CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 CRF01_AE *ENV* GENES DERIVED FROM RECENTLY INFECTED THAI INDIVIDUALS

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ABSTRACT

Transmitted/founder virus (T/F) is responsible for the establishment of human immunodeficiency virus type 1 (HIV-1) infection and induces primary anti-HIV-1 immune responses; therefore, it is important to study the viral HIV-1 population in an effort to understand the early phases of HIV-1 infection. We amplified HIV-1 *env* genes from sera derived from recently infected Thai individuals, and established an envelope of glycoproteins (Env)-recombinant viruses. Generated Env-recombinant viruses were tested for their neutralization susceptibility to neutralizing human monoclonal antibodies (NHMAbs) and entry inhibitors, as well as being subjected to genotypic analysis. Most recombinant viruses were susceptible to neutralization by NHMAbs to Env gp41, whereas approximately one-third of the recombinant viruses were susceptible to a NHMAb against the CD4 binding site of gp120. In addition, all *env* genes were classified into CRF01_AE genes and showed low genetic divergence. These results provide information for understanding the immunological and genetic characteristics of CRF01_AE Env that were derived from recently infected Thai individuals.

Fourteen Env-recombinant viruses were constructed to study phenotypic and genotypic *env* gene characterizations. There were thirteen R5-tropism, whereas one was X4R5-tropism that were susceptible to neutralization by 4E10, 2F5 and IgG1 b12; they were 100% (14/14), 64% (9/14) and 35% (5/14), respectively. However, all fourteen Env-recombinant viruses were resistant to 2G12. Additionally, the amino acid residues and potential N-linked glycosylation sites on V1/V2 have an inverse significant correlation with IC50 of 4E10, *p* value 0.012 and 0.029, respectively. In contrast, no correlation was found in IC50 of 2F5 and IgG1 b12. However, T/F viruses were moderately susceptible to IgG1 b12 which is potentially a CD4 binding site which induces the high levels of neutralization susceptibility. Early Env clones show varying neutralization susceptibility to specific viral entry whereas the length of V1/V2 amino acids is negatively significant correlation with TAK-779 susceptibility of the recombinant viruses, *p* value 0.048. Early *env* are classified into CRF01_AE *env* which show relatively low divergence compared to chronically CRF01_AE Env clones that infected Thai individuals. Finally, this study is the first report on the subtype CRF01_AE T/F Env-recombinant viruses that provide a valuable tool for understanding immunological and antigenic characterizations in early phases of HIV-1 infection.

KEY WORDS: TRANSMITTED/FOUNDER VIRUS/ RECENT HIV-1 INFECTION/ ENV-RECOMBINANT VIRUS/ VIRAL ENTRY/BED-ELISA

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การศึกษาลักษณะยีนเปลือกหุ้มภายนอก (Envelope genes) ของเชื้อไวรัสเอชไอวี-1 ในพลทหารกองประจำการกองทัพบกที่ติดเชื้อใหม่ และศึกษาความไวในการนิวทรอลไลเซชั่นของไวรัสที่มียีนเปลือกหุ้มภายนอก (Env-recombinant) ที่สร้างขึ้น
CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 CRF01_AE *ENV* GENES DERIVED FROM RECENTLY INFECTED THAI INDIVIDUALS

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บทคัดย่อ

เชื้อเอชไอวี-1 เป็นสาเหตุของโรคภูมิคุ้มกันบกพร่อง (AIDS) และไม่มีวัคซีนในการป้องกันการติดเชื้อเหมือนไวรัสชนิดอื่น ทำให้ไวรัส เอชไอวี-1 ยังเป็นปัญหาสาธารณสุขต่อประเทศต่างๆทั่วโลก ถึงแม้ว่าประสิทธิภาพของยารักษามีความสามารถสูง แต่ยังคงมีราคาแพง ทำให้เกิดการสูญเสียต่อภาวะเศรษฐกิจของประเทศเหล่านั้น เชื้อเอชไอวี-1 เป็นเชื้อที่ยีนมีการเปลี่ยนแปลงตลอดเวลาของการติดเชื้อ ดังนั้นการศึกษาและเข้าใจอย่างชัดเจนระหว่างปฏิกิริยาของระบบภูมิคุ้มกันของร่างกายต่อการติดเชื้อเอช ไอ วี-1ในระยะเริ่มแรกจะเป็นโอกาสในการหลีกเลี่ยงยีนที่มีการเปลี่ยนแปลงไปจากยีนต้นแบบ การศึกษานี้ได้นำซีรั่มจากทหารกองประจำการที่พบว่า ติดเชื้อเอชไอวี-1 และตรวจเพิ่มเติมโดยวิธี BED ELISA ซึ่งวินิจฉัยว่าเป็นผู้ที่ติดเชื้อใหม่ซึ่งติดเชื้อมาไม่เกิน 127 วัน นำมาศึกษาลักษณะยีนเปลือกหุ้มภายนอก (*env* gene) โดยสร้างไวรัสที่มียีนเปลือกหุ้มภายนอกของผู้ติดเชื้อใหม่ และศึกษาความสามารถของไวรัสที่เราสร้างขึ้นโดยวิธีนิวทรอลไลเซชั่นต่อโมโนโคลนอลแอนติบอดิ และยาต่าง ๆ ที่มีความสามารถในการยับยั้งการเข้าสู่เซลล์เป้าหมายของเชื้อเอชไอวี-1 ที่ใช้กันอย่างแพร่หลายในปัจจุบัน

ไวรัส 14 ไวรัสที่มียีนเปลือกหุ้มภายนอกที่สร้างขึ้นประกอบด้วย13ไวรัสเป็นไวรัส R5-tropism และ 1 ตัว เป็นไวรัส R5X4-tropism ไวรัสทั้งหมดเป็นชนิดซับไทป์ CRF01_AE และเมื่อนำมาศึกษาความไวต่อโมโนโคนอลแอนติบอดิ โดยวิธี neutralization assay พบว่า 100% (14/14), 64% (9/14) และ 35% (5/14) สามารถยับยั้งการเพิ่มจำนวนของไวรัสเมื่อใช้โมโนคลอนอลแอนติบอดิ 4E10, 2F5 และ IgG1 b12 ตามลำดับ แต่ไวรัสทั้งหมดสามารถแบ่งตัวได้ดีเมื่อใช้โมโนโคลนอลแอนติบอดิ 2G12 และเมื่อนำมาศึกษาความสัมพันธ์ระหว่างลักษณะของยีนเปลือกหุ้มภายนอกกับความสามารถของโมโนโคลนอลแอนติบอดิต่อการยับยั้งการเพิ่มจำนวนของไวรัส พบว่า การเรียงตัวความยาวของจำนวนโปรตีนของยีนเปลือกหุ้มภายนอกของผู้ติดเชื้อใหม่และจำนวนของ PNLG sites บนส่วนของ V1/V2 มีความสัมพันธ์ในทางตรงกันข้ามกับการเพิ่มจำนวนของไวรัสเมื่อใช้โมโนโคลนอล 4E10 อย่างมีนัยสำคัญทางสถิติ (p value= 0.012, 0.029 ตามลำดับ) นอกจากนี้ยังพบว่าไวรัส 14 ตัวแสดงถึงความแตกต่างความสามารถของยาที่ใช้ในการยับยั้งการเข้าสู่เซลล์ของเชื้อเอชไอวี-1 ได้อย่างหลากหลาย และการเรียงตัวความยาวของจำนวนโปรตีนบนส่วนยีนเปลือกหุ้มภายนอกส่วน V1/V2 มีความสัมพันธ์ในทางเดียวกันกับการเพิ่มจำนวนของไวรัสเมื่อใช้ยายับยั้งการเข้าสู่เซลล์ของไวรัส R5 tropism (TAK-779) อย่างมีนัยสำคัญทางสถิติ (p value = 0.048) ดังนั้นการเปลี่ยนแปลงลักษณะยีนเปลือกหุ้มภายนอกของเชื้อเอชไอวี-1 เกิดขึ้นตลอดเวลาของการติดเชื้อเอชไอวี-1 และวิธีที่ใช้ในการศึกษานี้สามารถทำให้ทราบลักษณะโปรตีนบนส่วน *env* ได้ชัดเจนยิ่งขึ้นเพื่อนำไปสู่การพัฒนาวัคซีนและยารักษาโรคต่อไป และสิ่งสำคัญมากที่สุดคือการศึกษานี้เป็นรายงานฉบับแรกของการศึกษาลักษณะ *env* ของผู้ที่ติดเชื้อใหม่ของซับไทป์ CRF01_AE ที่ระบาดมากที่สุดในประเทศไทย

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LIST OF ABBREVIATIONS

HIV-1	Human Immunodeficiency Virus type 1
SIV	Simian Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Disease Syndrome
Env	Viral envelope
CRFs	Circulating recombinant forms
MSM	Men who have sex with men
FSW	Female sex worker
IDU	Injection drug user
SU	Surface subunit
TM	Transmembrane subunit
gp120	Glycoprotein 120
gp41	Glycoprotein 41
CD4bs	CD4 binding site
T/F	Transmitted or founder virus
GALT	Gut associated lymphoid tissue
IFN-α	Interferon-α
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducible ligand
TLRs	Toll-like receptors
cDCs	Conventional dendritic cells
pDCs	plasmacytoid dendritic cells
NKs	Natural killer cells
PRRs	Pattern recognition receptors
IL12	Interleukin-12
IL-15	Interleukin-15
IL18	Interleukin-18
MPER	Membrane proximal external region

LIST OF ABBREVIATIONS (cont.)

msd	Membrane spanning domain
γ	Gamma
α	Alpha
β	Beta
gm	Gram
µl	Microliter
ml	Milliliter
mg	Milligram
µg	Microgram
M	Molar
nM	Nanomolar
FBS	Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffer Saline
RLU	Relative Light Unit
NHMAbs	Neutralizing human monoclonal antibodies
PNLGs	Potential N-linked glycosylation sites
IC50	50% inhibitory concentration for suppressing viral replication
ID50	50% inhibitory dilution for the reciprocal dilution for suppressed viral replication
ODn	Normalized optical cut off
IMC	Infectious molecular clone
PV	Pseudotype virus
LTR	Long terminal repeat
SDF-1	Stroma cell derived factor 1
MIP1-α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
CypA	Cyclosporine A

CHAPTER I
INTRODUCTION

For more than three decades, human immunodeficiency virus (HIV) has been a serious public health concern, predominantly affecting the social-economy in many countries. Effective vaccines with specific viral antibodies for protection against hepatitis, poliomyelitis and influenza have been developed; however, vaccine against HIV-1 has been unable to initiate the specific anti-viral immune responses. It is assuming that 34 million are affected with infected HIV-1 people in world-wide, 2.5 million are newly infected and 1.8 million eventually die with AIDS in 2011 (1). The majority of infected HIV-1 population is from the low- and middle-income regions, especially Sub-Saharan Africa, which has more than 69% living with infected HIV-1 of the total population as well as this was estimated 13% of the world's population (2). According to a study in South and South-East Asia region, it was estimated that HIV-1 affected approximately 4 million people and newly HIV-1infection was estimated 280,000 individuals in 2011. Besides, in Thailand, the statistics have shown that around 490,000 infected individuals were living with HIV-1 infection, while 23,000 infected HIV-1individuals died with AIDS in 2011, the prevalence of HIV-1 infection was estimated 1% and a quarter of HIV-1 early or acute infection cases were found in individuals under 25 years old, which affected healthy individuals, family members, society and economic growth in developing countries (3). Therefore, effective early screening and treatment should be given in the early stages of infection in order to reduce HIV-1 transmission.

The first HIV/AIDS case was reported in Thailand in 1984. 80-85% of the cases were heterosexuals and 5% were injected drug users (IDU) (4). HIV-1 subtype A and E were found in infected individuals from the heterosexual group, or men who have sex with men (MSM), whereas subtype B was mostly found in IDU (5). In 1992, several programs were launched for the protection and control of HIV-1 infections in Thailand, such as increasing the budget increasing the budget to promote the use of

condoms and programs providing HIV-1 knowledge.. This effort was successful in decreasing the number of HIV-1 infection to around 0.6% in female sex workers (FSFs). Unfortunately, the number of HIV-1 infection in MSM 25% of all infected cases and still remains at around 16% in 2010 (2,6). Moreover, 12.1% of MSM with HIV-1 infection were under the age of 25 (6). Although there are limitations in the diagnosis of early or acute HIV-1 infections in developing countries, Khunakorn et al. reported that the IgG-Capture BED-Enzyme immunoassay was able to diagnose 0.5-0.6% of recently HIV-1 infection in Royal Thai Army conscripts in 2005-2006 (7). This assay has indicated for recent HIV-1 infection that infected for no more than 127 days (8).

HIV is distinctively divided into two groups by non-human primate cross-species infected HIV-1 transmission, namely HIV-1 and HIV-2 (9). HIV-1 is the most abundant distribution and can be further divided into four groups, depending on the HIV-1 genetic divergence variables, namely M (major), O (outlier), N (non-M and non-O) and P (pending) (10). It is important to note that M group is the major HIV-1 group, which can be subdivided to A-D, F-H, J-K and CRF01_AE and this group was the differently variable *gag* and *env* genes around 15% and 25%, respectively (11). Group O is restricted to west central Africa and group N is a strain discovered in 1998 in Cameroon and is found in very rare regions. Group P is a new strain, closely related to gorilla simian immunodeficiency virus (SIV), and was discovered in a Cameroon woman in 2009. HIV-1 diversity is by the means of high rates of mutation, recombination of reverse transcriptase enzyme and viral replication. Viral infected populations develop recombinant forms of replications overtime, namely circulating recombinant forms (CRFs). Molecular and epidemiological studies demonstrated a recombinant form of HIV-1 called subtype E, which is composed of subtypes A and E *gag* and *env* genes, respectively. Subtype CRF01_AE has been extensively found in Thailand and other countries in Southeast Asia (12).

$CD4^+$ T cells destruction and deficient host immune responses lead to the immuno-pathogenesis of HIV-1 infection. After infection, the virus is unable to be detected in the blood stream. This is called the “eclipse phase”. The subsequent production HIV-1 specific antibodies in the first few weeks is called the “sero-conversion” phase (9). The typical course of HIV-1 infection is categorized into three

phases, namely acute, chronic and AIDS. The high level of viral RNAs or acute retroviral syndrome is defined as the acute phase, which usually shown non-specific clinical and abnormal laboratory results. This includes the presence of HIV-1 RNAs and core protein (p24). RNA viruses then decline to a lower steady level after several weeks. This is called the "viral set point" which marks the end of the acute phase and viral specific immune responses against HIV-1 infection can be seen. This is the initial mechanism of viral-host interactions which provides a window for generating potential AIDS vaccines and efficient microbicides mediated HIV-1 infection (13). Moreover, this early event will critically determine both clinical outcome and disease progression. Several successful interventions are facilitated during the acute phase and they are capable of blocking HIV-1 transmissions. Rapid loss of $CD4^+$ T cells and high peak viremia are important conditions in the acute phase. Decreasing viremia corresponds to HIV-1 specific $CD8^+$ T lymphocyte cell functions; however, there are no procedures to eradicate these viruses (14).

Env gene is the target gene for the generation of neutralizing antibodies. Highly diversified genes and glycosylated sites are mechanisms to avoid host immune responses over the course of HIV-1 infection (15,16). Gp120 (surface subunit, SU) and gp41 (transmembrane subunit, TM) are formed on the HIV-1 envelope structures (gp160) that is composed of five conserved and five variable regions which are placed between conserved regions (15). While entering susceptible target cells, HIV-1 binds to the CD4 and chemokine receptors with surface glycoprotein 120 (gp120) and directly develops sera against the CD4 binding site (CD4bs). Env proteins are the most important proteins with typical intrasubtype and intersubtype differences from 20% to 35%, respectively (19). Thus, humoral immune responses against potential Env proteins among the different subtypes are important considered in vaccine designs and therapeutic interventions (17,18).

Moreover, transmitted/founder virus is responsible for the establishment of persistent viral infected population in the early or acute phase and induces anti-HIV-1 host immune responses. This virus is first seen in the mucosa and transported into the lymph nodes and lymphoid organs, especially gut associated lymphoid tissue (GALT) (20,21)in which the potential of viral replication associated with high peak viral load leading to increase selective pressure on specific immune responses against HIV-1

infection. Moreover, the viral variant reveals the genetic and phenotypic quasispecies, but only a single virion is able to high produce the viral replication as well as to generate pathogenic HIV-1 infections (22). R_0 is the basic reproductive infected cell ratio in acute infections. This phase is exponentially viral productions with an estimated R_0 of 8, whereas the number of infections is reduced to below 1 in the chronic phase (23,24). Viremia can be detected in the first few weeks of infection and is commonly used as a crucial diagnostic tool. The early detections are defined by positively HIV-1 specific antigens, followed by anti HIV-1 specific antibodies into Fiebig stages I–VI (25). Fiebig I patients can be tested for viral RNAs by PCR. The level of viral plasma gradually increases and peaks around one month after which it will slightly decrease. The progression of HIV-1 infection from acute to early chronic stage is the end of Fiebig stage V corresponds to 100 days when the plasma viral load continues to plateaus (25). In the chronic phase, asymptomatic conditions can last up to ten years along with the gradual decrease in $CD4^+$T cells and increase in viral levels, until the eventual collapse of immune responses, leading to AIDS (9).

Almost all infected HIV-1 individuals produce specific anti-HIV-1 antibodies, but around 10-25% are specific to vulnerably flourish in broadly neutralizing antibodies against infections.(16). Due to its genetic diversity and inaccessibly conserved regions difficulty sustained the broadly neutralizing antibodies which are proposed to develop HIV-1 vaccine and efficient microbicides (26). Specific anti-HIV-1 neutralizing antibodies are obtained from conserved motifs on the envelope surfaces such as the CD4 binding site on gp120, outer domain, V3 base, quaternary V1/V2, and gp41 at the membrane-proximal epitope region (MPER) (16). Previous study has shown that the humoral immune responses can prevent against SIV infection in non-human primates (27). On the other hand, HIV-1 elicits a small number of broadly neutralizing antibodies in humans; hence, the potent neutralizing monoclonal antibodies are able to inhibit viral entry and control viral replications which are still developed in AIDS field.

Broadly neutralizing antibodies are derived from a specific single clone of HIV-1-infected individuals on susceptible cells and know how to inhibit viral replication.(28). Various broadly neutralizing antibodies have been derived from subtype B HIV-1 infection, namely IgG1 b12, which is directed to overlapping of CD4

binding site epitopes, and 2G12 which is against dominant epitopes in mannose clusters on outer domain at position 295, 332, 339, 386 and 392 of gp120 (29). Two monoclonal antibodies are recognized epitopes at the proximal membrane MPER on gp41, namely 2F5 (ELDKWA) and 4E10 (NWF(D/N)IT) (29,30). All of them are useful for the study of cross-clade properties and potential infectivity. Moreover, specific viral entry is useful for inhibiting viral entry and is a specific, one target therapeutic intervention. T-20 (enfavirdine) is the fusion inhibition and AMD3100 and TAK-779 are the small molecule that directly binds to CXCR4 and CCR5 co-receptor antagonists, respectively (31,32). Neutralization susceptibility measurement is required to appropriate receptor target cells, viral panels and vaccine candidate sera in vaccine evaluation. Primary isolations are difficult and inconsistent propagations leading to invalid measurements of neutralizing antibodies. Thus, many scientists recommended using DNA plasmid encoding full envelope length which is cloned into the infectious proviral DNA (pNL4-3) infectious molecular clone that is constructed a luciferase reporter gene at the downstream on HIV-1 gene that allows a single round of infection. The constructed Env-recombinant viruses examine the potential breaths of neutralizing antibodies and specific viral entries. The important advantages of using Env-recombinant viruses includes easy handling, simple to expand in mammalian cell lines such as HEK 293T cells, is precise, reproducible and easy to standardize in the laboratory (33).

