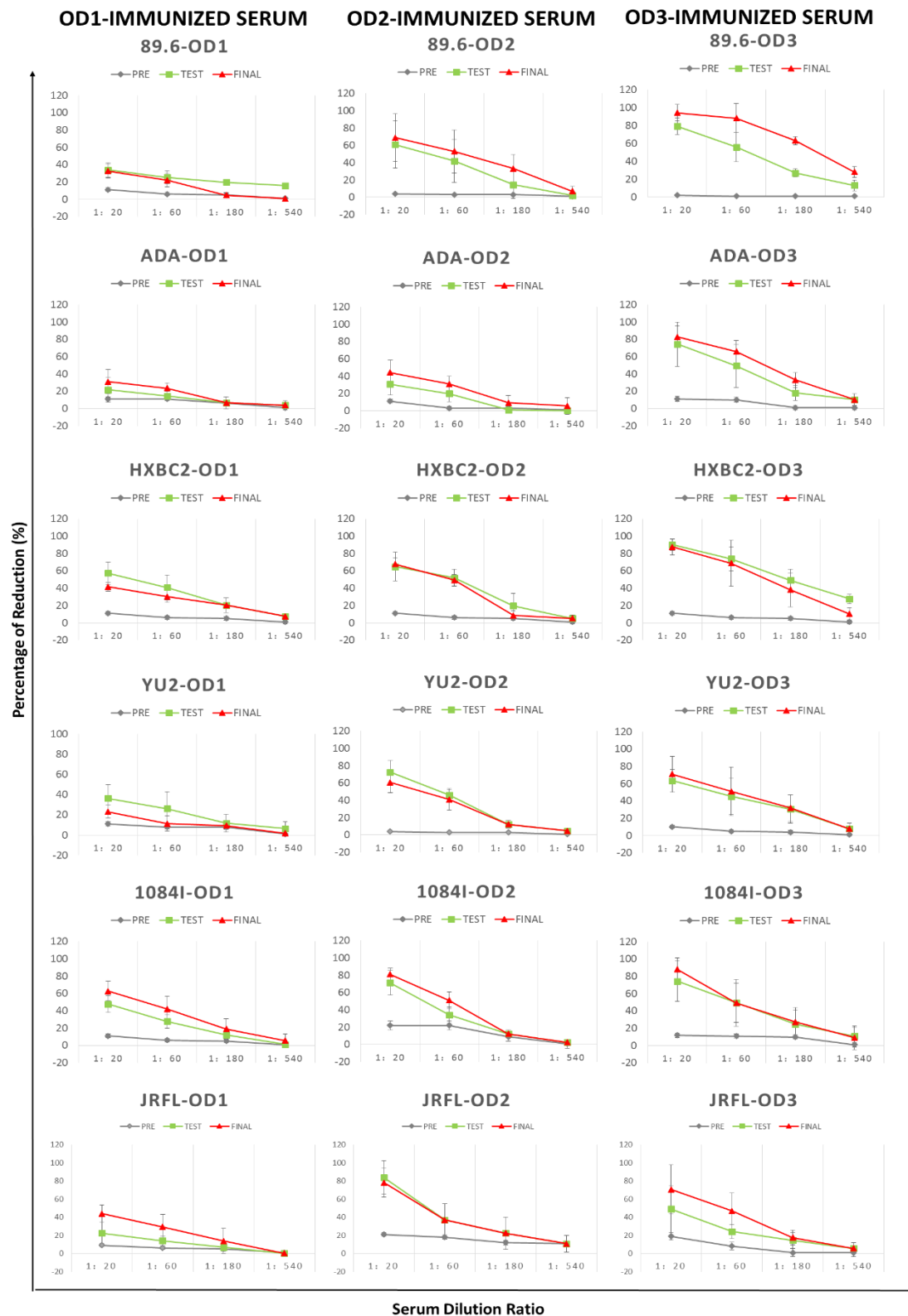
Notes: 96-well plates were coated with three antigens, then were probed with dilutions of pre-bleed serum (day 0), test bleed serum (day 35, or final bleed serum (day 63). Experiments were performed in triplicate and figures represent an average data of multiple experiments.

**Figure 14:** ELISA analysis of antisera from immunized guinea pigs comparing antigenic differences among three immunogens. [\[Back to text\]](#)



**Figure 15:** Virus neutralization assay of antisera from immunized guinea pigs

comparing serum neutralizing activities at different time points. [\[Back to text\]](#)