HIV-1 infection is an important problem in Thailand leading to the need to conduct phase III clinical vaccine trials (34). Based on good laboratory practices (GLP) and good clinical practices (GCP), these methods are uniquely practiced in the vaccine research studies (35). Therefore, effective measurements are required for high-throughput assays that are authorized, accurate, and reproducible in the immune responses. In this study, serum samples were collected from Royal Thai Army conscripts who were diagnosed with recent HIV-1 infection by BED-enzyme immune assay (8). Early full length *env* gene was subcloned into proviral DNA (pNL4-3), a molecular cloned luciferase reporter gene, to construct Env-recombinant viruses, which were defined as genotypic *env* characterizations to study neutralization susceptibility to neutralizing human monoclonal antibodies (NHMAbs), pooled patient’s serum, and specific viral entry.

CHAPTER II
OBJECTIVES

Specific anti-HIV-1 antibody measurements in vaccine trials must be accurate, reproducible and in accordance to good laboratory practices (GLPs). Encoding full-length *env* gene on viral plasmid DNA (proviral), it is co-transfected into target cells which are individually presented the different co-receptors either CCR5 or CXCR4. Env-recombinant viruses have been examined the potential viral infectivity and co-receptor usage. Therefore, the constructed Env-recombinant virus or pseudotype virus is a valuable tool for studying the neutralization susceptibility with neutralizing human monoclonal antibodies (NHMAbs) and specific viral entry (35,36). Importantly, it can be standardized to evaluate the specific anti HIV-1 humoral immune responses in vaccine candidates. Besides, the inaccurate humoral antibody measurements and the inconsistent and difficult HIV-1 primary isolate propagations allow to recommend using early *env* gene in the acute or primary phase of HIV-1 infection for facilitating to obviously understand the transmitted or founder virus and host immune response interactions and determined developed on therapeutic treatments and AIDS vaccine designs (33).

General objective

To characterize genotypic and phenotypic early *env* gene from acute HIV-1 infected Thai individuals.

Specific objectives

2.1 To extract full length HIV-1 *env* genes of peripheral blood serum samples from Royal Thai Army conscripts.

2.2 To construct Env-recombinant viruses

2.3 To study Env-recombinant virus infectivity

2.4 To study phenotypic and genotypic early *env* genes.

2.5 To study neutralization susceptibility to early Env-recombinant viruses with neutralizing human monoclonal antibodies (NHMAbs), specific antagonist co-receptors; CXCR4 antagonist, AMD3100, CCR5, TAK-779 and a fusion inhibition (T-20).

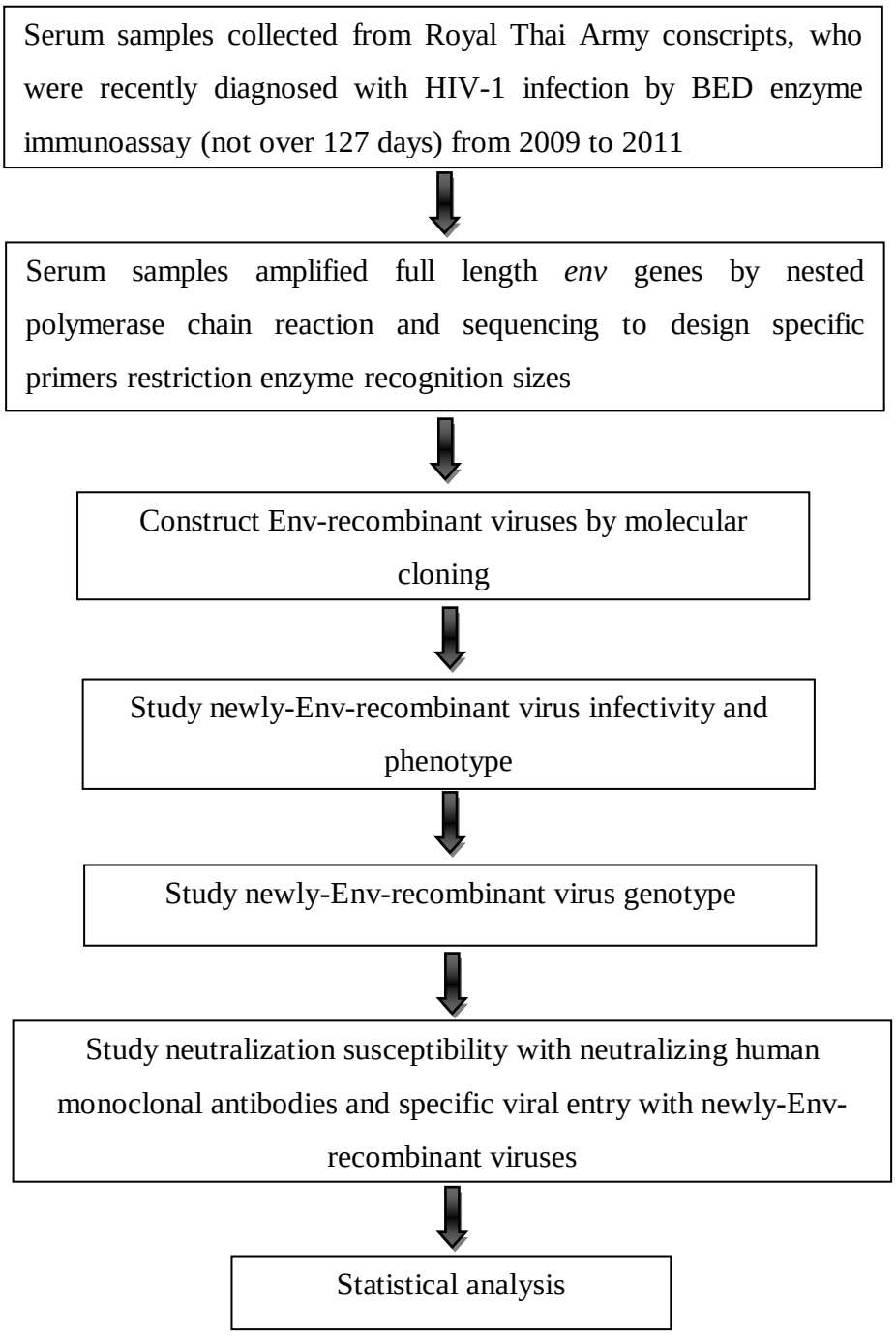
Research question:

Does early *env* gene on Env-recombinant virus show high infectivity and high neutralization susceptibility?

Research hypothesis:

The characteristic of early *env* gene correlates with neutralization susceptibility to neutralizing human monoclonal antibodies and specific viral entry functions.

Research frame work



CHAPTER III
LITERATURE REVIEW

3.1. Biology of Human Immunodeficiency Virus type 1

3.1.1 HIV-1 viral particle

HIV-1 particle is a roughly spherical shape with around 80-120 nanometer in diameter that is a different structure from the other retroviruses. The HIV-1 particle contains many components required for entry into the host cell for viral transcription, integration into the host's DNA and replication of virus particles. The identical single stranded RNA molecules associated with reverse transcriptase proteins are packed in viral particle as well as it encodes the nine genes enclosed by a conical capsid that consists of 2000 copies of the Gag protein p24 (37). This particle is surrounded with lipid membrane containing both cone-shaped capsid core proteins (p24) and matrix proteins (p17). Additionally, the trimeric gp41 and gp120 inserted into the lipid bilayers are called transmembrane glycoprotein and envelope glycoprotein, respectively. (Fig 3.1) (38) This envelope protein enables the viral to attach and fuse another target cell to start viral life cycle. Both of surface proteins are as the target to HIV treatment and vaccine design, especially, gp120 on envelope gene.

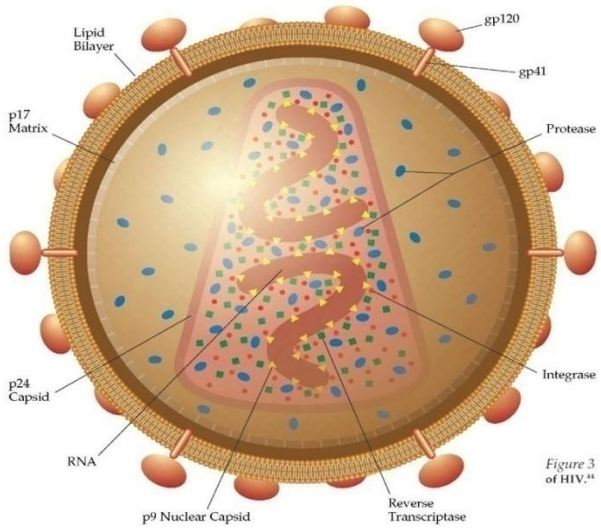


Figure 3.1. The structure of HIV-1 particle is around 80-120 nm in diameter and packed with identical genomic RNAs associated with reverse transcriptase, protease and integrase enzymes. The matrix proteins (p17) and capsid core proteins (p24) are enclosed by lipid bilayer derived from host cell membranes. Trimeric envelope glycoproteins composed of surface glycoproteins (SU, gp120) and transmembrane glycoproteins (TM, gp41) penetrated into the lipid membrane (38).

3.1.2 HIV-1 genome

HIV-1 genome is around 9 kb in length and is encoded from identical single stranded RNA by a reverse transcriptase enzyme. It contains the nine genes including three important structural genes, *gag* (*group specific antigen gene*) encoded assembly proteins which are nucleocapsid (NC), p17 (Matrix Antigen, MA) and p24 (Core Antigen, CA), *pol* (*polymerase gene*) encoded several enzymes which are reverse transcriptase, integrase and protease for viral replication and *env* (*envelop gene*) encodes proteins for binding and fusion between viral particle with cell-free target. The regulatory genes *tat* (*transactivator of transcription gene*) is required for the positive regulation of transcription and *rev* (*regulator of expressed virion*) allows for the transport of unspliced and partially spliced transcripts from the nucleus to the cytoplasm via nuclear pore complex. The accessory genes *vif* (*viral infectivity factor gene*) is required for partial infectivity, *vpu* (*viral protein U gene*) is the progenic virion maturation, *vpr* (*viral protein R gene*) transports DNA to the nucleus, increases virion productions and cell cycle arrest, and lastly *Nef* (*negative factor gene*) promotes

viral replication. LTRs (long terminal repeats) located at the two proximal viral DNA genome regions, control gene expression. (Fig 3.2) (39).

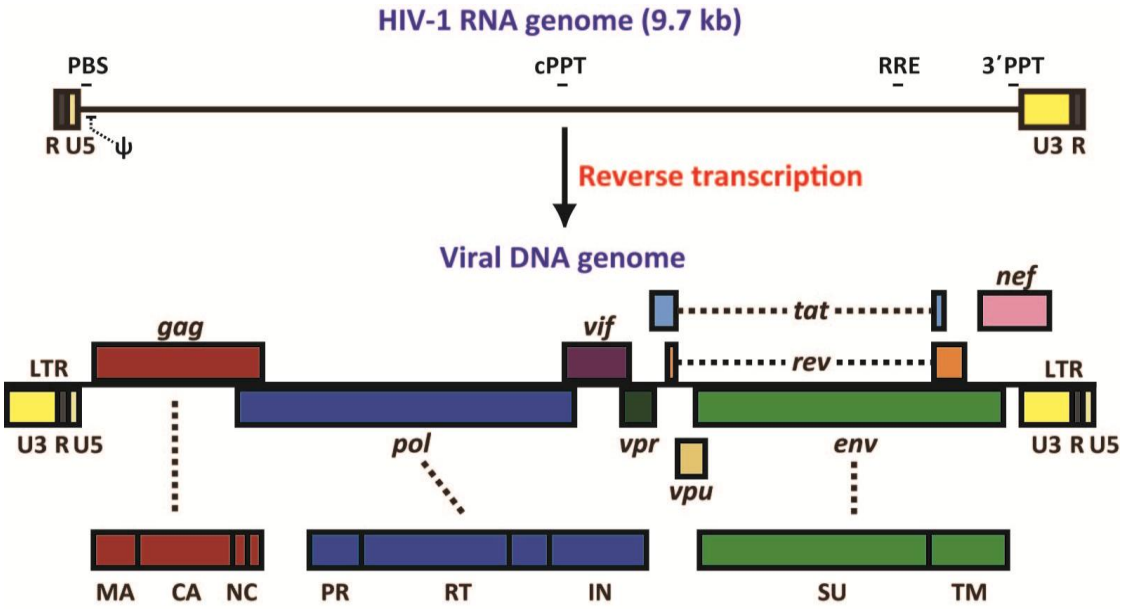


Figure 3.2. Viral DNA genome consists of the predominant structural proteins including *gag*, *env* and *pol*. Gag encodes matrix protein, core protein and nucleocapsid protein whereas Env encodes surface and transmembrane glycoprotein. Pol gene encodes three important enzymes for viral replication including protease, reverse transcriptase and integrase. The regulatory protein consists of *tat* and *rev* genes and accessory protein includes *vif*, *vpr*, *vpu* and *nef* genes. They have also played several mechanisms of gene transcriptions and translations (39).

3.1.2.1 Structural gene

3.1.2.1.1 Group specific antigen gene (p55)

Gag gene is 55-kilodalton-structural protein forming C to N terminus. Gag polyprotein domains are divided into the three conserved proteins in the retrovirus including matrix protein (MA, p17), capsid core protein (CA, p24) and nucleocapsid protein (NC, p9). They are cleaved by viral protease enzymes and rearranged to assemble viral infectious particle maturation (40).

3.1.2.1.2 Matrix protein (MA, p17)

MA protein is responsible for releasing HIV-1 protease in the late stages of infection. Its polypeptides attach to the inner surface of

viral lipid membranes and the functions are to stabilize viral particle core and to build the block of mature viral formations. MA molecule supports the cellular nuclear import machinery as a result of transported viral DNAs into infected nucleus target cells. The flexible C-terminal helices extended away from the membranes and closes to central viral variants (41).

3.1.2.1.3 Capsid protein (CA, p24)

CA is also called core protein. It is located at the central viral particle containing viral RNA and important enzymes. HIV-1 core protein, N-terminal domain two β-hairpins, C terminal domain seven α-helices and external loop, contains not only conserved amino acid proline residues but also buried aspartate residues as a result of a salt bridge formation. The proteolytic processing of the MA-CA interaction can form the N-terminal β-hairpins which are the important condensation of the core protein and capsid assembly (42). The oligomerization of C-terminal α-helices is determined the amino acid residues 146-231 and 151-231 which allow packing interactions between N-terminal of CA and entire HIV-1 CA dimer (43). In addition, CA binds to cyclosporine A (CypA) leading to packaging the copies of CypA into HIV-1 particle and this interaction are disrupted enhancing to inhibit viral replication (44) which are used for developing HIV-1 microbicides.

3.1.2.1.4 Nucleocapsid protein (NC, p9)

NC is composed of two domains which are amino-terminus (F1) and caboxyl-terminus (F2) linked by linker sequences (RAPRKKG) that are located next to the end of 5′ long terminal region viral DNA. These functional domains are the recognition and packaging of viral genomes (45). Its function is to important viral structure and to interact of the interprotein NC-NC formation (43).

3.1.2.2 Pol genes

3.1.2.2.1 Protease (PR)

Gag-Pol precursors contain the several viral enzymes namely protease, integrase, Rnase H and reverse transcriptase which are the fusion productions. PR is discovered in the first HIV-1 protein which is composed of a symmetric homodimer and stabilized by four stranded anti-parallel β-sheets from N to C terminal domain. The active sites are located at the surface connecting with two subunits including catalytic triad, Asp25-Thr26-Gly27, response for cleave PR

activities. Its structures can improve the important of HIV-1 treatments. N-terminal protease is close to the inner surface of envelope lipid membranes whereas its C-terminal proteins assemble into the core viral protein (45).

3.1.2.2.2 Reverse transcriptase (RT)

RT is encoded by *pol* gene which has RNA-dependent and DNA-dependent polymerase activities. The functional RT is to convert single-stranded RNA into complementary stranded of DNA in the cytosol during viral entry and Rnase H will remove the initial RNA template. The properties of RT are predominantly enhanced the error prone replication and no proof- reading functions leading to several point mutations in viral copies genome (46).

3.1.2.2.3 Integrase (IN)

Crystal IN protein structure consisting of N-terminus facilitates oligomerization, a central catalytic domain for catalytic activity and C- terminus for initially DNA binding domain (41,47). IN is a pivotal enzyme for incorporating viral DNA into chromosomal DNA of infected cells. This protein will recognize by new viral DNA at $5^{/}$ and $3^{/}$ end of LTR and cleave two or three bases at $3^{/}$ end into sticky end and then inserted viral DNA to cellular host DNA in nucleus which is called provirus (48).

3.1.2.3 Env gene

Env precursor glycoprotein (gp160) is generated and undergone N-link glycosylation in the endoplasmic reticulum. The protease protein cleaves *env* polyglycoproteins into trimeric glycoproteins namely transmembrane subunit (TM, gp41) and surface subunit (SU, gp120). Gp41 locates at the furin site whereas gp120 that contains five variable regions interspersed five conserved regions (Fig 3.3); therefore, trimeric *env* gene elicited on surface and infecte other adjacent cells by binding and fusion processes (25,49).

3.1.2.3.1 Transmembrane subunit (TM, gp41)

Gp41 is estimated 345 amino acid residues and is located at the cytoplasmic tail (CT). It is composed of an ectodomain, a single membrane spanning domain and a long C-terminal tail. CT domain contains several lentiviral lytic peptides namely LLP-1, LLP-2 and LLP-3 which are utilized for Env-fusogenicities, protein stabilities, multimerizations, cell-surface expressions and

incorporations. The important motifs are YXXL for endocytosis process. Kennedy sequences are the target of neutralizing monoclonal antibodies (50,51) (Fig 3.3). TM domain contains around 25 amino acid residues that hang on the lipid bilayers. The core of TM mutations can enhance Env-mediated fusion mechanism (52) containing 24 amino acid residues. Tryptophan-rich residues are called the membrane proximal external regions (MPERs) (53). N-terminal hydrophobic extracellular domain form two α helical coiled-coil structures which are so called HR1 and HR2, used for fusion process. Generally, fusion peptides are buried in quaternary complex gp120/gp41 that are exposed for fusion and penetration into target cell membrane.

3.1.2.3.2 Surface subunit (SU, gp120)

SU contains five variable regions (V1-V5) that intersperse with five conserved regions (C1-C5). From V1 to V4 elicits in the various loops which are linked by disulfide bonds at their bases for evading host immune responses. Its core protein consists of inner and outer domains connected by bridging β sheet which is from N to C terminus accumulating CD4 receptor complexion for generated neutralizing antibodies (54). Binding between gp120 and CD4 molecule of target cells enhances the exposure V3 loop gp120 for second binding with co-receptor leading to conformational changes of gp120 and signal to gp41 as a result of membrane fusion in the initially HIV-1 life cycle (55). Moreover, V3 loop, C2, C3 and C4 domains show changes in few envelope lengths. The potential *N*-linked glycosylation mechanism supports viral evasion of host immune responses (56). Env folding facilitates the important binding between viral cell surface and receptors on targeted cells (57). Interestingly, the recombinations, insertions, deletions and point mutations can occur on variable domains and the most N-linked glycosylation sites (PNLGs) may be elicited on V1/V2 loop. Not only the wide range of 50-90 amino acids but also the number of PNLGs on V1/V2 loop is related to disease progression evading the humoral immune responses (58). CD4 receptor initially interacts with the conserved regions on effective V3 epitopes with high affinity for generating neutralizing antibody (59), in addition, gp120 has around 25-30 *N*-linked glycosylation sites whereas around 3-5 *N*-linked glycosylation sites are on gp41.

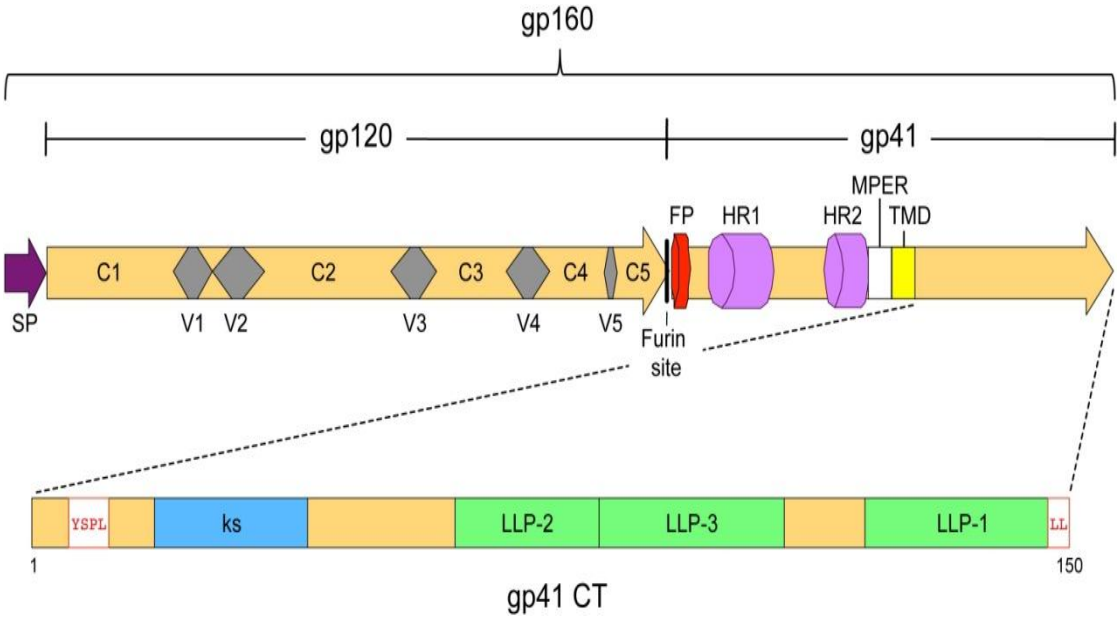


Figure 3.3. The linearization of gp160 has been predominantly shown in two regions containing surface (SU, gp120) and transmembrane subunits (TM, gp41). The protease cleaving site is located at furin as shown in red color. Gp120 consists of five variable regions (V1-V5) and five conserved regions (C1-C5). Moreover, HR1 and HR2 on gp41 serve as fusion mechanisms in early or acute phase of HIV-1 life cycle. The large section presented the part of gp41 containing the several lentiviral lytic peptides (LLP-1, LLP-2 and LLP-3), Kennedy sequences that is the independent motif (YXXL) for exerted endocytosis process (48,49).

3.1.2.4 Regulatory proteins

3.1.2.4.1 Tat gene (trans-activator of transcription gene)

Tat gene is initiated at the transcriptional proviral DNA (HIV-1 promoter) that is estimated 72-101 amino acid residues which is located at the end of 5′ LTR and exhibited in the early fully spliced mRNAs and late incompletely spliced HIV mRNAs which are the essential functions for HIV-1 replication. Tat function is able to enhance transcriptional elongation by recruiting the several transcriptional factors such as Tat-cyclin T, CDK 9 and P-TEFb incorporating with RNA polymerase II, NF-kb, SP1 and TBP (Fig 3.6) which bind to the *trans*-activating response element (TAR) leading to translate the functional proteins including tumor necrosis factor β (60,61), transforming growth factor β (62) and down-regulate Bcl-2 mediate HIV-1 pathogenesis (63).

3.1.2.4.2 Rev gene (regulator of expressed virion)

Rev gene is 13-kD specific-RNA sequences binding to Rev-response-element (RRE) which is located within unspliced viral mRNA leading to transcriptional unspliced and incomplete spliced viral mRNAs (containing introns) from nucleus to cytoplasm for generating viral productions. Rev protein transports the high number of full length and multiple spliced mRNAs (64) for translating viral proteins. Normally, Rev protein contains three important regions including arginine-rich domain allow to binding RRE (36), multimerized domains to stimulate Rev functions (65) and effecter domain for a specific nucleus export signal (NES) (66).

3.1.2.5 Accessory proteins

3.1.2.5.1 Nef gene (*negative factor gene*)

Nef protein can be recognized after HIV-1 infection. It is 27-kD, contains around 206 amino acid residues and extended at 3' LTR. It important achieves maintaining the high level of viral loads as a result of AIDS. These functions not only facilitate HIV-1 viral replication but also reduce the number of CD4 receptors expressions. It can perturb the T cells activation by the downregulation of MHC class I (67). Nef increases the CD4 endocytosis ratio and degradation process via directed binding with cytoplasmic tail CD4 receptor and eliminating premature Env-CD4 binding site. Nef protein supported the virion incorporations and promoted releasing infectious viral particles (68). Moreover, Nef-defective virions enhance with AIDS-like disease but onset of disease was delayed the disease progressions (69).

3.1.2.5.2 Vpr gene (*viral protein R gene*)

Vpr protein is the basic protein, 14-kD and is made up of around 96 amino acid residues. Each virion has approximately 100 copies and has functions in HIV-1 infected non-dividing cells and directly interacts with the carboxyl-terminus of p55- Gag correspondence of p6 protein proteolytic process (44). Vpr is presented in the Pre-integration complex (PIC) and acts as a nucleo-cytoplasmic transport factor by directly controlling the viral genome into nucleus infected cells via nuclear pore complex (NPC). Vpr protein prevented the cell cycle for entry into mitosis by activation of the p34cdc2/cyclin B complex (70).

3.1.2.5.3 Vpu gene (*viral protein U gene*)

Vpu polypeptide is 16-kD which is expressed on viral mRNA encoding *env* gene which is controlled by *rev* gene. Vpu functions are the down-modulation of CD4 receptor and increasingly secreting virions. It controls the viral envelope by stimulating the ubiquitin-mediated degradation of CD4 molecule complexed with *env* gene (71).

3.1.2.5.4 Vif gene (*viral infectivity factor gene*)

Vif gene is around 192 amino acid residues (23-kD) and is the predominant viral replication in peripheral blood mononuclear cells (PBMC), macrophages and certain cell lines (72). It makes from a single spliced mRNA in the late of HIV-1 replication and counteracts with host antiviral cellular factor. The apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) functions to increase viral particle incorporations and inhibits reverse transcriptase through a deamination-independent mechanism (73).

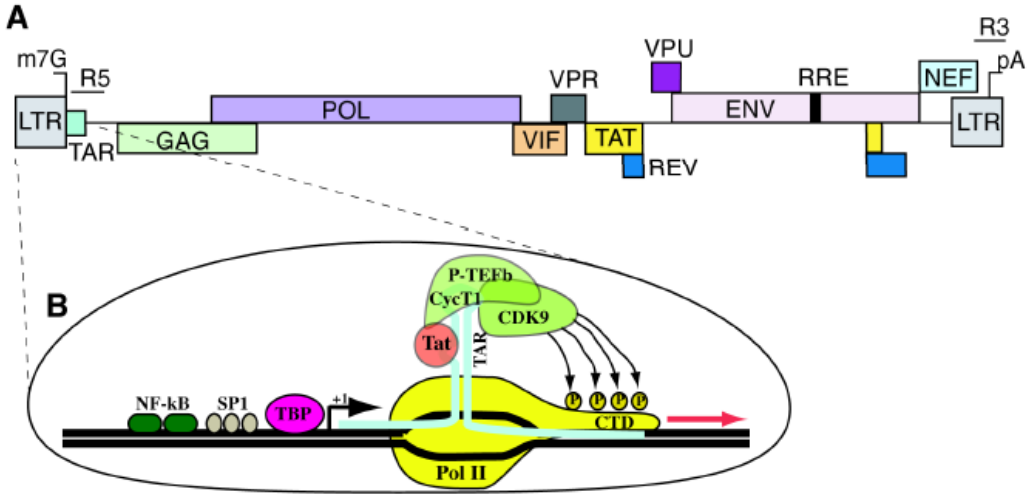


Figure 3.4. A) HIV-1 genomes have been shown the various HIV-1 genes as proviral genomes. B) A number of cellular transcription factors include two tandems of NF-kB and three tandems of SP1 that join onto 5′ long terminal repeat (LTR). Tat-Tar interaction promotes the recruitment of the cyclin T1, positive transcription elongation factor b (P-TEFb), and CDK9 which phosphorylate the C-terminal domain (CTD) of RNA Polymerase II facilitating elongation transcriptions and gene expressions (50).

3.1.3 HIV-1 life cycle

HIV-1 infections can infect various types of immune cells including macrophages, activated $CD4^+$ T cell, resting $CD4^+$ T cells, quiescent $CD4^+$ T cell, peripheral blood mononuclear cells and dendritic cells in the lymph nodes and hematopoietic stem cells (74,75) (Fig 3.5). Its life cycle divided into early and late phase. The predominant early phase is not only the entry into target cells but also the integrated into host genome. The late phase includes full proviral genome replications (76) (Fig 3.6).

3.1.3.1 Early or Acute phase

HIV-1 life cycle initially occurs after viral binding and fusion with cell membranes and requires both CD4 and co-receptors, which are either receptor type 5 (R5), receptor type 4 (X4) or makes it seem like HIV can use both X4 and R5 at same time. After binding between gp120 and CD4 receptor on target cells as a subsequent conformational change of gp120, V3 loop is exposed for binding co-receptors. As a result of the conformational changes of gp41, HR1 and HR2 are formed in the mutually six-helix bundle structures and to completely fuse with the host cell membranes, which releases HIV-1 core containing identical single stranded viral RNAs, tRNA as primers, viral integrase and reverse transcriptase into cytoplasm of target cells. Utilizing reverse transcriptase, uncoated RNAs are converted into linear double stranded complementary DNA (dscDNA) and this process takes place for more than three hours. Pre-integrated complex (PIC) is included linear dscDNA, integrase, matrix protein, retro-transcriptase, viral protein R (Vpr) and various types of host proteins such as HMG I(Y), the high-mobility group protein B1, which is an important protein in chromatin and supported to control transcription (77), LEDGF/p75, the lens epithelium-derived growth factor, facilitated viral integration by binding with integrase protein as a transcriptional co-activator (78). PICs are transmitted by passing to the nucleus of infected cells via nuclear pore complex (NPC) and then viral DNA integrates into host genome so called proviral.

3.1.3.2 Late phase

Binding between proviral and enhancer elements at 5′ LTR proximal promoter for gathering transcriptional host factors such as nuclear factor-kB (NF-kB) (79), nuclear factor of activated T cells (NFAT) (80) and specificity protein 1 (SP1) (81), initially induce basal factors and promotes RNA polymerase II for binding at TATA box and produce a number of viral mRNAs. Moreover, trans-activation response element (TAR) is a stem loop of around 59 amino acids at the 5′ end of the nascent viral transcript for binding of Tat protein promoting efficient elongation of viral transcription in the cytoplasm (82). Viral transcriptions compose of unspliced and multiple spliced mRNAs which create to genomic RNA and viral proteins, respectively. The viral proteins are synthesized to be a large precursor polyproteins which are cleaved by viral protease and assemble to newly mature virions and budding, infecting adjacent other target cells (Fig 3.6).

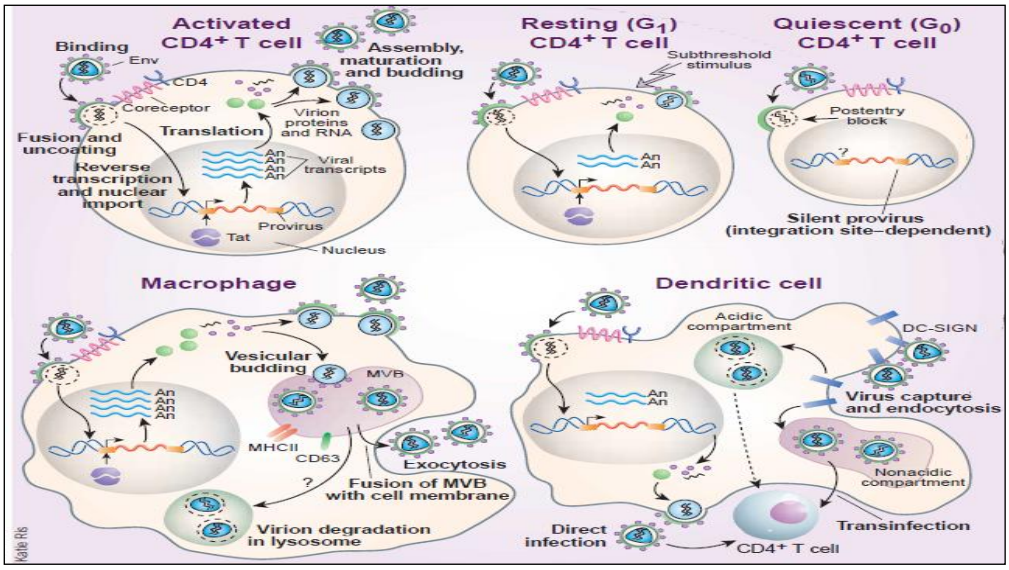


Figure 3.5. Viral replication is efficient and has rapid cytopathicity in an activated $CD4^+$ T cell that contains long terminal region for binding of the regulating host cellular factors and Tat protein enhances gene expression together with in macrophages. Resting $CD4^+$Tcell is entered to G_1 but it is subthreshold stimulations to activated cells and slowly decay in vivo. Tat is limited by rates in quiescent $CD4^+$Tcell (G_0) leading to transcriptional factor silencing. The level of basal transcriptional factors for gene expression is controlled by the site of proviral integration in these cells which occur before cells quiescence. Macrophages are obtained virions at membranes of multivesicular bodies which are cytoplasmic compartments enriched MHC class II. These are virions abundantly released after fusion between virions and plasma membrane of mutivesicualr bodies. They are then degraded by the late lysosome. Dendritic cells directly bind to virions by DC-SIGN entering the acidic compartment leading to degradation. Some virions escape degradation by entering in the non-acidic compartment. In addition, some virions initiate infection in adjacent CD4 T cells in trans by fusion with endosome containing virus at the plasma membrane (74).

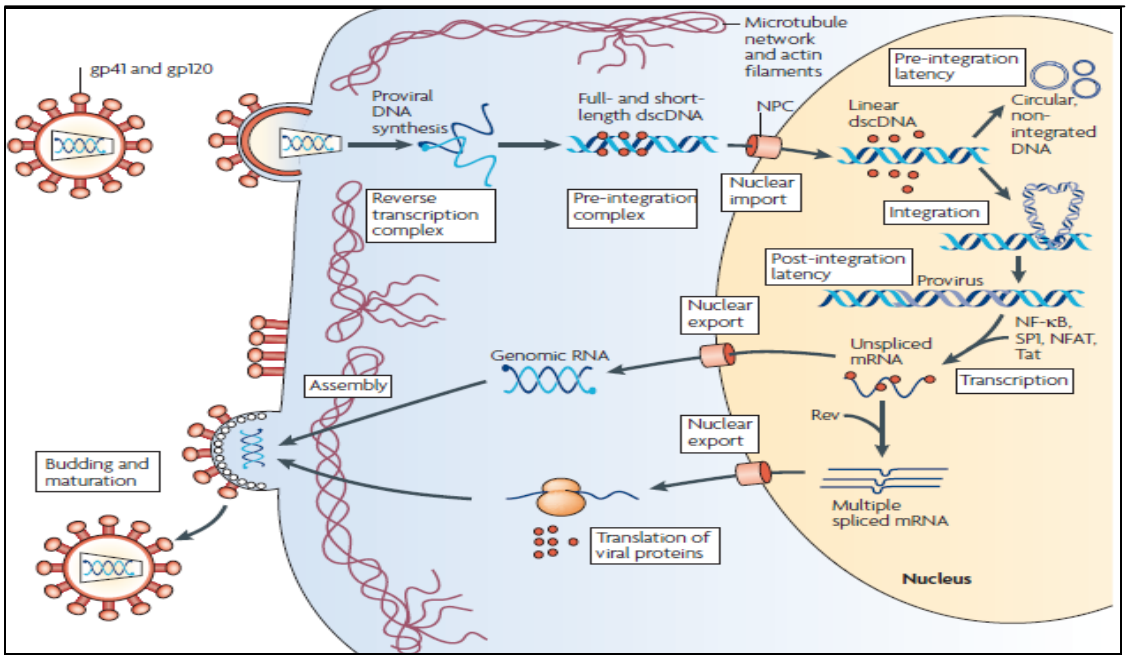


Figure 3 6. HIV-1 life cycle and latency are obtained in HIV-1 infection individuals. After binding and fusion between gp120 and CD4 and CC chemokine receptors including CCR5, CXCR4 and both which are determined to viral tropisms. The HIV-1 core proteins will be released into the cytosol of infected target cells and retro-transcribed to double-stranded cDNA which can either stay in the cytosol with high yields in transient forms or reversible pre-integrated latent forms, or pre-integrated complex (PIC) containing dscDNA so called post-intregration latent forms or provirus. Next, it is transcribed into a number of mRNAs in nuclear which consisted of unspliced mRNAs and multiple spliced mRNAs presenting Rev. Viral polyproteins are translated by translation in the cytoplasm in which these proteins are cleaved by viral protease into assembly viral proteins and budding mature virions for infection of adjacent cells (76).

3.1.4 Structural HIV-1 *env* genes

Approximately 845 to 870 amino acids are the precursor of envelope gene synthesis in HIV-1 infected cells in rough endoplasmic reticulum so called gp160 glycoproteins linked with asparagine and high mannose sugar chain adding in Golgi apparatus and cleaved into both gp120, surface subunit (SU), and gp41, transmembrane subunit (TM). Gp160 is grouped to oligomeric complexes as a trimer. Env predominantly incorporates binding and fusion with CD4 receptor and co-receptors entering target cells. Gp120 and gp41 are assembled the linked trimer with non-covalence and easy changeability. Env gene is located at position 6625-7758 nucleotides correspond with subtype B HXB2 whereas with subtype CRF01_AE. Gp120 consists of five variable regions (V1-V5) which intersperses with five conserved regions (C1-C5) (83) (Fig 3.3) and induce the conformational changes after viral binding with CD4 receptor leading to expose V3 and co-receptors (CCR5 and CXCR4) allowing fusion to occur (84).