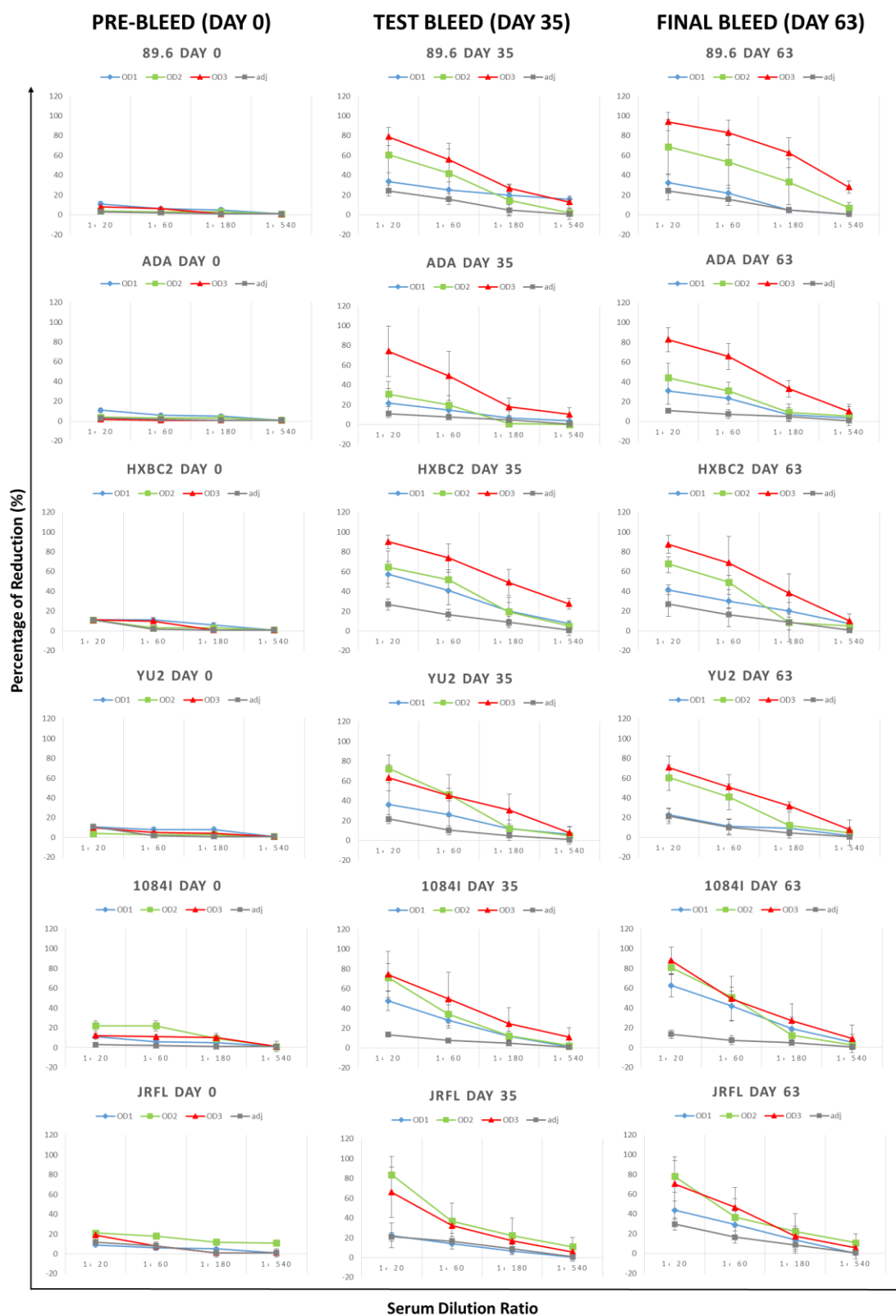




Notes: Neutralization assays of antisera from four groups of antigen-immunized or adjuvant-immunized groups. Neutralization activities were represented as the percentage (%) of reduction in relative luciferase units compared to wells with virus and cells only. Experiments were performed in triplicate and figures represent an average data of multiple experiments.

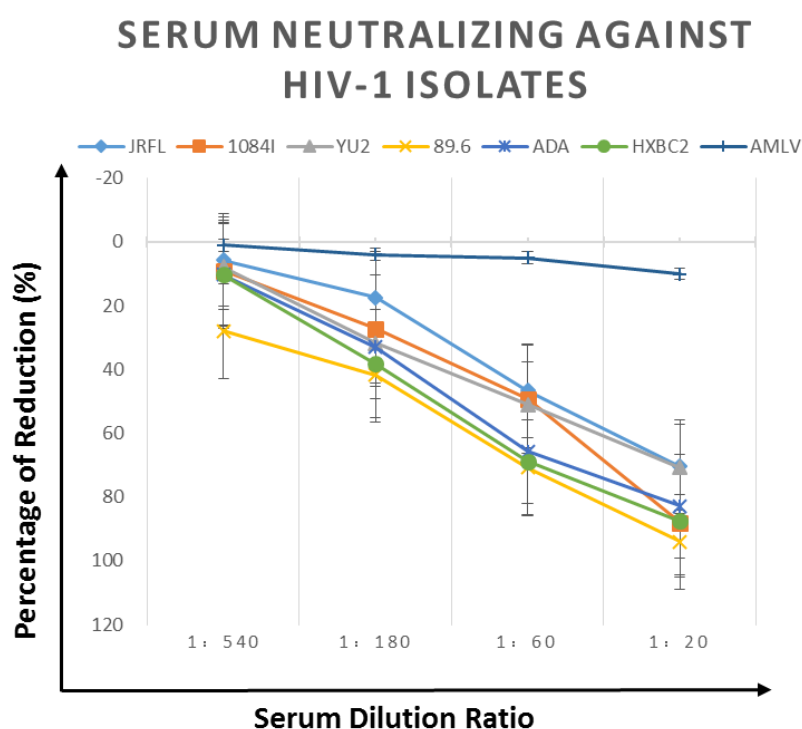
**Figure 16:** Virus neutralization assay of antisera from immunized guinea pigs

comparing serum neutralizing activities of three immunogens. [\[Back to text\]](#)



Notes: Neutralization assays of antisera from four groups of antigen-immunized or adjuvant-immunized groups. Neutralization activities were represented as percentage (%) of reduction in relative luciferase units compared to wells with virus and cells only. Experiments were performed in triplicates and figures represent an average data of multiple experiments.

**Figure 17:** Virus neutralization assay of antisera from OD3-immunized guinea pigs comparing serum neutralizing activities against diverse HIV-1-1 isolates and non-HIV-1-1 isolate. [\[Back to text\]](#)



Notes: Neutralization assays of antisera from four groups of antigen-immunized or adjuvant-immunized groups. Neutralization activities were represented as percentage (%) of reduction in relative luciferase units compared to wells with virus and cells

only. Experiments were performed in triplicates and figures represent an average data of multiple experiments.

## References

1. Gottlieb MS. 2006. Pneumocystis pneumonia--Los Angeles. 1981. *Am J Public Health* 96: 980-1; discussion 2-3
2. Friedman-Kien AE. 1981. Disseminated Kaposi's sarcoma syndrome in young homosexual men. *J Am Acad Dermatol* 5: 468-71
3. Hymes KB, Greene JB, Marcus A, William DC, Cheung T, Prose NS, Ballard H, Laubenstein LJ. 1981. Kaposi's Sarcoma in Homosexual Men - a Report of 8 Cases. *Lancet* 2: 598-600
4. Gallo RC. 2006. A reflection on HIV-1/AIDS research after 25 years. *Retrovirology* 3
5. Fact sheet 2015 | UNAIDS  
<http://www.unaids.org/en/resources/campaigns/HowAIDSchangedeverything/factsheet>  
 Retrieved February 4, 2016
6. Experts: MH17 Victims Could Have Remained Conscious During Fall | TIME  
<http://time.com/3028548/mh17-malaysia-airlines-victims-ukraine/> Retrieved March 21, 2016
7. "HIV-1 Classification: CDC and WHO Staging Systems". Guide for HIV-1/AIDS Clinical Care. AIDS Education and Training Center Program. Retrieved March 21, 2016
8. UNAIDS, WHO (December 2007). "2007 AIDS epidemic update" (PDF). p. 10. Retrieved February 03, 2016.
9. International Committee on Taxonomy of Viruses (2002). "61.0.6. Lentivirus". National Institutes of Health. Retrieved February 28, 2016
10. International Committee on Taxonomy of Viruses (2002). "61.0.6. Lentivirus". National Institutes of Health. Retrieved February 28, 2006.
11. International Committee on Taxonomy of Viruses (2002). "61. Retroviridae". National Institutes of Health. Retrieved February 28, 2006.
12. Gilbert PB, McKeague IW, Eisen G, Mullins C, Gueye NA, Mboup S, Kanki PJ. 2003. Comparison of HIV-1-1 and HIV-1-2 infectivity from a prospective cohort study in Senegal. *Stat Med* 22: 573-93
13. Douek DC, Roederer M, Koup RA. 2009. Emerging concepts in the immunopathogenesis of AIDS. *Annu Rev Med* 60: 471-84