3.1.4.1 HIV-1 gp120

3.1.4.1.1 Constant region 1 (C1). It is a conserved region 1 that is located at N-terminus gp120 and its plays an important role in furin recognition site and used for the initial drug interventions (85). C1 interacts with the various types of monoclonal antibodies such as 7E2/4 and M85 which its domains are exposed for binding around 31 to 40 amino acids (86).

3.1.4.1.2 Variable V1/V2. It is located at 137-211 and 316-370 amino acids (Fig 3.3) (48,87). V1 and V2 connect together via disulfide bond of which other variable regions form the independent loop of gp120. Moreover, their functions are effective infectivity, resistant neutralizing antibodies, viral phenotypes and co-receptor usages. Either partial or full V1/V2 deletion affect to viral infectivity and replication (87). Increasingly V1/V2 lengths and PNLG sites during HIV-1 infection are shown in chronically infected HIV-1 (88). In contrast, V1/V2 envelope lengths have been correlated in the early host immune responses (89).

3.1.4.1.3 Constant region 2 (C2). This region has been involved in Ca^{2+} specific dependent carbohydrate binding properties as a result of binding with alternative $CD4^{+}$cells. C2 region displays high calcium concentrated

oligomerizations eliciting both native and recombinant gp120. Furthermore, it also possesses fusogenic abilities in HIV-1 host cell ranges (90,91).

3.1.4.1.4 Variable region 3 (V3). Typically, V3 length is around 35 amino acids and ranges from 31 to 39 amino acids, which include α-helical, β-sheet and bridging sheet. V3 property contains highly variable regions, glycosylated sites and cysteine residues (92). V3 importantly determines viral entry, co-receptor usages and neutralizing antibodies (93). N-terminus of co-receptor binds to V3 base attaching to second extracellular loop of co-receptor, which highly contains conserved Pro-Gly residues, facilitating the conformational changes of gp41mediated viral fusion. V3 is required to balance between functional conservation and antigenic variations. V3 is a neutralizing antibody target for developing vaccines and drug target designs (94). Moreover, it determines the viral tropisms and disease progressions (93). However, two conserved motifs are mostly shown on V3 loop namely GPGR and GPGQ which are commonly elicited in subtype B and other subtypes, respectively (95).

3.1.4.1.5 Constant region 3 (C3). C3 occurs as an oligomeric gp120 complex, their conserved region mostly interacts with gp41, which is associated with co-receptor usage on target cells (90). Asn-368 and Glu-370 on C3 region are critical residues and binds to the CD4 receptor, blocks monoclonal antibodies and inhibits macrophage inflammatory protein 1 α (MIP-1α) (96,97).

3.1.4.1.6 Variable region 4 and Constant region 4 (V4, C4). The principal sequences, EVGKAMYAPP (429 to 438 residues) have been shown on the surface of C4 domain that poorly generates neutralizing antibodies especially compared with V3 loop. For instance, V4 polymorphisms elicit high point mutations include deletions and insertions as a consequence of changing amino acid patterns in HIV-1 proviral DNA and viral RNA plasma (98). This region is a highly glycosylated sites and quasispecies in the early chronic HIV-1 infection. The virally conformational change relies on defensive immune responses and immune selective pressures (99).

3.1.4.1.7 Constant region 5 (C5). C5 is locates at around 489-511 amino acid residues of HIV-1 strain HXB2 corresponding to C-terminus of gp120 and directly interacts with C1 and C2 domains forming the complex

for binding with gp41. Interestingly, gp41/gp120 complex is stabilized by C1 or C5 domain (100).

3.1.5 HIV-1 entry and fusion processes

Binding between viral surface membrane and cell target, requires of the multistep fusion processes and subsequent viral core transportation into the cytoplasm. The CD4 binding site on CD4 target cells and trimeric *env* gene interaction which induces gp120 conformational change lead to expose V3 loop for approaching co-receptors on the surface of target cells either CCR5, CXCR4 or both. The major HIV-1 coreceptors are the chemokine receptors requiring the permissiveness of the infected cells corresponding to CCR5-using (R5) or CXCR4-using (X4) HIV-1 strains. After binding between CD4 receptor, chemokine receptors and viral membrane, the major conformational change of domain in N- and C- terminal gp41 region (termed HR1 and HR2, respectively) formes to a six-helix bundle before fusion which brings both cellular membrane and virion close together, allowing the pore formation and subsequent virus internalization (101) (Fig 3.7).

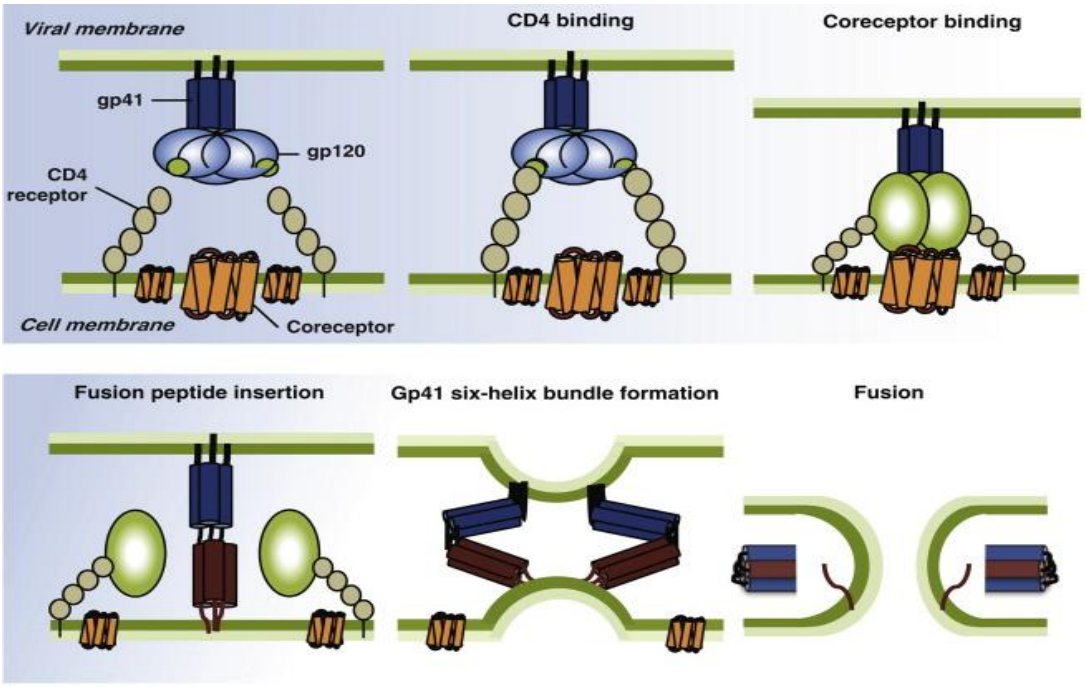


Figure 3.7. The following diagram shows how the virus enters the target cell. The process begins with the binding of gp120 and CD4 which induces a conformational change of gp120 and then exposes the V3 loop to co-receptors CCR5 or CXCR4 which encourages the viral entry to target cell. This binding process results in dissociation of gp120 from the gp41six-helix bundle formation and allows it to penetrate and enters the host cells (102).

3.1.6 Natural history of HIV-1infection

The balance between HIV-1 viral isolations and immune responses, determines the wide range of outcomes form rapid to long-term progressions. The directly exposed bloods or secretions on damaged skin are sites for transmission of viral HIV-1. Indeed, this virus is capable of surviving and replicating in living cells that transfer the viruses to the local immune cells such as T cells, macrophages, dendritic cells and the others on vaginal and mucosal anal rectum. Interestingly, $CD4^+$ lymphocytes and dendritic cells allow the virus to spread into regional lymph nodes and lymphatic tissues through receptor-dependent mechanisms. After replicating and spreading, they are subsequently entered into bloodstream defining on the early stage of infection (103). The viral particles can be found on memory and activating $CD4^+$ T-cells and which are the important cells empowering cytopathicity and persist in

permanent reservoirs, especially, macrophages, resting $CD4^+$ T cells and quiescent (G_0) $CD4^+$ T cells (Fig 3.5) (104).

The natural history of HIV infection starts in the first few weeks after eliciting viremia, providing detection and a potential transmission of viral infection. HIV-1 specific antibody appearance is so called “sero-conversion” whereas HIV-1 specific antibody is undetected in the window period. After several weeks, viral RNA declines to a lower steady level so called “viral set point” at the end of acute phase. From two to more than ten years, ranging in chronic infection or “asymptomatic phase” by high levels of $CD4^+$ lymphocytes corresponding to decreasing viremia. However, $CD4^+$ lymphocytes are unable to eradicate these viruses. During the asymptomatic stage, a number of $CD4^+$ lymphocytes are progressively lost and the functional immune impairments are the privation of $CD4^+$ lymphocytes including the direct killing of competent immune cells, the increased induction apoptosis of infected cells and direct killing the infected $CD4^+$ T cells (105). The progressively declined $CD4^+$ lymphocytes and increasingly viral variant leads to become opportunistic infections or “AIDS phase” (Fig 3.8) (106).

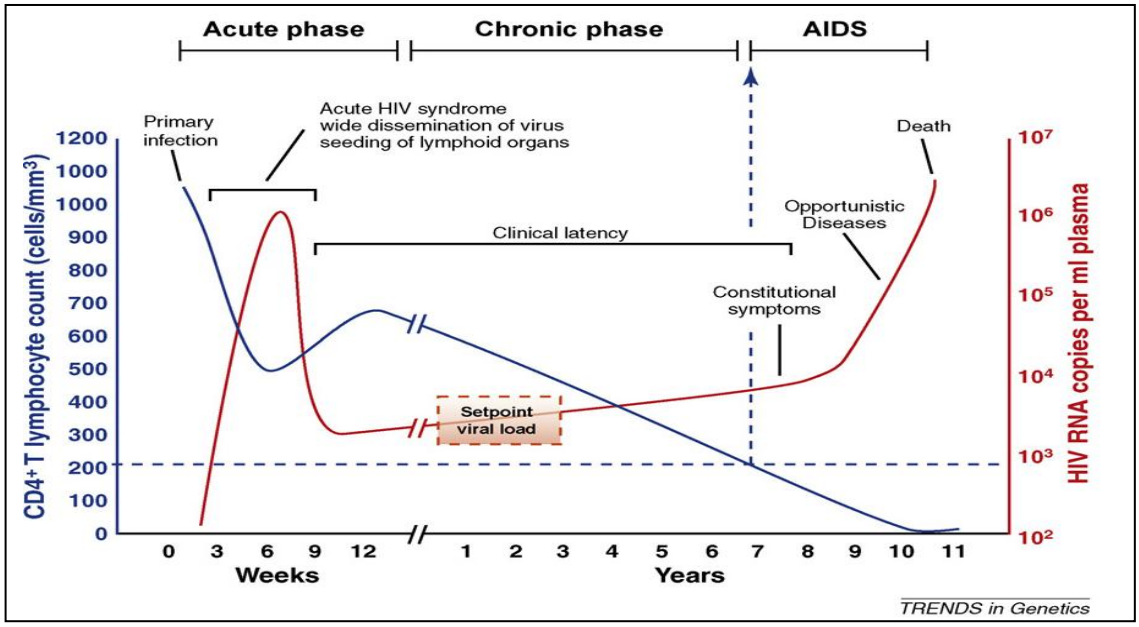


Figure 3.8. The peripheral blood CD4 T cells and plasma viral load in typical courses of HIV-1 infection are shown in three major phases namely primary infection (high viral load), clinical latency (low viral load, $CD4^+$ cells less than 200 cells/mm^3, and AIDS (opportunistic diseases are occurred by high viral load and low $CD4^+$ cells. The initial stage is called eclipsed phase, a stage before systemic viral dissemination. The acute phase is characterized by flu-like symptoms and a peak of viral load, followed by a drop of viral set point which is the end up of acute phase. During the second phase or an asymptomatic chronic phase, viral load is gradually increased while CD4 T cells are decreased to the level of 200 cells/ml; a point defining AIDS occurrence (106).

3.1.7 HIV-1 pathogenesis

HIV-1 pathogenesis is not only destroys of helper $CD4^+$ T-lymphocytes but also impairs immune responses relating to disease progressions and initial treatments. These viruses enter into infected cells requiring both CD4 molecule and co-receptors that determine HIV-1 strains. Arguably, the viral co-receptor usages are either CCR5, CXCR4, or both, which relate to progressive diseases (107). Nearly all viruses are CCR5 co-receptor usages in early phase, M-tropic, whereas CXCR4 co-receptor usages are T-tropic in the late phase (108). In particular, high HIV-1 variation between viral genomes and mutations are important antigenic shifts, major change of an antigenic on the surface of the pathogenic micro-organisms, and increasing virulence. Generally, viruses replicate in lympho-reticular tissues such as lymph nodes, spleen, gut-associated lymphoid cells, macrophages, follicular dendritic cells and activated $CD4^+$ T cells. HIV-1 is associated with strong cytopathicity, affecting the fusogenic capacity between viral envelope and Vpr protein which contributes to the direct of killing infected cells (109). Moreover, the most important replications of HIV infection have been found in apoptosis of bystander cells (110), especially, infected HIV-1 $CD4^+$ and $CD8^+$ T cells have undergone spontaneous apoptosis *in vitro* (111).

The recombinant forms occurring in infected cells are known as circulating recombinant forms (CRFs). They are the results of the template switching between two genetically different HIV genomes which remain fixed and extendable in the population (112). Rangsin R et al., studied CRF01_AE HIV-1 subtype distributed in Thailand reported a 3-year shorter median survival compared to other subtypes (113,114). Moreover, this subtype was strongly correlated to $CD4^+$T cells loss, especially a mean 58 cell/mm^3/year greater and faster time to treatment than subtype B (115). HIV-1 is transmitted via sexual intercourse, needle drug abuse, transfusion with contaminated blood and maternal-child transmissions. After 3 to 5 weeks infection, patients may present symptoms resembling flu-like illness, fever, maculopapular rash, oral ulcers, lymphadenopathy, arthralgia, pharyngitis, malaise, weight loss and myalgia (109). Viral replication continues to the chronic phase and pathogenic effects still persist in slow progression, but the reduction of $CD4^+$ lymphocytes and aberrant of immune responses facilitate to opportunistic infections by bacteria, fungi, viruses

and parasites, and tumors. The estimate time onset to AIDS-related death is around 11 years.

3.1.8 Immune response against HIV-1 infection

The specific anti-HIV-1 immune responses establish against infected HIV-1 requires of both humoral and cellular immunity. However, HIV-1 immune responses depend on many intricate factors such as persisting and killing immune competent cells and able to encumber functional immune responses. Owing to a high diversity and change virulent antigens over the time of infections, it enhances aberrant humoral and cell-mediated immunities, especially, the loss of functional antigen presenting cells and $CD4^+$ T cells depletions.

3.1.8.1 Innate Immune Responses.

Innate immune responses is the first line of defense against HIV-1 infection, and also play two pivotal roles including replicated containments and inducing adaptive immune responses. The major innate immune responses are the antigen-presenting cells (APCs) namely interferon producing cells (IPCs), natural killer (NK) cells, gamma-delta (γδ) T cells, Langerhans cells, macrophages and dendritic cells (DCs). Initially, dendritic cell is divided into two major subsets, namely conventional CD11c+ myeloid dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs, $CD11c^-$). cDCs located in skin are namely Langerhans cells and dermal dendritic cells, genital or gut mucosa and in blood. pDCs are found in blood, thymus, inflamed skin, mucosa and lymph nodes. Both dendritic cells express CD4, CCR5 and CXCR4 and they are enhanced infectivities. Virus production are less than 5%, cDCs may also express apolipoprotein B mRNA editing enzyme catalytic polypeptides (APOBECs) that deaminate cytidine to uridine in nascent minus-strand viral DNA enhancing to block HIV replications (116). Antigen-specific T cells are enhanced by dendritic cells maturation, cDCs transmit to T cells through virologic synapse formation or via exosomes follow by direct viral replication, (117) whereas pDCs also transmitted HIV-1 infection to T cells via interferon type 1 production which limits viral replication in the latter by means of the induction of tumor necrotic factor-related apoptosis-inducing ligand (TRAIL) and Fas/Fas ligand on $CD4^+$ T cells (118). Moreover, NK cells and NKT cells are members of innate lymphocytes which

function as antibody-dependent cell-mediated cytotoxicity and produce antiviral cytokines or chemokines, namely IFNα, IL-12, IL-15 and IL-18. The effective NK cells perform in balancing between activating and inhibitory receptors that may play roles in controlling viral replication (119).

3.1.8.2 Adaptive Immune Responses

Adaptive immune responses consist of humoral and cell-mediated immune responses which are the lymphocytes from B and T cell lineages. B cells derived from bone marrow, while T cells leave from bone marrow to mature in the thymus. T cells are determined by the presence of $CD4^+$ or $CD8^+$, which can bind epitopes of the class II and the class I histocompatibility, respectively. Viral mutation allows for escaping recognition of the immune responses, including neutralizing antibodies or cytotoxic $CD8^+$ T cells. $CD8^+$ T cell plays the central role in early HIV-1 infection by controlling the number of viruses but undetectable antibodies. The initial responsive T cells are frequently found in the specific for Env and Nef, whereas Gag p24 and Pol proteins tend to peak at the late stage of T cells responses which maintain the viral load at the set point more than controlling viremia (120). $CD8^+$ T cells recognizes MHC class I expression epitopes on transmitted or founder viruses. The most mutant viruses are detected by neutralizing antibodies that revealed around 12 weeks; therefore, the rapid loss of the founder viruses and mutant viral replacements, indicates that the complete loss of $CD8^+$ T cell mediated-inhibition of viral proteins which contribute to killing 4-6% of virus-infected cell per day. For instance, $CD4^+$ T cells are composed of memory T cells, and HIV-1-specific $CD4^+$ T cells which are useful to both humoral and cell-mediated immune responses. $CD4^+$ T cells bind to epitopes by APCs and then releases cytokine/chemokine productions as results of producing inflammatory responses and accumulating $CD4^+$ T cells. The natural history HIV-1infection establishes the immune responses around 3-6 weeks after viral infection. The first weeks, HIV-1 transmission is highly dynamic including frequently viral cytopathicity and bystander effects to damageable immune cell microenvironments. Viral detection can be seen at 14-21 days after SIV infection in macaques whereas it can be seen at 21-28 days of HIV-1 infection in humans. A number of $CD4^+$ T cells elicit low level whereas viruses show a high peak and nearly all of their numbers return to normal levels in the blood but not eradication. B cells are

not depleted during early phase but their responses are impairments that account for the destruction of other cells and developing in germinal centers. The immune activation against HIV-1 mediated B and T cells are apoptosis because they release apoptotic microparticles leading to express tumor necrotic factor and also related to apoptosis-inducing ligand (TRAIL) as well as Fas ligand expression as results of killing bystander cells, immune-suppression and the long term failure of immune systems (121,122).

3.1.8.3 Neutralizing Antibodies

During natural HIV-1 infection, a high number of the neutralizing antibodies (NAbs) are elicited against the primary HIV-1 infection and requires of conserved regions on *env* gene. Recently viral variants are specifically directed neutralizing antibodies providing window opportunities for ideal vaccines and microbicides. Broadly neutralizing antibodies allow neutralizing of most viral isolates and cross-clade. Antibody mechanisms against HIV-1 infection consist of neutralization susceptibility, complement fixation, opsonization and phagocytosis and other functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI) (Fig 3.9) (17,123,126). Neutralizing antibodies are explained by blocking CD4 receptor and co-receptor (CCR5 or CXCR4) inhibited viral replications as well as a high ability to bind with free virus particles via the synapse controlling viral infections (124).

Moreover, antibody-dependent cellular cytotoxicity (ADCC) is as a bridge between innate and adaptive immune responses and is detected within the first few weeks after onset of symptoms in acute phase. Fc domain of antibody bound to between Fc receptor on immuno-competent cells and Fc receptor on infected cell leading to destroy infected cells by releasing the cytotoxic granules (perforins and granzymes), antiviral cytokine (TNF), chemokines, proteases, nitric oxide, and reactive oxygen radicals or Fas/FasL interaction. IgG and IgA can trigger ADCC which connected with several cell types, especially, natural killer cells (NK cells), macrophages and monocytes. ADCC-mediated antibodies can eradicate virions, thus, it can reduce the production of progeny and limit cell-to-cell spreading. Neutralizing antibodies and non-neutralizing antibodies are able to elevate ADCC activity which prevail all stages (125,126). At present, neutralizing antibodies is the main interest in

developing vaccines and *env* gene is the main target for generating specific anti-Env antibodies, which control the wide ranges of viral replications. Antibodies against viral proteins are created within few weeks after infection but neutralizing antibodies (NAbs) are created within weeks of infection and initially responds to the autologous but not heterologous viral target (127).

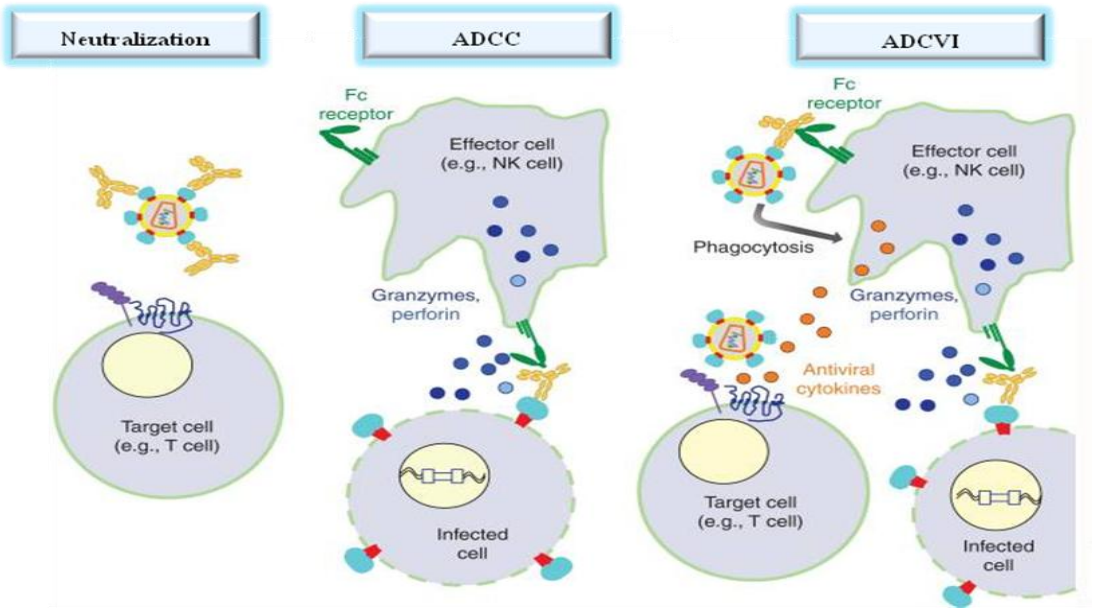


Figure 3.9. The mechanism of antibodies acts through neutralizing antibodies, ADCC (antibody-dependent cellular cytotoxicity) and ADVCI (antibody-dependent cell-mediated virus inhibition). Neutralizing antibodies bind to HIV-1 envelope glycoproteins and block infections. Complexion of binding among Fab-Neutralizing antibodies, envelope glycoproteins and Fc receptors on effector cells lead to lysis of infected cells. Another process, ADVCI measures the effectors of ADCC-mediated cell killing and reduces viral production by secreting antiviral cytokines, and other effects of FcR-virus interaction such as phagocytosis (126).

3.2. Broadly Neutralizing Monoclonal Antibodies

Almost all neutralizing antibodies are recognized in the first year, but broadly neutralizing antibodies (bnAbs) will occur a few years after sero-conversion (128). Neutralizing antibodies are divided into two categories: antibodies bind to variable regions and conserve regions that neutralize relatively narrow groups and a wide range of isolations. The highly functional *env* genes have the main targets for neutralizing antibodies such as CD4 binding site, CD4 inducible site, V3 region on gp120 and the membrane-proximal epitope region (MPER). Up to date, HIV-1 monoclonal antibodies are constructed from human who have been generated effectively neutralizing antibodies (Fig 3.10).

3.2.1 The monoclonal antibody IgG1b12

In 1992, IgG1b12 was isolated in the first broadly anitibody by Fab phage technique, it derived from the bone marrow of an asymptomatic long-term infected HIV-1 (129). It is able to neutralize around 50% of clade B HIV-1 infected individuals and around 30% in non-clade B viruses (130) and completely protected SIV infections. It showed highly affinity with overlapping CD4 binding sites both monomeric and trimeric of gp120 (131) and switched to show potently whole IgG1 neutralization susceptibility (132). After binding between crystal structure of b12 and gp120, it accounted for a feature binding by protruding finger-like of a long complementarily-determining region (CDR) H3 fitting to CD4 binding site of gp120 which extends of V1/V2 loop with Trp^{100} at the tip of CDR H3 penetrating the Phe^{43} pocket (133).

3.2.2 The monoclonal antibody 447-52D

447-52D is obtained from a heterohybridoma isolations in subtype B HIV-1 infection and determines the specific V3 loop (around 35 residues) of gp120 leading to potentially generating neutralizing antibodies. However, its region has conserved amino acids in the apex, requiring co-receptor binding namely GPxR motif (amino acid 312-315). Subtype B is GPGR motif whereas GPGQ is a common in non-subtype B HIV-1 infection (134). In addition, monoclonal antibodies can neutralize

more than 50% in subtype B whereas at least 7% in the others (135). This is able to neutralize both X4 and R5 tropism. The β hairpin structure is 16-mer peptides on V3 which forms a three-stranded mixed β sheet and recognized by CDR H3 of Fab 447-52D. Moreover, GPGR residues were the specific sequences of V3 hairpin apex with the base of CDR H3 (136).

3.2.3 The monoclonal antibody 2G12

This antibody recognizes at high affinity of carbohydrate epitopes cluster on gp120 and characterizes the potent inhibiting syncytium formations in the AA-2 cells. 2G12 will abolish neutralization activities namely complement fixation and antibody-dependent cellular cytotoxicity because of removed N-link carbohydrates especially, in the C2, C3, V4 and C4 domains on gp120 (137). Taken together, 2G12 binding sites are remarkable residues for N-link glycosylation sites at N295, N332, N339, N386 and N392 on *env* genes (138) and may relate to the mannose-dependent attachment to DC sign on antigen presenting cells or lectin receptor enhancing viral entry. Its Fab crystal structure is Manα1-2Man and with the oligosaccharide $Man_9GlcNAc_2$ that exposes Fab dimerization or assemble into an interlocked V_H domain-swapped dimer (139). Besides, 2G12 can neutralize 41% in clade B viruses whereas none form clade C and E (Fig 3.10) (130).

3.2.4 The monoclonal antibody 2F5

At present, gp41 divides into two clusters as a consequence of cluster I: antibodies react with a predominant part of gp41 amino acids 579-613 generating non-neutralizing, cluster II: antibodies react the membrane proximal external region (MPER) amino acids 644-667 generating either neutralizing or non-neutralizing (140). The linear gp41 sequences are the main target of 2F5, 4E10 and Z13 around 30 amino acids (141). 2F5 monoclonal antibody recognizes six conserved sequences namely Glu-Leu-Asp-Lys-Trp-Ala (ELDKWA) located at the domain of gp41 as well as approximately find in 72% highly variable HIV-1 isolates (142). Nevertheless, this epitopes immunization is failed to express neutralizing antibodies, assumed that generated antibodies depended on presenting incomplete trimeric gp41 epitopes (143).

This monoclonal antibody can neutralize around 67% of viruses but not from clade C viruses (Fig 3.10) (136).

3.2.5 The monoclonal antibody 4E10 and Z13

4E10 and Z13 recognize an adjacent to the 2F5 epitopes and conserved sequences are NWFDIT and NWF(D/N)IT, respectively (144). 4E10 is able to enhance protection in macaque after against HIV-1 challenge (145) and Z13 made from phage display library. Both mAbs compete to synthesis peptides from gp41 and can bind to HIV-1_{mn} virion which is a laboratory subtype B HIV-1 strain. Even though, Env recombinant protein denature reduces effectively binding two monoclonal antibodies, the conformation change is an important requirement for full epitopes expression (122) and enhancement of neutralizing antibodies to clade B, C and E. This suggests that gp41 is the predominant domain closely to access for neutralizing antibodies and vaccine designs (Fig 3.10) (146).

3.2.6 Soluble CD4 (sCD4)

The CD4 binding site (CD4bs) is the first receptor enhancing conformational changes and V1/V2 and V3 loop exposure for binding with co-receptors and viral entry. Therefore, broadly neutralizing anti-CD4bs monoclonal have been generated all the times and employs effectively structural and biological properties as consequences of viral entry inhibitions, viral replications (147) and syncytium formations (148). CD4bs binds to inner domain, outer domain and four-stranded bridging sheet gp120 which five disulphides and four cavity-altering substitutions are created to interdomain movements and stabilized bridging sheet formations. When CD4 compared to IgG1 b12 (mAb react to overlapping of CD4 receptor) in the atomic levels, they found that b12 restricted to gp120 inducing conformation by one-half of inner domains whereas the outer domains are fixed, in part of CD4 entirely fixed core, as consequences of both domains have a similar atomic level (149).

3.2.7 The monoclonal antibody sCD4-17b

The sCD4-17b is composed of domain 1 and 2 of chimeric proteins which connect to a flexible polypeptide linker and a single chain variable fragment of 17b. A human monoclonal antibody masks at a conserved CD4-induced epitope on gp120 overlapping co-receptor binding site. The high inhibition of R5 clade B viruses depended on its concentrations such as 50% inhibition in the concentration of 3.2 nM whereas 95% inhibition in the concentration of 32 nM (150). Moreover, sCD4-17b can enhance to neutralize a diverse genetic HIV-1 primary isolates and apply to antiviral therapy fields against HIV-1 infection (151).

3.2.8 The monoclonal antibody PG9 and PG16

Two broadly neutralizing antibodies had been generated from non-subtype B HIV-1 infection which were African donors and initially failed to neutralize HIV-1_{SF162}. They are unable to bind with gp120 and gp41 but they recognize the glycosylation regions of native viral *env* trimer (152). However, their functions were tested by multiclade Env-pseudotype virus, they found that both mAbs showed greater breath neutralizations than b12 and 2G12, 73% and 79% respectively. The analysis of variable genes elicited a unique long complementary determining region (CDR) H3 structure which affects to potently neutralizing antibodies (153). Furthermore, these antibodies recognized conserved epitopes of V2 and V3 against non clade B isolations (154).

3.2.9 The monoclonal antibody VRC01, VRC02 and VRC03

HIV/AIDS Vaccine Research found newly monoclonal antibodies namely VRC01 and VRC02 which were naturally found in infected HIV-1 blood. They designed proteins exposed with surface residues of SIV homologues that retained and stabilized the core proteins gp120 (155). After cloning into IgG expression vectors, three antibodies namely VRC01, VRC02 and VRC03 were strongly bound to RSC3 at CD4 binding site (CD4bs). The CDR3 of VRC03 contained 16 amino acids as well as three monoclonal antibodies shared common sequence motifs in CDR1, CDR2 and CDR3 of heavy chain. In addition, the potency and breath neutralizations of both VRC01 and VRC02 were around 91% and a geometric mean value is 0.33 μg/ml. Of notice, these monoclonal antibodies were derived from subtype B HIV-1 infected but can neutralize all genetic subtypes of HIV-1. Broadly monoclonal neutralization of VRC03 is approximate 57% (156).

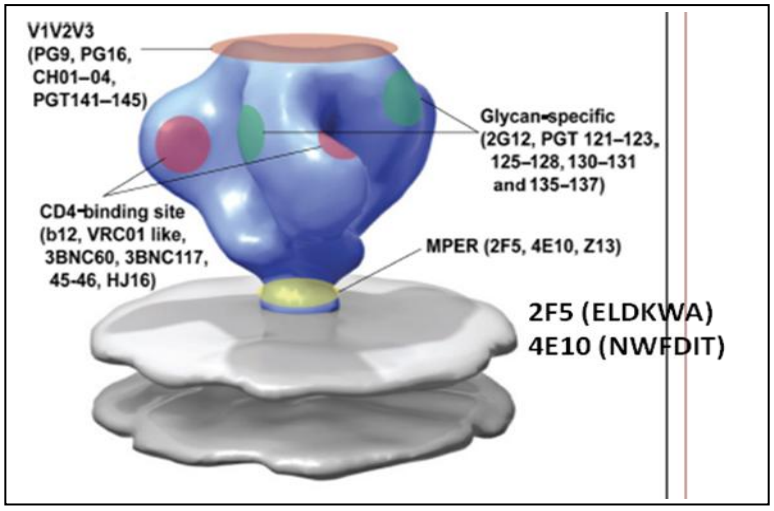


Figure 3.10. IgG1 b12 is the first neutralizing antibodies which recognized at CD4-binding site on envelope whereas 2G12 recognized mannose glycan shield. 2F5 and 4E10 recognized specific epitopes at membrane proximal external region of gp41 (MPER) which are ELDKWA and NWFDIT, respectively (157).

3.3. Significant of acute HIV-1 infection

Due to the natural history of HIV-1 infections, it is divided into three phases including primary/acute, latent/chronic and AIDS phases. The acute phase can provide the understanding of transmitted or founder virus (T/F) and host immune response interactions. T/F virus occurs in the first few weeks of early HIV-1 infections. Genetic diversities are significantly reduced and there is loss in the number of viral variants (122). Moreover, newly viral variants are understood in a number of transmitted viruses, viral mechanisms and rapidly viral genetic diversities, which contribute vaccine discovery or therapeutic treatments (122,163). Identifying the recent HIV-1 infection is importantly to conceive the viral transmitted dynamics, high risk reduction and preventive programs in populations. However, it is difficult to understand the characteristic appearance of the viral markers of acute HIV-1 infection because of its complexicity and multiple routes of viral infection, including cervicovaginal, in utero, penile, rectal, oral, percutaneous and intravenous. Langerhans' cells (subset of DCs) are as the first target cells in acute HIV-1 infection which resides at gut-associated lymphoid tissue (GALT), furthermore, monocyte-derived macrophages as a target are poorer than $CD4^{+}$T cells (164). Due to genetic studies, they are used to distinguish between chronic and acute infection by their complex genetic quasi-species (165). Most of T/F viruses utilized CD4 receptor and C-C chemokine receptor 5 (CCR5) requiring viral entry so called R5-tropic. Approximately, 80% transmission is a result of heterosexual (166), 60% from men who have sex men (168) and 20% from injection drug user. In addition, identified newly HIV-1 infections, they are explored short V1/V2 envelope lengths and less V3 net charge (169). Nevertheless, a number of PNLG sites were not significant difference between acute and chronic HIV-1 infection. Interestingly, these viruses contain motif epitopes generating neutralizing antibodies (166).

Typically, HIV-1 acute infection can characterize following step by step of HIV-1 antigen and specific antibodies (170) (Fig 3.11). The initially HIV-1 infection is able to detect viral RNA in plasma so called eclipse phase. Viral plasma increases at around 21-28 days and then followed by a decrease. Acute phase is categorized by Fiebig stages (I-VI) which rely on the sequence of detection assays (viral RNA by PCR, p24 and p31 by enzyme-linked immunosorbent assay (ELISA) and HIV-1

specific antibodies by ELISA and western blot, respectively (164) (Fig 3.11). The steady viremia can be seen at around 100 days at the end of Fiebig stage. The initial pathogenic events are rising tumour necrosis factors related apoptosis-inducing ligand (TRAIL is also called TNFSF10) in plasma before viremia occurrence and increasing TRAIL correspond to high level of interferon-α (IFN-α). Activated T and B cells, release a number of apoptotic microparticles in the blood circulation include soluble TNF receptor 2 (TNFR2) and FAS ligand (CD95L) (122,165) (Fig 3.12) as results of inducing $CD4^+$ T cell and bystander cell death leading to immune-suppressions (165). Moreover, microparticles have been shown a portion which initiates CC-chemokine receptor 5 (CCR5) expression as an early HIV-1 target cells (166).

Transmitted or founder virus is predominantly identified to homogenous genotype and non-syncytium inducing phenotype which is required viral properties in developing vaccines (167,168). Non-neutralizing antibodies are initially depicted against gp41 envelope whereas a trimeric gp120 is rarely exposed for functionally stimulated neutralizing antibodies (169). Likewise, the antibody-dependent cellular cytotoxicity (ADCC) or antibody- dependent cell-mediated viral inhibition (ADCVI) is increased within 3 weeks after infection (170). The effective antibodies is developed against HIV-1 infection whereas $CD8^+$ T cells firstly respond to short Nef and Gag protein epitopes (171) presented by HLA molecules before high peak viremia. These cells secrete perforins and granzymes for killing infected cells. The first T cells respond to escape viral mutation correspond to decreasing viral load in acute phase established viral set point (172). Depleted $CD4^+$ T cells from gastrointestinal tract in early phase affect to HIV-1 replications (Fig 3.12).

According to increasing viremia, cytokine storm is able to evoke early innate immune responses leading to release proinflammatory cytokines such as interleukin-1 (IL-1), various types of cytokines and chemokines (118) (Fig 3.14) namely interleukin-15 (IL-15), type 1 interferon (IFN) and CXC-chemokine ligand 10 (CXCL10) as well as the high peak of viremia and interleukin -10 (IL-10). Taken together, interferon-γ, interleukin-18 (IL-18), tumour necrotic factor (TNF) and interleukin-22 (IL-22) are increased. Some cytokines such as type 1 IFN is act as an antiviral for inhibits viral replication and IL-15 as IL-18 initiates the immune-pathogenesis in acute HIV-1 infection (122,173).