14. Zhu T, Wang N, Carr A, Nam DS, Moor-Jankowski R, Cooper DA, Ho DD. 1996. Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission. *J Virol* 70: 3098-107
15. Markowitz, edited by William N. Rom ; associate editor, Steven B. (2007). Environmental and occupational medicine (4th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins. p. 745. ISBN 978-0-7817-6299-1.
16. van der Kuyl AC, Cornelissen M. 2007. Identifying HIV-1-1 dual infections. *Retrovirology* 4: 67
17. Markowitz, edited by William N. Rom ; associate editor, Steven B. (2007). Environmental and occupational medicine (4th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins. p. 745. ISBN 978-0-7817-6299-1.
18. Boily MC, Baggaley RF, Wang L, Masse B, White RG, Hayes RJ, Alary M. 2009. Heterosexual risk of HIV-1-1 infection per sexual act: systematic review and meta-analysis of observational studies. *Lancet Infect Dis* 9: 118-29
19. Yu MK, Vajdy M. 2010. Mucosal HIV-1 transmission and vaccination strategies through oral compared with vaginal and rectal routes. *Expert Opinion on Biological Therapy* 10: 1181-95
20. Ng BE, Butler LM, Horvath T, Rutherford GW. 2011. Population-based biomedical sexually transmitted infection control interventions for reducing HIV-1 infection. *Cochrane Database of Systematic Reviews*
21. Crosby R, Bounse S. 2012. Condom effectiveness: where are we now? *Sex Health* 9: 10-7
22. "Will I need a blood transfusion?" (PDF). NHS patient information. National Health Services. 2011. Retrieved February 29, 2016
23. Markowitz, edited by William N. Rom ; associate editor, Steven B. (2007). Environmental and occupational medicine (4th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins. p. 745. ISBN 978-0-7817-6299-1.
24. "Blood safety ... for too few". WHO. 2001. ArchHIV-1ed from the original on February 17, 2016.
25. Coutoudis A, Kwaan L, Thomson M. 2010. Prevention of vertical transmission of HIV-1-1 in resource-limited settings. *Expert Rev Anti Infect Ther* 8: 1163-75
26. McGovern SL, Caselli E, Grigorieff N, Shoichet BK. 2002. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J Med Chem* 45: 1712-22
27. Compared with overview in: Fisher, Bruce; Harvey, Richard P.; Champe, Pamela C. (2007). Lippincott's Illustrated Reviews: Microbiology (Lippincott's Illustrated Reviews Series). Hagerstown, MD: Lippincott Williams & Wilkins. ISBN 0-7817-8215-5. Page 3
28. Various (2008). HIV-1 Sequence Compendium 2008 Introduction (PDF). Retrieved March 21, 2016
29. National Institute of Health (June 17, 1998). "Crystal structure of key HIV-1 protein reveals new prevention, treatment targets" (Press release). ArchHIV-1ed from the original on February 19, 2006. Retrieved February 14, 2016
30. Klein JS, Webster A, Gnanapragasam PNP, Galimidi RP, Bjorkman PJ. 2010. A dimeric form of the HIV-1-1 antibody 2G12 elicits potent antibody-dependent cellular cytotoxicity (vol 24, pg 1633, 2010). *Aids* 24: 2601-
31. Chan DC, Fass D, Berger JM, Kim PS. 1997. Core structure of gp41 from the HIV-1 envelope glycoprotein. *Cell* 89: 263-73

32. Crispin M, Doores KJ. 2015. Targeting host-derived glycans on enveloped viruses for antibody-based vaccine design. *Curr Opin Virol* 11: 63-9
33. de Taeye SW, Ozorowski G, Torrents de la Pena A, Guttman M, Julien JP, van den Kerkhof TL, Burger JA, Pritchard LK, Pugach P, Yasmeen A, Crampton J, Hu J, Bontjer I, Torres JL, Arendt H, DeStefano J, Koff WC, Schuitemaker H, Eggink D, Berkhout B, Dean H, LaBranche C, Crotty S, Crispin M, Montefiori DC, Klasse PJ, Lee KK, Moore JP, Wilson IA, Ward AB, Sanders RW. 2015. Immunogenicity of Stabilized HIV-1-1 Envelope Trimers with Reduced Exposure of Non-neutralizing Epitopes. *Cell* 163: 1702-15
34. Chan DC, Kim PS. 1998. HIV-1 entry and its inhibition. *Cell* 93: 681-4
35. Wyatt R, Sodroski J. 1998. The HIV-1-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 280: 1884-8
36. Pope M, Haase AT. 2003. Transmission, acute HIV-1-1 infection and the quest for strategies to prevent infection. *Nat Med* 9: 847-52
37. Haedicke J, Brown C, Naghavi MH. 2009. The brain-specific factor FEZ1 is a determinant of neuronal susceptibility to HIV-1-1 infection. *Proc Natl Acad Sci U S A* 106: 14040-5
38. Pollard VW, Malim MH. 1998. The HIV-1-1 Rev protein. *Annual Review of Microbiology* 52: 491-532
39. Zheng YH, Lovsin N, Peterlin BM. 2005. Newly identified host factors modulate HIV-1 replication. *Immunol Lett* 97: 225-34
40. Butsch M, Boris-Lawrie K. 2002. Destiny of unspliced retroviral RNA: ribosome and/or virion? *J Virol* 76: 3089-94
41. Ricci EP, Herbreteau CH, Decimo D, Schaupp A, Datta SA, Rein A, Darlix JL, Ohlmann T. 2008. In vitro expression of the HIV-1-2 genomic RNA is controlled by three distinct internal ribosome entry segments that are regulated by the HIV-1 protease and the Gag polyprotein. *RNA* 14: 1443-55
42. Mandell, Bennett, and Dolan (2010). Chapter 121.
43. WHO case definitions of HIV-1 for surveillance and revised clinical staging and immunological classification of HIV-1-related disease in adults and children. Geneva: World Health Organization. 2007. pp. 6–16. ISBN 978-92-4-159562-9
44. Diseases and disorders. Tarrytown, NY: Marshall Cavendish. 2008. p. 25. ISBN 978-0-7614-7771-6.
45. Mandell, Bennett, and Dolan (2010). Chapter 118.
46. WHO case definitions of HIV-1 for surveillance and revised clinical staging and immunological classification of HIV-1-related disease in adults and children. Geneva: World Health Organization. 2007. pp. 6–16. ISBN 978-92-4-159562-9.
47. Mandell, Bennett, and Dolan (2010). Chapter 118.
48. Mandell, Bennett, and Dolan (2010). Chapter 118.
49. "Stages of HIV-1". U.S. Department of Health & Human Services. Dec 2010. Retrieved February 13, 2016.
50. Evian, Clive (2006). Primary HIV-1/AIDS care: a practical guide for primary health care personnel in a clinical and supportive setting (Updated 4th ed.). Houghton [South Africa]: Jacana. p. 29. ISBN 978-1-77009-198-6.
51. Charles B. Hicks, MD (2001). Jacques W. A. J. Reenders & Philip Charles Goodman, ed. Radiology of AIDS. Berlin [u.a.]: Springer. p. 19. ISBN 978-3-540-66510-6.