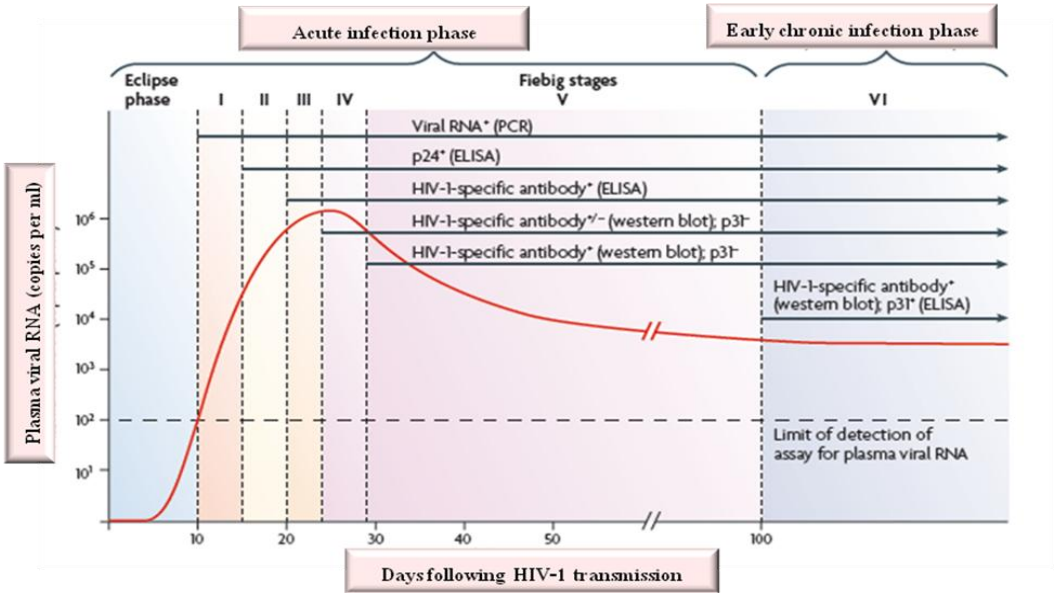


Figure 3.11. This diagram shows several parameters which detects in the early HIV-1 infection include viral RNA and specific antibodies. Based on standard clinical detection, it consists of six stages following by Roman numerals (Fiebig stages, I-IV). Initially, viral RNA was detected by PCR so called eclipse phase and consequently elicited specific antibodies which identified by enzyme link immunosorbent (ELISA) and western blot. Until finally acute stage, patients will further to develop chronic HIV-1 infection, which takes approximately 100 days (122).

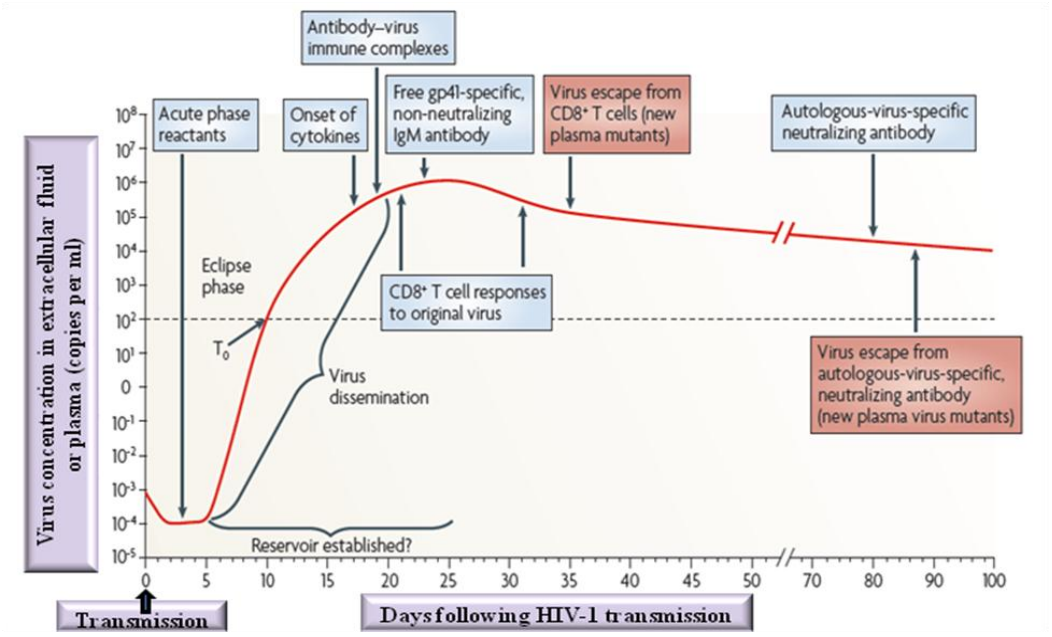


Figure 3.12. The early innate and adaptive immune responses are shown in the acute phases. After HIV-1 infection, viral replication occurs at mucosal tissues and then it drains to lymph nodes called eclipse phase. T_0 is the first viral detection in plasma. Viral levels are gradually rising within days, various types of cytokine and chemokine levels will be set up as observed in figure 8. Initially, specific antibody complexes are detected include non-neutralizing IgM antibodies glycoprotein 41 as well as $CD8^+$ T cells will be expansion against HIV-1 infection as a results of decreasing plasma viremia. Moreover, autologous-virus-specific neutralizing antibodies are elicited within 80 days as a subsequent of viral escape these antibodies and generated new plasma virus mutants (122).

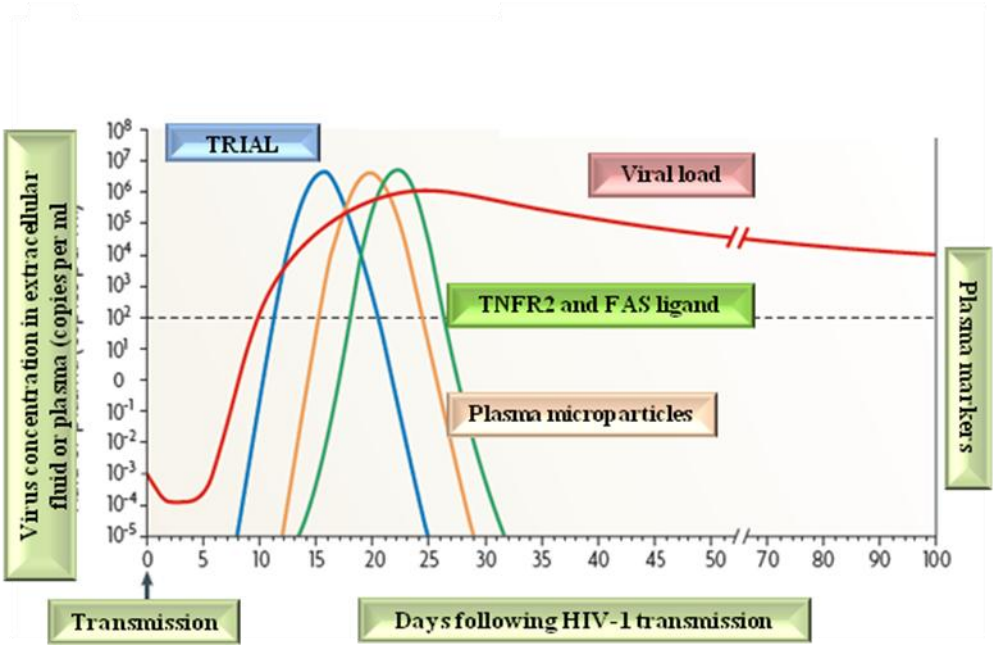


Figure 3.13. Before the high peaks of viremia, it shows the high level of soluble tumour necrotic factor-related apoptotic ligand in the plasma (TRAIL, also known as TNFSF10) which are initially induced apoptosis and immune activation. The high TRAIL levels also coincide to increasing interferon-α correspondence to inducible presented apoptotic microparticles, soluble TNF receptor (TNFR2 also known as TNFSF1B), soluble FAS ligand. The portion of microparticles presented CC-chemokine receptor 5 which are targets of HIV-1 infection. All of them are elicited in plasma during early phase lead to $CD4^{+}$T cell death (122).

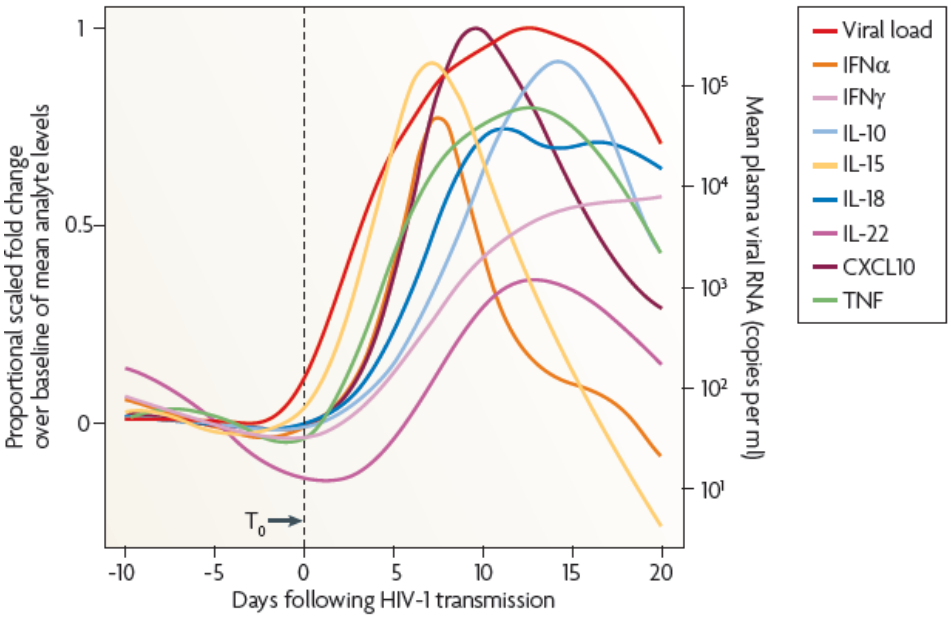


Figure 3.14. Cytokine storm occurs in acute HIV-1 infection**,** T_0 is the first viral RNA detection which is approximately shown 100 copies per ml. Kinetic of cytokine and chemokine stroms are depicted by two waves of interleukin-15 (IL-15) and interferon-α (IFN-α), subsequently, tumor necrosis factor (TNF), IL-18, IL-10 and CXCL10 (CXC-chemokine ligand 10) (122).

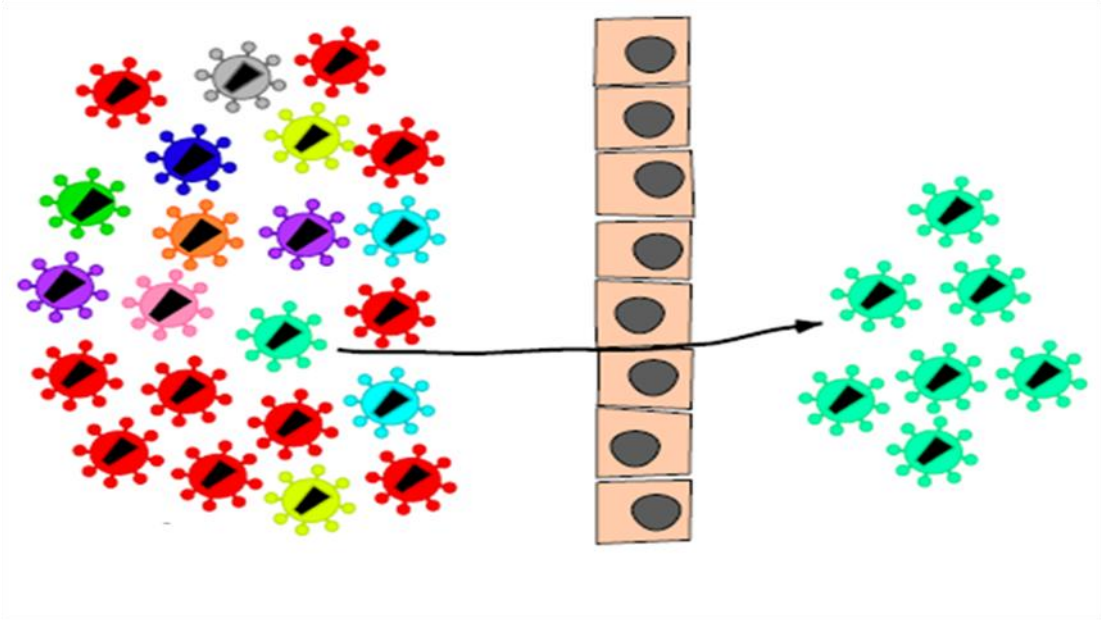


Figure 3.15. Genital tract derives from virus population at the time of sampling contains a large subpopulation of virus and numerous other variants. However, only one of these variants distinct from the large variant populations; successfully crosses the mucosal epidermal barrier of the genitals. To generate an infection, they are capable of spreading to all lymphoid tissues of the recipient. The model of HIV-1virus transmission shows both diverse quasispecies and a single virus in semen, cervico-vaginal secretions and chronic blood patients. All of them may penetrate into mucosal and cutaneous surface but some of them can replicate into productive infections. R_0 represented to estimate viral growth and basic reproductive rate. It is calculated by using $CD4^+$ T cell count and viral load. The reproductive T/F virus is estimated R_0 of 8 in the early or acute stage of HIV-1 infection (166,167).

CHAPTER IV
MATERIALS AND METHODS

In Thailand, various high risk groups have been evaluated for HIV-1 infection prevalence under the National HIV-1 Sero-Surveillance since 1989. One of the surveillance populations was the Royal Thai Army conscripts who were approximately inducted 60,000-21-year-old annually. They represented young Thai men and serves as the epidemic HIV-1 prevalence in Thailand. Although the National HIV-1 Sero-Surveillance was an important program for the prevention and improvement of HIV-1 infections, it was limited in the monitoring infections. Several methods have been proposed to detect viral RNA and HIV-1 p24 antigen in early HIV-1 infections (174). In 2002, Parekh et al., developed an assay to distinguish between acute HIV-1 infection and chronic infection using Captured BED-ELISA (HIV-1 subtypes B, E and D, IgG-Capture enzyme immunoassay, Catypte HIV-1 BED incidence EIA, Calypte Biomedical Corporation, Maryland, USA).This assay detects the increasing proportion of specific anti-HIV-1 immunoglobulin G (IgG) from total anti-HIV-1 IgG (8). A threshold cutoff based on a calibrator specimen and low titer of antibodies to HIV-1 antigens is used to classify recent sero-conversion. This assay protocol must be strictly adhered to obtain accurate, precise and reproducible results. This procedure can be used in populations with various subtypes, using uniform criteria such as cutoff, sero-conversion duration, and window period (175,176).

The specific features of this assay are dilution of specimen at 1:101 and increased in non-specific HIV IgG in the diluted specimen. The branched gp41 antigen derived from multiple subtypes permits equal detection of different specific HIV-1 subtypes and shows the immune-dominant antigen which appears in the early stages of HIV-1 infection. Moreover, the wells on the microplate are coated with goat anti-human IgG and after added the serum into the 96 well-plate, both anti-HIV-IgG and non-anti-HIV-IgG will be captured on the goat anti-human IgG coated wells. The relative amounts of anti-HIV-IgG and non-anti-HIV-IgG captured represent IgG

antibody populations. The results are calculated from the proportion of HIV-1 specific IgG to total IgG. The early sero-converters show a lower proportion of HIV specific IgG in the serum or plasma than those with long term infection. Previous studies have indicated that HIV-specific-IgG may continue to increase for more than 2 years after sero-conversion (177). The normalized optical density (ODn) are calculated by optical density (OD) of specimen divided by median OD of calibrator, using a calibrator specimen, demonstrating that ODn of 0.8 corresponds to mean sero-conversion duration of no more than 127 days. This experiment offers the best combination of sensitivity and specificity for classifying acute or chronic. The cut off at 0.8 also yields less false recent infections in AIDS patients who are provided a better predictive value of newly HIV-1 infection (8).

Around 60,000 young Thai men (21-year-olds) are recruited to Royal Thai Army (RTA) conscripts in April every year and placement takes place twice a year in May and November. Since 1989, Army Institute of Pathology (AIP) and Armed Forces Research Institute of Medical Sciences (AFRIMS) has cooperated with the 37 military hospitals for screening RTA conscripts to assess the prevalence of HIV-1 infection in young Thai men. HIV-1 screening was performed in 30,000 newly inducted RTA conscripts bi-annually in May and November using the HIV-positive antibody test. The results were obtained using ELISA and confirmed by western blot analysis, using licensed commercial reagents. Recent HIV-1 infections were detected by BED ELISA.

Experiment 4.1: Determination of full length *env* gene

4.1.1 Serum samples

Individual peripheral blood samples were collected from RTA conscripts enrolled in military from 2009 to 2011.They were tested for HIV-1 infection screening by ELISA and confirmed by western blot analysis. The positive HIV-1 infection serum was tested for acute HIV-1 infection by BED ELISA (169). The protocol has been approved by Institutional Review Broad, Royal Thai Army Medical Department.

4.1.2 Full length *env* gene preparation

Serum samples were separated from whole blood and kept under -80°C until used. Before RNA extraction, viral particles were concentrated by ultracentrifugation at 65,000 rpm for 1-3 hr using TLA-100.3 rotor with Optima TLX ultracentrifuge (Beckman Coulter). Viral RNAs were extracted using a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) and transformed to cDNA by using the SuperScript III First-Stand Synthesis kit (Invitrogen, Carlsbad, California, USA), together with the reverse primer, K-env-R1,5′-CCAATCAGGGAAGAAGCCTTG-3′ corresponding to nucleotide (nt) 8736 to 8716 of CRF_01AE reference strain CM240 (Genbank accession no U54771), according to manufacturer's instructions. PCR product was obtained as the full length *env* gene but it included *rev*, *vpu* gene and the partial *tat* and *nef* gene fragments by nested PCR using BIO-X-ACT DNA polymerase (Bioline) or PrimeSTAR GXL DNA Polymerase (TAKARA Biotechnology, Japan) and these primers: outer sense primer N-env-F1, 5′-TTAGAGGAGCTT-AAAAATGAAGC-3′, nt 5193 to 5215; outer antisense primer N-env-R1, 5′-TTAAAAAGAAGCTAAGATCAAAAGC-3′, nt 8638 to 8614 and inner sense primer N-env-F2, 5′-GAATTGGGTGTCAAC-ATAGCAGAATAGGC-3′, nt 5344 to 5372; inner antisense primer N-env-R2, 5′-TATCTAGATCTTGAGATACTGCTCC -3′, nt 8485 to 8461. 10 µl cDNA is carried out in the first round and 5 µl was used in the second round for 50 µl. PCR mixture containing 0.2 µM for each primer, 0.8 mM dNTPs and 0.03 unit of polymerase enzyme and PCR amplification was carried out in the first round for 1 cycle at 94°C for denaturation for 1 min, 10 cycles at 94°C for

denaturation for 20 sec, annealing at 48°C for 30 sec, elongation at 68°C for 5 min, followed by 20 cycles of which each cycle at 94°C for 10 sec, 48°C for 30 sec, extension at 68°C for 5 min, cycle elongation of 10 sec for each cycle, and prolonged extension at 68°C for 10 min. The PCR cycle of the second round was 1 cycle of denaturation at 94° C for 1 min, 30 cycles of which each cycle is 94°C for 10 sec, 52°C for 30 sec, followed by extension at 68°C for 5 min, cycle elongation of 5 sec for each cycle and prolonged extension at 68°C for 5 min as according to Boonchawalit S et al,. (178). PCR products were approximately yield 3 kb.

4.1.3 Direct DNA sequencing preparation

PCR products were purified by QIAmp PCR purification kit (QIAGEN,) or by GENECLEAN SPIN kit (qbiogene, Japan) as well as sequencing. Briefly, 200 ng *env* DNA, 2µM each primer and 1.5 µl BigDye Terminator reaction mixture were carried out in 1 cycle of 94°C denature for 1 min, 25 cycles of 94°C denaturation for 10 sec, annealing at 50°C for 5 sec and elongation at 60°C for 4 min and end up with 4°C of PCR cycle. The nucleotide sequences of *env* gene were determined by cycle-sequencing dideoxy chain termination method on an automated DNA sequence (ABI PRISM 3100). The data nucleotides were aligned with HIV-1 strains of CM240 reference subtypes CRF01_AE which obtained from the Los Alamos HIV database (http://hiv-web.land.gov/).

4.1.4 DNA cloning preparation

Each specific primer was designed and synthesized individually, based on sequencing results. They were amplified the gp160 open reading frame and the forward primer contained recognition site for BspEI, immediately upstream of the Env signal peptide, the corresponding reverse primer contained NotI, immediately downstream of the stop codon of *env* gene. 2.5 kb of PCR product was cloned into pCI-envCT shuttle vector (Promega, City, Country). Briefly, 15 µl of purified DNA was cut by BspEI and NotI as well as 2µg pCI-envCT and incubated at 37°C for 2-3 hr. Purified 2 µl of shuttle vector backbone and 14 µl of Env DNA were ligated using ligase enzyme (BioLabs) and incubated at 19°C for 30 min. The ligation mixture was

transformed into *Escherichia coli* (*E. coli*) HB101 by heat shock method. 1 ml of SOC was added in ligation mixture and incubated at 37°C on shaker for 30 min. After that, it was centrifuged at 2000 rpm for 2 min. Around 100µl of ligated reaction was spread onto Luria-Bertani medium (LB)-agar plated with ampicillin 100 µg/ml and incubated at 37°C overnight.

4.1.5 Identification of recent *env* gene in shuttle vector by restriction enzymes

Env gene was transposed into pCI-envCT. The colonies containing the recombinant genes were selected around ten clone individuals from agar plate and exponentially grew plasmid DNA in *E.coli* LB broth containing 100 µg/ml ampicillin and incubated at 37°C on shaker at 200 rpm overnight. Plasmid DNA was purified by GenElute^{TM} plasmid miniprep kit (SIGMA-Aldrich, Missouri, United States) and diluted by 100 µl of 0.1 Tris-EDTA (TE) buffer. The NotI/BspEI and NotI/BlpI restriction enzymes were used to identify the selected clones following manufacturer's instructions for using NEB3 and NEB2 buffer, respectively.

4.1.6 Preparation of *env* gene on pCI-envCT cloned into pNL-Luc-EnvCT (expression vector)

The infectious SalI/NotI fragment of pCI-envCT containing the recently *env* gene was cloned into pNL4-3 (179) derived luciferase reporter proviral construct, pNL-Luc-envCT (180), constructed Env-recombinant viruses. In brief, 2 µg of the SalI/NotI fragment of pCI-envCT containing new *env* gene was cut by SalI and NotI, as well as 2 µg pNL-Luc-envCT and incubated at 37°C for 2-3 hr. 8 µl of purified recent *env* gene and 2 µl of pNL-Luc-envCT backbone were ligated by using ligase enzyme (BioLabs) and incubated at 19°C for 30 min. The reaction was transformed into *E. coli* HB101, 1 ml of SOC was added in ligation mixture, and incubated at 37°C on shaker for 30 min. After that, it was centrifuged at 2000 rpm for 2 min. 100µl of ligated reaction was spread onto LB medium-agar plated with ampicillin 100 µg/ml and incubated at 37°C overnight.

4.1.7 Identification newly *env* gene in pNL-Luc-envCT by restriction enzymes

The four clone individuals were selected from the agar plate and picked up into exponentially growing plasmid DNA in *E. coli* LB broth with 100 µg/ml ampicillin which was then incubated at 37°C on a shaker at 200 rpm overnight. Plasmid DNA was purified by GenElute^{TM} plasmid miniprep kit (SIGMA-Aldrich) and diluted by 100 µl of 0.1 TE buffer. The NotI/SalI and NotI/BlpI restriction enzymes were used to identify the selected clones following the manufacturer's instructions.

Experiment 4.2: The construction of Env-recombinant viruses

4.2.1 Preparation Env-recombinant viruses

HEK 239T cells were transfected with proviral DNAs using FuGENE HD (Roche) described previously reports (181,182). Briefly, $2X10^5$ 239T cells in 2 ml of growth media were prepared. 2 µg Env-recombinant viruses were added to newly *env* plasmid DNA in pNL-Luc-Env-CT and 6 µl of FUGENE HD as the transfection reagent. The supernatant Env-recombinant viruses were harvested 48 hr after transfection, aliquoted at 500 µl per tube and immediately stored at -80°C until used. Env-recombinant virus concentration was determined by measuring gag protein p24 ELISA (ABL) following manufacturer's instructions.

4.2.2 Study of phenotypic Env-recombinant viruses

U87.CD4.CXCR4 and U87.CD4.CCR5 were used to study phenotypic Env-recombinant viruses and infectivities as previous report (182). Env-recombinant viruses were acquired using 10 ng of Env-recombinant virus transfected into $3X10^4$ U87.CD4.CCR5 and U87.CD4.CXCR4 and incubated in 5% CO_2 at 37°C for 48 hr. The value of relative light unit (RLU) was measured by luciferase activity which was measured in infected cells using Steady Glo Luciferase assay kit (Promega) using LB 960 microplate luminometer (Berthold, Bas Wildbad, Germany) (182).

4.2.3 Proviral DNA sequencing preparation

Proviral *env* DNAs were purified by GenElute™ plasmid miniprep kit (SIGMA-Aldrich). The nucleotide sequences of the full length *env* gene were determined by cycle-sequencing dideoxy chain termination on an automated DNA sequence (ABI PRISM 3100). Nucleotide sequences were aligned with CM240 CRF01_AF HIV-1 which was obtained from the Los Alamos HIV database (http://hiv-web.land.gov/). In brief, 10 µl sequencing reaction mixture was added to 100 ng of template, 2 µM on each primer and 1.5 µl of BigDye® terminator that was ready mixed reaction, and followed by 1 cycle of denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, elongation at 60°C for 4 min, and hold at 4°C (181).

Experiment 4.3: To study neutralizing susceptibility of Env-recombinant viruses with neutralizing human monoclonal antibodies (NHMAbs), pooled patient's serum, specific co-receptor antagonists and a fusion inhibition.

4.3.1 Neutralization susceptibility of Env-recombinant viruses to NHMAb

$5X10^3$/100µl of U87.CD4.CCR5 and U87.CD4.CXCR4 cells were incubated for 24 hr for neutralization assay, measured by the reduction Luc reporter gene expression in the single round of virus replication in these cells (168). IgG1 b12, 2G12, 2F5 and 4E10 were used to determine neutralization susceptibility. Env-recombinant viruses were diluted into 2ng/50µl and incubated with 2-fold serially diluted NHMAbs at 37°C in 5% CO_2 for 1 hr, followed by addition into U87.CD4.CCR5 or U87.CD4.CXCR4, depending on the phenotypic Env-recombinant viruses. They were then incubated at 37°C in CO_2 for 48 hr and luciferase activity of infected cells was then measured by Steady Glo Luciferase assay kit (Promega) using LB960 microplate luminometer (Berthold). The 50% inhibitory concentration (IC50)

of NHMAbs was measured by dose-response curve using a standard function of GraphPad Prism 5 software (GraphPad Software, San Diego. CA).

4.3.2 Neutralization susceptibility of Env-recombinant viruses to specific entry

$5X10^3$/50µl of U87.CD4.CCR5 and U87.CD4.CXCR4 cells were incubated for 24 hr. CXCR4 antagonist (AMD3100) (180), CCR5 antagonist (TAK-779) (Sigma-Aldrich) (181) and a fusion inhibition (T-20) (182) were used. U87.CD4.CCR5 or U87.CD4.CXCR4 were treated with 2-fold serially diluted AMD3100, TAK-779 and T-20, which were incubated in a CO_2 incubator at 37°C for 1 hr, followed by addition of 4ng/100 µl Env-recombinant viruses, and incubated in CO_2 at 37°C for 48 hr. Luciferase activity of infected cells was measured by Steady Glo Luciferase assay kit (Promega) using LB960 microplate luminometer (Berthold). The IC50 of viral entry (AMD3100, TAK-779) and a fusion inhibition (T-20) for suppressing viral replication were measured by dose-response curve using a standard function of GraphPad Prism 5 software (GraphPad Software).

4.3.3 Neutralization susceptibility of Env-recombinant viruses to pooled patient's serum

$5X10^3$/100µl of U87.CD4.CCR5 and U87.CD4.CXCR4 cells were incubated for 24 hr. The pooled patient's serum was the final concentration 1:20 with 2 fold serial dilution. Env-recombinant virus was diluted to 2ng/50µl and incubated with 2-fold serially diluted pooled patient's serum which was activated by heat at 56°C for 1 hr and was incubated in CO_2 at 37°C for 1 hr. Env-recombinant virus was then added to U87.CD4.CCR5 or U87.CD4.CXCR4 that was incubated inCO_2 at 37°C for 48 hr. Luciferase activity of infected cells was measured by Steady Glo Luciferase assay kit (Promega) using LB960 microplate luminometer (Berthold). The ID50 of reciprocal serum dilution was measured by dose-response curve using a standard function of GraphPad Prism 5 software (GraphPad Software)

4.4 Statistical analysis:

Statistical analysis was carried out using the standard function of GraphPad Prism 5 software (GraphPad Software). Correlation between was determined by the Spearman's rank correlation test and significance between two groups was determined by unpaired *t*-test. *P*-value of less than 0.05 was used as the significant level for all analysis (183).

CHAPTER V
RESULTS

5.1 Identification of full length *env* gene of recent HIV-1 infection from Royal Thai Army conscripts

Serum samples were obtained from RTA conscripts who were enrolled to the military from 2009 to 2011. Serum samples were not only positive for HIV-1 infection, but also showed low proportion of specific HIV-1 IgG (OD n <0.8) and was interpreted as recent HIV-1 infections by BED-ELISA (8). Eighteen early *env* gene samples were obtained and their yields were 3 kb including the full length *env*, *rev*, *vpu*, and partial *tat* and *nef* genes. Additionally, the acute HIV-1 infection was determined in this study by BED codes namely AF1087, AF1106, AF1136, AF1220, AF1349, AF1423, AF1428, AF1436, AF1451, AF1577, AF1911, AF1336, AF1434, AF1919, AF1419 and AF1784.

For direct *env* gene sequencing, the eighteen early *env* genes of recent HIV-1 infection subtype CRF01_AE was determined by nucleotide residues on HIV database using RIP 3.0 (http://www.hiv.lanl.gov/RIP). The specific primers were designed and synthesized for amplified early *env* gene individuals. The forward primer contained the gp160 open reading frame and recognition site for BspEI, immediately upstream of the Env signal peptide and the corresponding reverse primer contained NotI, immediately downstream of the stop codon of *env* gene. After amplification, PCR products were 2.5 kb.

5.2 Information of constructed early CRF01_AE Env-recombinant virus function and property

All of 118 of constructed early CRF01_AE Env-recombinant viruses were examined for viral infectivity and co-receptor usage. The infectious molecular clones were defined by the high relative light unit (RLU) which measures the reduction in luciferase activity in infected cells and compared with pNL4-3-infected U87.CXCR4, restricted with the 100 RLU in luciferase activity (Table 5.1). Twenty-seven infectious Env-recombinant viruses showed high RLU of over 100,000 and low infectivity was shown in ninety-one of those. This demonstrated that early CRF01_AE Env-recombinant viruses may contain the defective *env* gene enhancing the loss infectivity, is consistent with a previous study on chronically CRF01_AE HIV-1 infections by Utachee et al., (181,182). Due to similar protein sharing structures of *env* genes (data not shown), 14 out of 27 early CRF01_AE Env-recombinant viruses were selected for performing neutralization susceptibility to neutralizing human monoclonal antibodies (NHMAbs), specific co-receptor antagonists, fusion inhibition, and pooled patient's serum. The fourteen infectious Env-recombinant viruses revealed co-receptor usage and RLU on table 5.1 as results of one X4R5 and thirteen R5 co-receptor usage which obtained from 10 patients (Table 5.1). The constructed Env-recombinant viruses were R5-tropisms which is the consistent with previous studies, demonstrating that viral variants utilized the chemokine receptor 5 (R5-tropism) in the primary or acute HIV-1 infection, so called transmitted or founder (T/F) virus (163,184). They showed various levels (35-206 fold) of infectivity relative to pNL-envCT (pNL4-3) (Table 5.1).

Table 5.1 Phenotypic properties of 14 T/F *env* genes derived from recently infected Thai individuals.

BED code[a]	Env clone	*Relative infectivity*[b]		Phenotype[c]
		U87.CXCR4	*U87.CCR5*	
AF1087	RTA2	0	87	R5
AF1106	RTA3	105	39	X4R5
AF1136	RTA4	0	75	R5
AF1136	RTA5	0	128	R5
AF1136	RTA6	0	149	R5
AF1428	RTA8	0	168	R5
AF1451	RTA9	0	36	R5
AF1911	RTA11	0	134	R5
AF1784	RTA13	0	206	R5
AF1919	RTA16	0	117	R5
AF1451	RTA21	0	36	R5
AF1577	RTA23	0	100	R5
AF1451	RTA24	0	185	R5
AF1336	RTA27	0	143	R5

[a.] BED code presented on infected HIV-1 individuals by BED ELISA.

[b.] Relative light unit was compared with luciferase activity of pNL4-3-infected U87.CD4.CXCR4 (U87.X4).

[c.] Phenotypes were defined by the high value of relative light unit on U87.CD4.CXCR4 or U87.CD4.CCR5 or both.

5.3. Characterization of early CRF01_AE *env* genes infected HIV-1 individuals.

5.3.1 Fourteen early *env* gene characterizations

The full-length gp160 nucleotide sequence confirmed that 14 infectious molecular clones were subtype CRF01_AF. This was determined using RIP 3.0 (www.hiv.lanl.gov). The nucleotide sequences were assembled, translated and aligned with reference strain (CM240_AY736838). Cysteine residues were formed in variable regions including V1, V2, V3, V4 envelope loops and conserved amino acid residues on gp120 region of 14 infectious molecular clones. The potential N-linked glycosylation sites (PNLGs) were manually edited on *env* gene individuals together with N-glycosite (www.hiv.lanl.gov) tool, which were examined by NXT and NXS motifs of which X was any amino acid residue. The average numbers of amino acid residues were determined in V1, V2, V3, V4 and V5 regions. Among them, 14 early CRF01_AE Env gp120 were 27, 42, 35, 27, 8 amino acids, respectively (Table 5.2). The number of amino acid residues in V1 and V2 regions on early CRF01_AE Env gp120 were significantly lower than those of 35 CRF01_AE Env gp120 derived from chronically infected Thai individuals (181,182) (Fig. 5.16). The number of amino acid residues in V3, V4 and V5 regions were comparable between the CRF01_AE Env gp120 derived from recently and chronically infected Thai individuals (data not shown). In addition, the average number of PNLG sites in gp160 among 14 early CRF01_AE Env was 28 compared to that in CRF01_AE Env gp160 as described in previous report (182), indicating that the length of V1 and V2 amino acids may support the role of susceptible neutralizing antibody in acute or primary phase HIV-1 infection (87,122).

Table 5.2 Protein structure-related properties of 14 T/F *env* genes derived from recently infected Thai individuals

Env gene	Envelope lengths[a]					PNLGs[a,b]
	V1	V2	V3	V4	V5	gp160
RTA2	25	43	35	27	7	27
RTA3	28	41	35	21	7	28
RTA4	31	44	35	27	9	30
RTA5	31	44	35	27	9	30
RTA6	25	43	35	27	7	27
RTA8	27	38	35	29	12	28
RTA9	30	43	35	26	7	28
RTA11	27	38	35	29	12	28
RTA13	27	43	35	27	8	30
RTA16	13	46	35	29	6	27
RTA21	25	43	35	27	7	27
RTA23	30	43	35	26	7	28
RTA24	25	43	35	27	7	27
RTA27	27	35	35	29	12	28
Mean	26.5	42.1	35	27	8.4	28.1

a. Amino acid residues in gp120 variable regions, V1, V2, V3, V4 and V5 and PNLG sites in the deduced amino acid sequence of 14 CRF01_AE *env* genes were counted manually

b. PNLG sites were examined using N-glycosite(www.hiv.lanl.gov).

5.3.2. Deduced amino acid diversity on early CRF01_AE *env* genes.

Genetic diversities were differently determined by deduced amino acid sequences on 14 newly subtype CRF01_AE *env* genes including gp160 (including signal peptide), gp120, gp41, C2-V5 (C2, V3, C3, V4, C4, V5) together with V3 (Table 5.3). Pairwise distance matrices were defined by using the PROTDIST program from the Phylogeny Inference Package (PHYLIP) version 3.6, incorporating the Dayhoff PAM matrix a gift from Dr. Joe Felsenstein, Department of Genome Sciences, University of Washington, Seattle). The mean interclone distances of amino acid sequences on gp160, gp120, gp41, C2-V5 and V3 were 10.95%, 13.37%, 7.42%, 12.85% and 11.44%, respectively (Table 5.3). In addition, the highest amino acid difference was observed in the C2-V5 region of gp120 (Table 5.3). However, the overall diversity of amino acid sequences among 14 early CRF01_AE *env* gene was markedly lower than that among 35 CRF01_AE Env proteins derived from chronically infected Thai individuals as described in previous report (181).

Table 5.3 Deduced amino acid diversity among 14 T/F CRF01_AE *env* genes

		% *Difference*[a]		
	n	*Min*	*Mean*	*Max*
gp160[b]	14	0.1	10.95	19.7
gp120	14	0.2	13.37	24.1
gp41	14	0	7.42	14.4
C2-V5	14	0.4	12.85	25.7
V3	14	0	11.44	22.9

a. Deduced amino acid sequences calculated the interclone distances by pairwise comparisons

b. Including Env signal peptide

5.3.3 Deduced amino acid sequences on 14 early CRF01_AE *env* genes.

Fourteen early CRF01_AE Env-recombinant viral nucleotide sequences were assembled, translated and aligned comparing with consensus sequence (CM240_AY736838). The first deduced amino acids of gp120 excluded the signal peptide (Fig 5.16). The dot denoted sequence identity while dash line denoted gaps introduced to optimize alignment. Arrows above the sequence denoted cysteine residues that form variable loops, V1, V2, V3, and V4 on gp120, in particular, two arrowheads on V4 region represented an extra loop structure. The potential N-linked glycosylation sites (NXT and NXS motif, where X is any amino acid) were shown by underlining as well as the membrane proximal external region.