52. Elliott, Tom (2012). Lecture Notes: Medical Microbiology and Infection. John Wiley & Sons. p. 273. ISBN 978-1-118-37226-5.
53. "Stages of HIV-1". U.S. Department of Health & Human Services. Dec 2010. Retrieved February 13, 2016.
54. Mandell, Bennett, and Dolan (2010). Chapter 118.
55. Blankson JN. 2010. Control of HIV-1 replication in elite suppressors. *Discov Med* 9: 261-6
56. Mandell, Bennett, and Dolan (2010). Chapter 118.
57. Holmes CB, Losina E, Walensky RP, Yazdanpanah Y, Freedberg KA. 2003. Review of human immunodeficiency virus type 1-related opportunistic infections in sub-Saharan Africa. *Clin Infect Dis* 36: 652-62
58. Mandell, Bennett, and Dolan (2010). Chapter 169.
59. Mandell, Bennett, and Dolan (2010). Chapter 169.
60. Freed EO, Gale M, Jr. 2014. Antiviral innate immunity: editorial overview. *J Mol Biol* 426: 1129-32
61. Rustagi A, Gale M, Jr. 2014. Innate antiviral immune signaling, viral evasion and modulation by HIV-1-1. *J Mol Biol* 426: 1161-77
62. Loo YM, Gale M, Jr. 2007. Viral regulation and evasion of the host response. *Curr Top Microbiol Immunol* 316: 295-313
63. Pichlmair A, Reis e Sousa C. 2007. Innate recognition of viruses. *Immunity* 27: 370-83
64. van Montfoort N, Olganier D, Hiscott J. 2014. Unmasking immune sensing of retroviruses: interplay between innate sensors and host effectors. *Cytokine Growth Factor Rev* 25: 657-68
65. Towers GJ, Noursadeghi M. 2014. Interactions between HIV-1-1 and the Cell-Autonomous Innate Immune System. *Cell Host & Microbe* 16: 10-8
66. Jakobsen MR, Bak RO, Andersen A, Berg RK, Jensen SB, Tengchuan J, Laustsen A, Hansen K, Ostergaard L, Fitzgerald KA, Xiao TS, Mikkelsen JG, Mogensen TH, Paludan SR. 2013. IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1-1 replication. *Proc Natl Acad Sci U S A* 110: E4571-80
67. Li XD, Wu J, Gao D, Wang H, Sun L, Chen ZJ. 2013. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* 341: 1390-4
68. Sun LJ, Wu JX, Chen ZJ. 2013. Cyclic GMP-AMP Synthase (cGAS) is a cytosolic DNA sensor that activates type I interferon pathway by generating a second messenger. *Journal of Immunology* 190
69. Gao DX, Wu JX, Wu YT, Du FH, Aroh C, Yan N, Sun LJ, Chen ZJJ. 2013. Cyclic GMP-AMP Synthase Is an Innate Immune Sensor of HIV-1 and Other Retroviruses. *Science* 341: 903-6
70. Loo YM, Gale M, Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34: 680-92
71. Wang Y, Wang X, Li J, Zhou Y, Ho W. 2013. RIG-I activation inhibits HIV-1 replication in macrophages. *J Leukoc Biol* 94: 337-41
72. Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ. 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339: 826-30
73. Malim MH, Emerman M. 2008. HIV-1-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host Microbe* 3: 388-98
74. Li M, Kao E, Gao X, Sandig H, Limmer K, Pavon-Eternod M, Jones TE, Landry S, Pan T, Weitzman MD, David M. 2012. Codon-usage-based inhibition of HIV-1 protein synthesis by human schlafen 11. *Nature* 491: 125-8

75. Lu J, Pan Q, Rong L, He W, Liu SL, Liang C. 2011. The IFITM proteins inhibit HIV-1 infection. *J Virol* 85: 2126-37
76. Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, Schoggins JW, Rice CM, Yamashita M, Hatzioannou T, Bieniasz PD. 2013. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature* 502: 563-+
77. Goujon C, Moncorge O, Bauby H, Doyle T, Ward CC, Schaller T, Hue S, Barclay WS, Schulz R, Malim MH. 2013. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature* 502: 559-+
78. Norman JM, Mashiba M, McNamara LA, Onafuwa-Nuga A, Chiari-Fort E, Shen W, Collins KL. 2011. The antiviral factor APOBEC3G enhances the recognition of HIV-1-infected primary T cells by natural killer cells. *Nat Immunol* 12: 975-83
79. Park SY, Waheed AA, Zhang ZR, Freed EO, Bonifacino JS. 2014. HIV-1-1 Vpu Accessory Protein Induces Caspase-mediated Cleavage of IRF3 Transcription Factor. *Journal of Biological Chemistry* 289: 35102-10
80. Geijtenbeek TBH, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GCF, Middel J, Cornelissen ILMHA, Nottet HSLM, KewalRamani VN, Littman DR, Figdor CG, van Kooyk Y. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100: 587-97
81. Hubner W, McNerney GP, Chen P, Dale BM, Gordon RE, Chuang FY, Li XD, Asmuth DM, Huser T, Chen BK. 2009. Quantitative 3D video microscopy of HIV-1 transfer across T cell virological synapses. *Science* 323: 1743-7
82. Jolly C, Sattentau QJ. 2004. Retroviral spread by induction of virological synapses. *Traffic* 5: 643-50
83. Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RM, Kupfer A. 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV-1- and SIV-infected lymph nodes. *Nat Med* 1: 129-34
84. Brennan TP, Woods JO, Sedaghat AR, Siliciano JD, Siliciano RF, Wilke CO. 2009. Analysis of human immunodeficiency virus type 1 viremia and provirus in resting CD4+ T cells reveals a novel source of residual viremia in patients on antiretroviral therapy. *J Virol* 83: 8470-81
85. Prince HE, Jensen ER. 1991. Three-color cytofluorometric analysis of CD8 cell subsets in HIV-1 infection. *J Acquir Immune Defic Syndr* 4: 1227-32
86. Giorgi JV, Detels R. 1989. T-cell subset alterations in HIV-1-infected homosexual men: NIAID Multicenter AIDS cohort study. *Clin Immunol Immunopathol* 52: 10-8
87. Hazenberg MD, Stuart JW, Otto SA, Borleffs JC, Boucher CA, de Boer RJ, Miedema F, Hamann D. 2000. T-cell division in human immunodeficiency virus (HIV-1)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* 95: 249-55
88. Chakrabarti LA, Lewin SR, Zhang L, Gettie A, Luckay A, Martin LN, Skulsky E, Ho DD, Cheng-Mayer C, Marx PA. 2000. Normal T-cell turnover in sooty mangabeys harboring active simian immunodeficiency virus infection. *J Virol* 74: 1209-23
89. Silvestri G, Sodora DL, Koup RA, Paiardini M, O'Neil SP, McClure HM, Staprans SI, Feinberg MB. 2003. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* 18: 441-52



90. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68: 6103-10
91. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68: 4650-5
92. Wilson JD, Ogg GS, Allen RL, Davis C, Shaunak S, Downie J, Dyer W, Workman C, Sullivan S, McMichael AJ, Rowland-Jones SL. 2000. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* 14: 225-33
93. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM. 2009. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med* 206: 1273-89
94. Bernardin F, Kong D, Peddada L, Baxter-Lowe LA, Delwart E. 2005. Human immunodeficiency virus mutations during the first month of infection are preferentially found in known cytotoxic T-lymphocyte epitopes. *J Virol* 79: 11523-8
95. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, Keele BF, Learn GH, Turnbull EL, Salazar MG, Weinhold KJ, Moore S, B CCC, Letvin N, Haynes BF, Cohen MS, Hraber P, Bhattacharya T, Borrow P, Perelson AS, Hahn BH, Shaw GM, Korber BT, McMichael AJ. 2009. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* 206: 1253-72
96. Michael NL, Nelson JA, KewalRamani VN, Chang G, O'Brien SJ, Mascola JR, Volsky B, Louder M, White GC, 2nd, Littman DR, Swanstrom R, O'Brien TR. Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 delta32. *J Virol* 1998; 72:6040-7.
97. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. Antibody neutralization and escape by HIV-1. *Nature* 2003; 422:307-12.
98. Strain MC, Gunthard HF, Havlir DV, Ignacio CC, Smith DM, Leigh-Brown AJ, Macaranas TR, Lam RY, Daly OA, Fischer M, Opravil M, Levine H, Bacheler L, Spina CA, Richman DD, Wong JK. 2003. Heterogeneous clearance rates of long-lived lymphocytes infected with HIV-1: intrinsic stability predicts lifelong persistence. *Proc Natl Acad Sci U S A* 100: 4819-24
99. Learmont J, Tindall B, Evans L, Cunningham A, Cunningham P, Wells J, Penny R, Kaldor J, Cooper DA. 1992. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* 340: 863-7
100. Gurusinghe AD, Land SA, Birch C, McGavin C, Hooker DJ, Tachedjian G, Doherty R, Deacon NJ. 1995. Reverse transcriptase mutations in sequential HIV-1 isolates in a patient with AIDS. *J Med Virol* 46: 238-43
101. Quinones-Mateu ME, Mas A, Lain de Lera T, Soriano V, Alcamí J, Lederman MM, Domingo E. 1998. LTR and tat variability of HIV-1 isolates from patients with divergent rates of disease progression. *Virus Res* 57: 11-20

102. Zhang L, Huang Y, Yuan H, Chen BK, Ip J, Ho DD. 1997. Genotypic and phenotypic characterization of long terminal repeat sequences from long-term survivors of human immunodeficiency virus type 1 infection. *J Virol* 71: 5608-13
103. Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK, Grant RM. 2001. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-1-infected patients with detectable viremia. *N Engl J Med* 344: 472-80
104. Deeks SG, Hoh R, Grant RM, Wrin T, Barbour JD, Narvaez A, Cesar D, Abe K, Hanley MB, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK. 2002. CD4+ T cell kinetics and activation in human immunodeficiency virus-infected patients who remain viremic despite long-term treatment with protease inhibitor-based therapy. *J Infect Dis* 185: 315-23
105. Guo X, Kameoka M, Wei X, Roques B, Gotte M, Liang C, Wainberg MA. 2003. Suppression of an intrinsic strand transfer activity of HIV-1-1 Tat protein by its second-exon sequences. *Virology* 307: 154-63
106. Richman DD, Wrin T, Little SJ, Petropoulos CJ. 2003. Rapid evolution of the neutralizing antibody response to HIV-1 type 1 infection. *Proc Natl Acad Sci U S A* 100: 4144-9
107. Gray ES, et al. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *J. Virol.* 2007; 81:6187–6196. [PubMed: 17409164]
108. Stamatatos L, Morris L, Burton DR, Mascola JR. Neutralizing antibodies generated during natural HIV-1-1 infection: good news for an HIV-1-1 vaccine? *Nature Med.* 2009; 15:866–870. [PubMed: 19525964]
109. Gray ES, et al. Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region. *J. Virol.* 2009; 83:11265–11274. [PubMed: 19692477]
110. Wei X, et al. Antibody neutralization and escape by HIV-1-1. *Nature.* 2003; 422:307–312. [PubMed: 12646921] The timing of the first antibody-selected escape mutations of HIV-1-1 in acute infection highlights the role of the glycan shield
111. Gray ES, Madiga MC, Moore PL, Mlisana K, Abdool Karim SS, Binley JM, Shaw GM, Mascola JR, Morris L. 2009. Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region. *J Virol* 83: 11265-74
112. Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL, Decamp AC, Parks RJ, Ashley VC, Lucas JT, Cohen M, Eron J, Hicks CB, Liao HX, Self SG, Landucci G, Forthal DN, Weinhold KJ, Keele BF, Hahn BH, Greenberg ML, Morris L, Karim SS, Blattner WA, Montefiori DC, Shaw GM, Perelson AS, Haynes BF. 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. *J Virol* 82: 12449-63
113. Alam SM, Searce RM, Parks RJ, Plonk K, Plonk SG, Sutherland LL, Gorny MK, Zolla-Pazner S, Vanleeuwen S, Moody MA, Xia SM, Montefiori DC, Tomaras GD, Weinhold KJ, Karim SA, Hicks CB, Liao HX, Robinson J, Shaw GM, Haynes BF. 2008. Human immunodeficiency virus type 1 gp41 antibodies that mask membrane proximal region epitopes: antibody binding kinetics, induction, and potential for regulation in acute infection. *J Virol* 82: 115-25
114. Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA, Heil ML, Kasolo F, Musonda R, Hahn BH, Shaw GM, Korber BT, Allen S, Hunter E. 2004. Envelope-constrained neutralization-sensitive HIV-1-1 after heterosexual transmission. *Science* 303: 2019-22

115. Alam SM, McAdams M, Boren D, Rak M, Searce RM, Gao F, Camacho ZT, Gewirth D, Kelsoe G, Chen P, Haynes BF. 2007. The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J Immunol* 178: 4424-35
116. Crooks ET, Moore PL, Franti M, Cayan CS, Zhu P, Jiang P, de Vries RP, Wiley C, Zharkikh I, Schulke N, Roux KH, Montefiori DC, Burton DR, Binley JM. 2007. A comparative immunogenicity study of HIV-1-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology* 366: 245-62
117. Tobin GJ, Trujillo JD, Bushnell RV, Lin G, Chaudhuri AR, Long J, Barrera J, Pena L, Grubman MJ, Nara PL. 2008. Deceptive imprinting and immune refocusing in vaccine design. *Vaccine* 26: 6189-99
118. Forthal DN, Landucci G, Daar ES. 2001. Antibody from patients with acute human immunodeficiency virus (HIV-1) infection inhibits primary strains of HIV-1 type 1 in the presence of natural-killer effector cells. *J Virol* 75: 6953-61
119. Aasa-Chapman MM, Holuigue S, Aubin K, Wong M, Jones NA, Cornforth D, Pellegrino P, Newton P, Williams I, Borrow P, McKnight A. 2005. Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection. *J Virol* 79: 2823-30
120. Burrer R, Haessig-Einius S, Aubertin AM, Moog C. 2005. Neutralizing as well as non-neutralizing polyclonal immunoglobulin (Ig)G from infected patients capture HIV-1-1 via antibodies directed against the principal immunodominant domain of gp41. *Virology* 333: 102-13
121. Burns DP, Desrosiers RC. 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J Virol* 65: 1843-54
122. Johnson WE, Desrosiers RC. 2002. Viral persistence: HIV-1's strategies of immune system evasion. *Annu Rev Med* 53: 499-518
123. What Is HAART? | National Institute on Drug Abuse (NIDA) <https://www.drugabuse.gov/publications/research-reports/HIV-1aids/what-haart> Retrieved February 12, 2016
124. Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M. 1991. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1-1 infection. *Science* 254: 423-7
125. Chou CS, Ramilo O, Vitetta ES. 1997. Highly purified CD25- resting T cells cannot be infected de novo with HIV-1-1. *Proc Natl Acad Sci U S A* 94: 1361-5
126. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. 1999. Cytokine signals are sufficient for HIV-1-1 infection of resting human T lymphocytes. *J Exp Med* 189: 1735-46
127. Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS. 1998. Induction of HIV-1-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med* 188: 83-91
128. Crampton N, Bonass WA, Kirkham J, Rivetti C, Thomson NH. 2006. Collision events between RNA polymerases in convergent transcription studied by atomic force microscopy. *Nucleic Acids Res* 34: 5416-25
129. Callen BP, Shearwin KE, Egan JB. 2004. Transcriptional interference between convergent promoters caused by elongation over the promoter. *Mol Cell* 14: 647-56
130. WHO | Mother-to-child transmission of HIV-1 <http://www.who.int/HIV-1/topics/mtct/en/> Retrieved February 23, 2016

131. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med*. Mar 30 2000;342(13):921-929.
132. Cohen MS, Chen YQ, McCauley M, et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med*. Aug 11 2011;365(6):493-505.
133. Overview of HIV-1 Treatments <https://www.aids.gov/HIV-1-aids-basics/just-diagnosed-with-HIV-1-aids/treatment-options/overview-of-HIV-1-treatments/> Retrieved February 22, 2016
134. Side Effects <https://www.aids.gov/HIV-1-aids-basics/just-diagnosed-with-HIV-1-aids/treatment-options/side-effects/index.html>. Retrieved February 22, 2016
135. Mehndru S, Vcelar B, Wrin T, Stiegler G, Joos B, Mohri H, Boden D, Galovich J, Tenner-Racz K, Racz P, Carrington M, Petropoulos C, Katinger H, Markowitz M. 2007. Adjunctive passive immunotherapy in human immunodeficiency virus type 1-infected individuals treated with antiviral therapy during acute and early infection. *J Virol* 81: 11016-31
136. Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, Manrique A, Huber M, Rehr M, Oxenius A, Weber R, Stiegler G, Vcelar B, Katinger H, Aceto L, Gunthard HF. 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat Med* 11: 615-22
137. Armbruster C, Stiegler GM, Vcelar BA, Jager W, Koller U, Jilch R, Ammann CG, Pruenster M, Stoiber H, Katinger HW. 2004. Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. *J Antimicrob Chemother* 54: 915-20
138. Caskey M, Klein F, Lorenzi JC, Seaman MS, West AP, Jr., Buckley N, Kremer G, Nogueira L, Braunschweig M, Scheid JF, Horwitz JA, Shimeliovich I, Ben-Avraham S, Witmer-Pack M, Platten M, Lehmann C, Burke LA, Hawthorne T, Gorelick RJ, Walker BD, Keler T, Gulick RM, Fatkenheuer G, Schlesinger SJ, Nussenzweig MC. 2015. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 522: 487-91
139. Scheid JF, Mouquet H, Feldhahn N, Walker BD, Pereyra F, Cutrell E, Seaman MS, Mascola JR, Wyatt RT, Wardemann H, Nussenzweig MC. 2009. A method for identification of HIV-1 gp140 binding memory B cells in human blood. *J Immunol Methods* 343: 65-7
140. Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, Ott RG, Anthony RM, Zebroski H, Hurley A, Phogat A, Chakrabarti B, Li Y, Connors M, Pereyra F, Walker BD, Wardemann H, Ho D, Wyatt RT, Mascola JR, Ravetch JV, Nussenzweig MC. 2009. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-1-infected individuals. *Nature* 458: 636-40
141. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 329: 112-24
142. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301: 1374-7
143. Klein F, Mouquet H, Dosenovic P, Scheid JF, Scharf L, Nussenzweig MC. 2013. Antibodies in HIV-1 vaccine development and therapy. *Science* 341: 1199-204
144. West AP, Jr., Scharf L, Scheid JF, Klein F, Bjorkman PJ, Nussenzweig MC. 2014. Structural insights on the role of antibodies in HIV-1 vaccine and therapy. *Cell* 156: 633-48

145. Klein F, Halper-Stromberg A, Horwitz JA, Gruell H, Scheid JF, Bournazos S, Mouquet H, Spatz LA, Diskin R, Abadir A, Zang T, Dorner M, Billerbeck E, Labitt RN, Gaebler C, Marcovecchio PM, Incesu RB, Eisenreich TR, Bieniasz PD, Seaman MS, Bjorkman PJ, Ravetch JV, Ploss A, Nussenzweig MC. 2012. HIV-1 therapy by a combination of broadly neutralizing antibodies in humanized mice. *Nature* 492: 118-22
146. Haigwood NL, Nara PL, Brooks E, Van Nest GA, Ott G, Higgins KW, Dunlop N, Scandella CJ, Eichberg JW, Steimer KS. 1992. Native but not denatured recombinant human immunodeficiency virus type 1 gp120 generates broad-spectrum neutralizing antibodies in baboons. *J Virol* 66: 172-82
147. Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, Hu D, Tappero JW, Choopanya K, Bangkok Vaccine Evaluation G. 2006. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* 194: 1661-71
148. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, Defawe OD, Carter DK, Hural J, Akondy R, Buchbinder SP, Robertson MN, Mehrotra DV, Self SG, Corey L, SHIV-1er JW, Casimiro DR, Step Study Protocol T. 2008. HIV-1-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 372: 1894-905
149. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB, Lama JR, Marmor M, Del Rio C, McElrath MJ, Casimiro DR, Gottesdiener KM, Chodakewitz JA, Corey L, Robertson MN, Step Study Protocol T. 2008. Efficacy assessment of a cell-mediated immunity HIV-1-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372: 1881-93
150. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Prensri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH, Investigators M-T. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1-1 infection in Thailand. *N Engl J Med* 361: 2209-20
151. Xu L, Liu Y, Chen Z, Li W, Liu Y, Wang L, Ma L, Shao Y, Zhao Y, Chen C. 2013. Morphologically virus-like fullerene nanoparticles act as the dual-functional nanoadjuvant for HIV-1-1 vaccine. *Adv Mater* 25: 5928-36
152. Chung AW, Kumar MP, Arnold KB, Yu WH, Schoen MK, Dunphy LJ, Suscovich TJ, Frahm N, Linde C, Mahan AE, Hoffner M, Streeck H, Ackerman ME, McElrath MJ, Schuitemaker H, Pau MG, Baden LR, Kim JH, Michael NL, Barouch DH, Lauffenburger DA, Alter G. 2015. Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV-1 Using Systems Serology. *Cell* 163: 988-98
153. Kwon YD, Pancera M, Acharya P, Georgiev IS, Crooks ET, Gorman J, Joyce MG, Guttman M, Ma X, Narpala S, Soto C, Terry DS, Yang Y, Zhou T, Ahlsen G, Bailer RT, Chambers M, Chuang GY, Doria-Rose NA, Druz A, Hallen MA, Harned A, Kirys T, Louder MK, O'Dell S, Ofek G, Osawa K, Prabhakaran M, Sastry M, Stewart-Jones GB, Stuckey J, Thomas PV, Tittley T, Williams C, Zhang B, Zhao H, Zhou Z, Donald BR, Lee LK, Zolla-Pazner S, Baxa U, Schon A, Freire E, Shapiro L, Lee KK, Arthos J, Munro JB, Blanchard SC, Mothes W, Binley JM, McDermott AB, Mascola JR, Kwong PD. 2015. Crystal structure, conformational fixation and entry-related interactions of mature ligand-free HIV-1-1 Env. *Nat Struct Mol Biol* 22: 522-31

154. Baden LR, Karita E, Mutua G, Bekker LG, Gray G, Page-Shipp L, Walsh SR, Nyombayire J, Anzala O, Roux S, Laher F, Innes C, Seaman MS, Cohen YZ, Peter L, Frahm N, McElrath MJ, Hayes P, Swann E, Grunenberg N, Grazia-Pau M, Weijtens M, Sadoff J, Dally L, Lombardo A, Gilmour J, Cox J, Dolin R, Fast P, Barouch DH, Laufer DS, Group BIHS. 2016. Assessment of the Safety and Immunogenicity of 2 Novel Vaccine Platforms for HIV-1-1 Prevention: A Randomized Trial. *Ann Intern Med* 164: 313-22
155. Veronese FD, DeVico AL, Copeland TD, Oroszlan S, Gallo RC, Sarngadharan MG. 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. *Science* 229: 1402-5
156. Earl PL, Doms RW, Moss B. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc Natl Acad Sci U S A* 87: 648-52
157. Earl PL, Moss B, Doms RW. 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J Virol* 65: 2047-55
158. Robey WG, Safai B, Oroszlan S, Arthur LO, Gonda MA, Gallo RC, Fischinger PJ. 1985. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* 228: 593-5
159. Lee JH, Ozorowski G, Ward AB. 2016. Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1-1 envelope trimer. *Science* 351: 1043-8
160. Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA. 2013. Crystal structure of a soluble cleaved HIV-1-1 envelope trimer. *Science* 342: 1477-83
161. Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB. 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1-1 envelope trimer. *Science* 342: 1484-90
162. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. 1998. Structure of an HIV-1 gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393: 648-59
163. Buzon V, Natrajan G, Schibli D, Campelo F, Kozlov MM, Weissenhorn W. 2010. Crystal structure of HIV-1-1 gp41 including both fusion peptide and membrane proximal external regions. *PLoS Pathog* 6: e1000880
164. Connor RI, Korber BT, Graham BS, Hahn BH, Ho DD, Walker BD, Neumann AU, Vermund SH, Mestecky J, Jackson S, Fenamore E, Cao Y, Gao F, Kalams S, Kunstman KJ, McDonald D, McWilliams N, Trkola A, Moore JP, Wolinsky SM. 1998. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. *J Virol* 72: 1552-76
165. Dolin R, Graham BS, Greenberg SB, Tacket CO, Belshe RB, Midthun K, Clements ML, Gorse GJ, Horgan BW, Atmar RL, et al. 1991. The safety and immunogenicity of a human immunodeficiency virus type 1 (HIV-1-1) recombinant gp160 candidate vaccine in humans. NIAID AIDS Vaccine Clinical Trials Network. *Ann Intern Med* 114: 119-27
166. Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV-1 gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393:648–659.

167. Kwong, P. D., R. Wyatt, Q. J. Sattentau, J. Sodroski, and W. A. Hendrickson. 2000. Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. *J. Virol.* 74:1961–1972.
168. Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski. 1998. The antigenic structure of the HIV-1 gp120 envelope glycoprotein. *Nature* 393:705–711.
169. Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. 1990. Protection of chimpanzees from infection by HIV-1-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 345:622–625.
170. Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson, and R. T. Wyatt. 2004. HIV-1 vaccine design and the neutralizing antibody problem. *Nat. Immunol.* 5:233–236
171. Mascola JR, Snyder SW, Weislow OS, Belay SM, Belshe RB, Schwartz DH, Clements ML, Dolin R, Graham BS, Gorse GJ, Keefer MC, McElrath MJ, Walker MC, Wagner KF, McNeil JG, McCutchan FE, Burke DS. 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. *J Infect Dis* 173: 340-8
172. Rusche JR, Lynn DL, Robert-Guroff M, Langlois AJ, Lysterly HK, Carson H, Krohn K, Ranki A, Gallo RC, Bolognesi DP, et al. 1987. Humoral immune response to the entire human immunodeficiency virus envelope glycoprotein made in insect cells. *Proc Natl Acad Sci U S A* 84: 6924-8
173. Wrin T, Nunberg JH. 1994. HIV-1-1MN recombinant gp120 vaccine serum, which fails to neutralize primary isolates of HIV-1-1, does not antagonize neutralization by antibodies from infected individuals. *AIDS* 8: 1622-3
174. Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H. 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *J Virol* 61: 570-8
175. Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC, et al. 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* 45: 637-48
176. Willey RL, Rutledge RA, Dias S, Folks T, Theodore T, Buckler CE, Martin MA. 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. *Proc Natl Acad Sci U S A* 83: 5038-42
177. Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, Sawyer LS, Hendry RM, Dunlop N, Nara PL, et al. 1994. Efficient neutralization of primary isolates of HIV-1-1 by a recombinant human monoclonal antibody. *Science* 266: 1024-7
178. Gorny MK, Xu JY, Karwowska S, Buchbinder A, Zolla-Pazner S. 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1-1 gp120. *J Immunol* 150: 635-43
179. Goudsmit J, Debouck C, Meloen RH, Smit L, Bakker M, Asher DM, Wolff AV, Gibbs CJ, Jr., Gajdusek DC. 1988. Human immunodeficiency virus type 1 neutralization epitope with

- conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc Natl Acad Sci U S A* 85: 4478-82
180. Broliden PA, von Gegerfelt A, Clapham P, Rosen J, Fenyo EM, Wahren B, Broliden K. 1992. Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. *Proc Natl Acad Sci U S A* 89: 461-5
  181. Binley JM, Sanders RW, Clas B, Schuelke N, Master A, Guo Y, Kajumo F, Anselma DJ, Maddon PJ, Olson WC, Moore JP. 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. *J Virol* 74: 627-43
  182. Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, Wurm FM, Hershberg RD, Cobb EK, Eichberg JW. 1990. Protection of chimpanzees from infection by HIV-1-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 345: 622-5
  183. Belshe RB, Gorse GJ, Mulligan MJ, Evans TG, Keefer MC, Excler JL, Duliege AM, Tartaglia J, Cox WI, McNamara J, Hwang KL, Bradney A, Montefiori D, Weinhold KJ. 1998. Induction of immune responses to HIV-1-1 by canarypox virus (ALVAC) HIV-1-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. *AIDS* 12: 2407-15
  184. Beddows S, Louisirotchanaikul S, Cheingsong-Popov R, Easterbrook PJ, Simmonds P, Weber J. 1998. Neutralization of primary and T-cell line adapted isolates of human immunodeficiency virus type 1: role of V3-specific antibodies. *J Gen Virol* 79 ( Pt 1): 77-82
  185. Gorny MK, VanCott TC, Hioe C, Israel ZR, Michael NL, Conley AJ, Williams C, Kessler JA, 2nd, Chigurupati P, Burda S, Zolla-Pazner S. 1997. Human monoclonal antibodies to the V3 loop of HIV-1-1 with intra- and interclade cross-reactivity. *J Immunol* 159: 5114-22
  186. Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, Ruker F, Katinger H. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* 67: 6642-7
  187. Moore JP, Trkola A, Korber B, Boots LJ, Kessler JA, 2nd, McCutchan FE, Mascola J, Ho DD, Robinson J, Conley AJ. 1995. A human monoclonal antibody to a complex epitope in the V3 region of gp120 of human immunodeficiency virus type 1 has broad reactivity within and outside clade B. *J Virol* 69: 122-30
  188. Vancott TC, Polonis VR, Loomis LD, Michael NL, Nara PL, Birx DL. 1995. Differential role of V3-specific antibodies in neutralization assays involving primary and laboratory-adapted isolates of HIV-1 type 1. *AIDS Res Hum Retroviruses* 11: 1379-91
  189. Posner MR, Cavacini LA, Emes CL, Power J, Byrn R. 1993. Neutralization of HIV-1-1 by F105, a human monoclonal antibody to the CD4 binding site of gp120. *J Acquir Immune Defic Syndr* 6: 7-14
  190. Thali M, Olshevsky U, Furman C, Gabuzda D, Posner M, Sodroski J. 1991. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. *J Virol* 65: 6188-93
  191. Kwong PD, Wyatt R, Majeed S, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. 2000. Structures of HIV-1-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. *Structure* 8: 1329-39



192. Richard Wyatt,<sup>1,2</sup> Peter D. Kwong,<sup>3</sup> Wayne A. Hendrickson,<sup>3,4</sup> and Joseph G. Sodroski<sup>1,4</sup> Structure of the Core of the HIV-1-1 gp120 Exterior Envelope Glycoprotein Retrieved online <http://www.HIV-1.lanl.gov/content/sequence/HIV-1/COMPENDIUM/1998/III/Sodroski.pdf>
193. Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J. 2002. HIV-1-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* 420: 678-82
194. Kwong PD, Wyatt R, Sattentau QJ, Sodroski J, Hendrickson WA. 2000. Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. *J Virol* 74: 1961-72
195. Xiang SH, Doka N, Choudhary RK, Sodroski J, Robinson JE. 2002. Characterization of CD4-induced epitopes on the HIV-1 type 1 gp120 envelope glycoprotein recognized by neutralizing human monoclonal antibodies. *AIDS Res Hum Retroviruses* 18: 1207-17
196. Jesse Thompson, Pankaj Kumar, Jizu Yi, Dane Bowder, Charles Wood, Shi-Hua Xiang, Stabilization of Outer Domain of gp120 from HIV-1-1 Subtype C for Vaccine Immunogen Design, *Procedia in Vaccinology*, Volume 9, 2015, Pages 6-15, ISSN 1877-282X
197. Madani N, Schon A, Princiotto AM, Lalonde JM, Courter JR, Soeta T, Ng D, Wang L, Brower ET, Xiang SH, Kwon YD, Huang CC, Wyatt R, Kwong PD, Freire E, Smith AB, 3rd, Sodroski J. 2008. Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1-1 gp120. *Structure* 16: 1689-701
198. Xiang SH, Finzi A, Pacheco B, Alexander K, Yuan W, Rizzuto C, Huang CC, Kwong PD, Sodroski J. 2010. A V3 loop-dependent gp120 element disrupted by CD4 binding stabilizes the human immunodeficiency virus envelope glycoprotein trimer. *J Virol* 84: 3147-61
199. Thompson J, Kumar P, Yi JZ, Bowder D, Wood C, Xiang SH. 2015. Stabilization of Outer Domain of gp120 from HIV-1-1 Subtype C for Vaccine Immunogen Design. *Procedia of the 8th Vaccine & Isv Congress* 9: 6-15
200. Xiang SH, Kwong PD, Gupta R, Rizzuto CD, Casper DJ, Wyatt R, Wang L, Hendrickson WA, Doyle ML, Sodroski J. 2002. Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein. *J Virol* 76: 9888-99
201. Zhang H, Hoffmann F, He J, He X, Kankasa C, West JT, Mitchell CD, Ruprecht RM, Orti G, Wood C. 2006. Characterization of HIV-1-1 subtype C envelope glycoproteins from perinatally infected children with different courses of disease. *Retrovirology* 3: 73
202. Rho HM, Poiesz B, Ruscetti FW, Gallo RC. 1981. Characterization of the reverse transcriptase from a new retrovirus (HTLV) produced by a human cutaneous T-cell lymphoma cell line. *Virology* 112: 355-60
203. Montefiori, D.C. (2004) Evaluating neutralizing antibodies against HIV-1, SIV and SHIV-1 in luciferase reporter gene assays. *Current Protocols in Immunology*, (Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, and R. Coico, eds.), John Wiley & Sons, 12.11.1-12.11.15.
204. Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, Sodroski JG. 1998. The antigenic structure of the HIV-1 gp120 envelope glycoprotein. *Nature* 393: 705-11
205. Joyce MG, Kanekiyo M, Xu L, Biertumpfel C, Boyington JC, Moquin S, Shi W, Wu X, Yang Y, Yang ZY, Zhang B, Zheng A, Zhou T, Zhu J, Mascola JR, Kwong PD, Nabel GJ. 2013. Outer domain of

- HIV-1-1 gp120: antigenic optimization, structural malleability, and crystal structure with antibody VRC-PG04. *J Virol* 87: 2294-306
206. Holzwarth G, Doty P. 1965. The Ultraviolet Circular Dichroism of Polypeptides. *J Am Chem Soc* 87: 218-28
207. Greenfield N, Fasman GD. 1969. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 8: 4108-16
208. Venyaminov S, Baikalov IA, Shen ZM, Wu CS, Yang JT. 1993. Circular dichroic analysis of denatured proteins: inclusion of denatured proteins in the reference set. *Anal Biochem* 214: 17-24
209. Korber B, Foley BT, Kuiken C, Pillai SK, & Sodroski JG. 1998 Numbering positions in HIV-1 relative to HXB2CG. *Human retroviruses and AIDS* 3:102-111