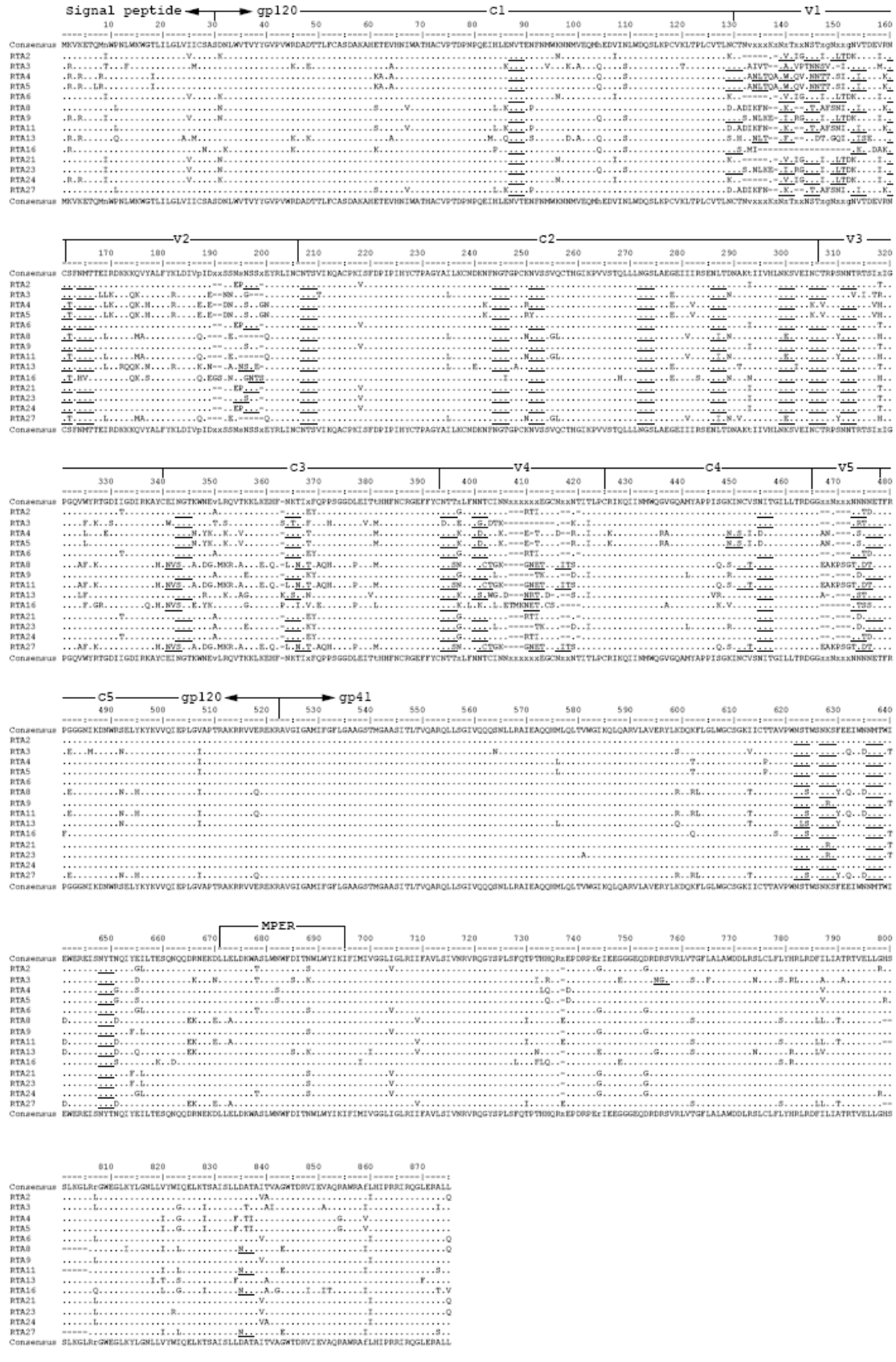

Signal peptide
gp120
C1
V1
V2
C2
V3
C3
V4
C4
V5
C5
gp120
gp41
MPER
Consensus
RTA2
RTA3
RTA4
RTA5
RTA6
RTA8
RTA9
RTA11
RTA13
RTA16
RTA21
RTA23
RTA24
RTA27

Figure 5.16 Deduced gp160 amino acid sequences of 14 T/F CRF01_AE *env* genes. Nucleotide sequence of 14 CRF01_AE *env* genes was translated, aligned and compared with the consensus sequence, as described in Materials and Methods. Positions of Env signal peptide, gp120 and gp41, as well as the variable (V1, V2, V3, V4 and V5) and conserved (C1, C2, C3, C4 and C5) regions of gp120 are denoted above the aligned sequences. In addition, the membrane-proximal external region (MPER) of gp41 is indicated. The numbering of amino acid residues begins with the first residue of the Env signal peptide. Dots denote amino acid identity, whereas dashes represent gaps introduced to optimize alignment. PNLG sites are shown by underlining.

5.4. Neutralization susceptibility of 14 early CRF01_AE Env-recombinant viruses.

5.4.1 Fourteen early CRF01_AE Env-recombinant viruses to neutralization susceptibility with NHMAbs and pooled patient's serum.

Fourteen early CRF01_AE Env-recombinant viruses were examined for their neutralization susceptibility to NHMAbs namely IgG1 b12, 2G12, 2F5, and 4E10 and pooled patients' serum. The 50% inhibitory concentration (IC50) of NHMAbs for suppressing viral replication whereas the reciprocal serum dilution, at which viral replication was suppressed by 50% (50% inhibitory dilution, ID50), were calculated by the dose-response curve using the standard function of GraphPad Prism 5 software (GraphPad Software). The results showed that all recombinant viruses were susceptible to 4E10 (144) whereas 9 of 14 (64%) recombinant viruses were susceptible to 2F5 (142) (Table 5.4). In contrast, recombinant viruses were resistant to neutralization by a NHMAb recognizing the mannose cluster of Env gp120, 2G12 (137) (Table 5.4). Similar tendency was seen on the neutralization susceptibility of 14 early CRF01_AE Env (Table 5.4) and 35 CRF01_AE Env (182) to 4E10, 2F5 and 2G12. Moreover, 5 out of 14 (36%) recombinant viruses were susceptible to IgG1 b12, a NHMAb recognizing the epitope overlapped with CD4 binding site on gp120 (126) (Table 5.4). In contrast, 34 of 35 (97%) CRF01_AE Env recombinant viruses were resistant to neutralization by IgG1b12 as indicated in a previous report (182), suggesting that the difference in the IgG1 b12 susceptibility between CRF01_AE Env derived from recently and chronically infected Thai individuals. The five PNLG sites that were susceptible to 2G12 epitopes was absent on recently CRF01_AE Env, but present in the 2F5 and 4E10 epitopes on gp41 (Table 5.5). In addition, the pooled patient's serum derived from 10 recently infected Thai individuals was not inhibited by early CRF01_AE *env* genes or T/F Env-recombinant viruses (Table 5.4). Moreover, the pooled patient serum showed no neutralizing activity to pNL-envCT and pNL-BaLenv (Table 5.4), while the replication of selected recombinant viruses containing early CRF01_AE *env* genes was efficiently inhibited in serum derived from HIV-1 chronically infected individuals, suggesting that the serum derived from recently

infected individuals had no or quite low anti-HIV-1 neutralizing activity and a one possibility was that serum samples were collected from the individuals in an acute HIV-1 infected phase prior the induction of anti-HIV-1 humoral immune responses.

5.4.2 Fourteen early CRF01_AE Env-recombinant viruses to neutralization susceptibility with specific viral entry

Fourteen early CRF01_AE Env-recombinant viruses were examined for neutralization susceptibility to HIV-1 entry inhibitors. The replication of all recombinant viruses containingX4 or X4R5 early CRF01_AE *env* genes was inhibited by a CXCR4 antagonist, AMD3100 (185), while that of all recombinant viruses containing R5 and X4R5 env genes was inhibited by a CCR5 antagonist, TAK-779 (186) at various levels (Table 5.6). In addition, the replication of most recombinant viruses was inhibited at various levels by a fusion inhibitor, T20 (187), whereas a recombinant virus containing early CRF01_AE *env* gene, RTA24 was T-20 resistant (Table 5.6). These compounds were effectively suppressing viral replication of 14 early CRF01_AE Env-recombinant viruses which were approximately shown 21.6-, 108- to susceptibility to TAK-779 and T-20, respectively (Table 5.6). Furthermore, dual co-receptor usage (RTA3_X4R5) showed higher susceptibility to AMD3100 than pNL (pNL4-3) whereas RTA3_X4 showed higher susceptibility to T-20 than RTA3_R5, indicating that TAK-779 may confer to test the potentially early Env-recombinant viruses, and T-20 may also be used for microbicidal treatments.

Table 5.4. Neutralization susceptibility of 14 T/F CRF01_AE Env-recombinant viruses to NHMAbs and pooled patient's serum[a]

Env gene	Co-receptor usages	Target cells[a]	IC50 of NHMAbs(10µg/ml)				ID50 of pooled patient serum
			2F5	4E10	2G12	b12	
pNL4-3[b]	X4	U87.X4	4.05	8.43	1.92	0.38	<20[d]
pBa-L[b]	R5	U87.R5	3.44	3.84	0.92	0.27	<20
RTA2	R5	U87.R5	>10	2.36	>10	9.1	<20
RTA3	X4R5	U87.R5	>10	2.75	>10	>10	<20
		U87.X4	>10	2.22	>10	>10	<20
RTA4	R5	U87.R5	8.32	2.28	>10	>10	<20
RTA5	R5	U87.R5	>10	2.49	>10	>10	<20
RTA6	R5	U87.R5	>10	0.85	>10	>10	<20
RTA8	R5	U87.R5	4.35	3.57	>10	>10	<20
RTA9	R5	U87.R5	7.1	2.29	>10	5.47	<20
RTA11	R5	U87.R5	5.02	4.47	>10	>10	<20
RTA13	R5	U87.R5	3.59	2.3	>10	>10	<20
RTA16	R5	U87.R5	3.35	2.53	>10	0.93	<20
RTA21	R5	U87.R5	7.6	2.93	>10	>10	<20
RTA23	R5	U87.R5	2.59	1.78	>10	5.12	<20
RTA24	R5	U87.R5	>10	3.47	>10	2.83	<20
RTA27	R5	U87.R5	3.75	5.46	>10	>10	<20

[a.] Neutralization susceptibility was performed by using U87.CD4.CXCR4 for X4 virus whereas U87.CD4.R5 for R5 virus, in addition, X4R5 was performed by using both cell lines.

[b.] Recombinant virus containing subtype B X4 (pNL4-3) and R5 (pBaL) Env.

[c.] IC50 was >10 µg/ml.

[d.] They have been defined as pooled patient serum at which viral replication was not suppressed by 50% inhibition at low level concentration.

Table 5.5 Deduced amino acid sequences of 14 T/F CRF01_AE Env-recombinant viruses related to 2G12, 2F5 and 4E10 epitopes.

	2G12 epitope[a]					2F5 epitope[b]						4E10 epitope[b]					
env gene	295N	332N	339N	386N	392N	E	L	D	K	W	A	N	W	F	D	I	T
RTA2	-	-	343	-	394	.	.	.	.	.	T	.	.	.	.	.	.
RTA3	-	-	343	-	-	.	.	.	.	.	T	.	.	.	S	.	.
RTA4	-	-	343	-	394	.	.	.	.	.	.	S	.	.	.	.	.
RTA5	-	-	343	-	394	.	.	.	.	.	.	S	.	.	.	.	.
RTA6	-	-	343	-	394	.	.	.	.	.	T	.	.	.	.	.	.
RTA8	-	-	341	-	394	A	.	.	.	.	.	.	.	.	.	.	.
RTA9	-	-	343	-	394	.	.	.	.	.	.	.	.	.	.	.	.
RTA11	-	-	341	-	394	A	.	.	.	.	.	.	.	.	.	.	.
RTA13	-	-	343	-	394	.	.	.	.	.	.	.	.	.	S	.	.
RTA16	-	-	341	-	394	.	.	.	.	.	.	.	.	.	.	.	.
RTA21	-	-	343	-	394	.	.	.	.	.	.	.	.	.	.	.	.
RTA23	-	-	343	-	394	.	.	.	.	.	.	.	.	.	.	.	.
RTA24	-	-	343	-	394	.	.	.	.	.	T	.	.	.	.	.	.
RTA27	-	-	341	-	394	A	.	.	.	.	.	.	.	.	.	.	.

[a.] The five potential N-link glycosylation sites at 295, 332, 339, 386 and 392 were importantly recognized by 2G12. A minus indicates that PNLGs is not found and a shift PNLGs position is shown by the amino acid position.

[b.] Dot represented the preserved amino acid residues on 2F5 and 4E10 epitopes correspondence to reference CRF0_AE strain and the amino acid substitution is shown on each clone.

Table 5.6 Neutralization susceptibility of 14 T/F CRF01_AE Env-recombinant viruses to coreceptor antagonists, AMD3100 and TAK-779, and a fusion inhibition, T-20[a]

Env gene	Coreceptor usage	Target cells	IC50 (nM)[b]		
			AMD3100	TAK-779	T20
pNL4-3[c]	X4	U87.X4	8.9	ND[d]	92
pBaL[c]	R5	U87.R5	ND	4.5	12.2
RTA2	R5	U87.R5	ND	5.9	17.6
RTA3	X4R5	U87.R5	ND	12.5	9.36
		U87.X4	2.1	ND	25.6
RTA4	R5	U87.R5	ND	8.9	4.7
RTA5	R5	U87.R5	ND	4.1	16.8
RTA6	R5	U87.R5	ND	5.4	16.4
RTA8	R5	U87.R5	ND	3.3	33.6
RTA9	R5	U87.R5	ND	8.9	21.9
RTA11	R5	U87.R5	ND	6.1	18.6
RTA13	R5	U87.R5	ND	5.5	2.9
RTA16	R5	U87.R5	ND	3.2	5.9
RTA21	R5	U87.R5	ND	3.6	137
RTA23	R5	U87.R5	ND	13.1	49.8
RTA24	R5	U87.R5	ND	3.7	>175nM
RTA27	R5	U87.R5	ND	21.6	315

a. Neutralization susceptibility of X4 or R5 virus was examined using U87.CD4.CCR5 and U87.CD4.CXCR4 cells, respectively, whereas that of X4R5 virus was examined using both cell lines

b. IC50 of AMD3100, TAK-779 and T-20 for suppressing viral replication was calculated using GraphPad Prism 5 software

c. Recombinant virus containg subtype B X4 (pNL4-3) or R5 (pBaL) Env

d. Not done.

5.5. Correlations between NHMAbs and amino acid sequences of early CRF01_AE proteins.

Overall, the length of variable regions of Env gp120 and N-linked glycosylation of particular amino acid residues affected the protein structure and neutralization susceptibility of HIV-1 Env proteins (183,184); therefore, correlations were evaluated between the neutralization susceptibility and the amino acid sequences of the early CRF01_AE Env proteins. Correlations were studied among the number of amino acid residues in gp120 variable region, the number of PNLG sites in particular Env regions, amino acid mutations in epitope regions and the neutralization susceptibility of 14 early CRF01_AE Env-recombinant viruses. As a result, IC50 values of IgG1 b12 in inhibiting viral replication showed no significant correlation with the total length of gp120 V1/V2/V3/V4/V5 (V1+V2+V3+V4+V5), *p* value 0.4 and PNLG sites in V1/V2/V3/V4/V5, *p* value 0.23 and no correlations among IgG1 b12 IC50 and the length of V1/V2 amino acids, PNLG sites in V1/V2, *p* value 0.4 and 0.2 respectively (data not shown). However, a correlation was observed between the lengths of gp120 V1/V2 regions and the IC50 values of TAK-779 in inhibiting viral replication (Fig. 5.18A), indicating that the length of V1/V2 regions was negatively correlated with TAK-779 susceptibility of the recombinant viruses. In addition, the IC50 values of 4E10 in inhibiting viral replication were correlated with the lengths of the gp120 V4 region, indicating a negative correlation between the 4E10 susceptibility of recombinant viruses and the length of the gp120 V4 region (Fig. 5.18B). In contrast, no correlations were observed among V1/V2/V3/V4/V5 envelope lengths and V1/V2, the PNLG sites in V1/V2/V3/V4/V5 amino acids with IC50 value of TAK-779. Moreover, the length of V1/V2 and PNLG sites on V1/V2/V3/V4/V5 were inversely significant correlations with IC50 values of 4E10 in inhibiting viral replication, *p* value 0.012 and 0.0285, respectively (Fig 5.18C, D). The results suggested that length of V1-V2 regions and the total number of PNLG sites in V1 and V2 regions was correlated with 4E10 susceptibility of the recombinant viruses. In addition, no mutations were found in the N-terminal heptad repeat of gp41.

In RTA24 (Fig 5.16),T-20 resistance of recombinant virus containing RTA24 could not be explained by the introduction of mutation in this region. In contrast, a single amino acid mutation, A to T, was detected in the position 678 in the 2F5 core epitope, ELDKWA (143), on the membrane-proximal external region (MPER) of gp1 in RTA2, 3, 6 and 24 (Table 5.5), whereas the recombinant viruses containing these *env* genes were resistant to 2F5-mediated neutralization, suggesting an important role of this point mutation in conferring 2F5 resistance. Although a single amino acid mutation, E to A, was detected at the position 673 in the 2F5 core epitope in RTA4 (Fig 5.16), the Env clone was susceptible to neutralization by 2F5 (Table 5.4). In addition, no mutation was found in the core epitope in RTA5 (Table 5.5), although the Env clone was resistant to neutralization by 2F5 (Table 5.4), suggesting the existence of other determinant affecting the 2F5 susceptibility of certain *env* clones. Finally, although a single mutation, D to S or N to S, was detected in the 4E10 core epitope, NWFDIT (139), in RTA3, 4, 5 and 13 (Table 5.5, Fig 5.16), the recombinant virus containing these *env* clones were susceptible to neutralization by 4E10 (Table 5.4), indicating that such mutation has no major effect on 4E10 susceptibility of the *env* clones.

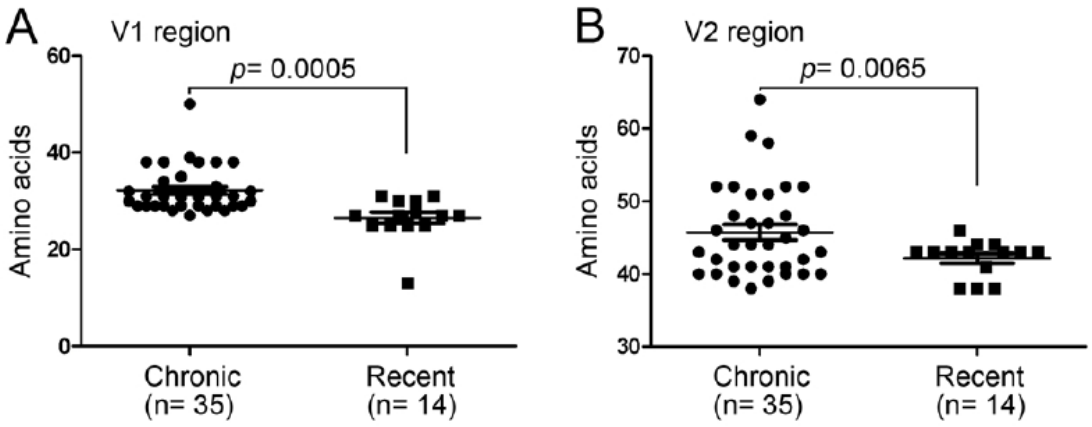


Figure 5.17 Comparison of the lengths of V1 and V2 regions between CRF01_AE Env gp120 derived from chronically and recently infected Thai individuals. The numbers of amino acid residues in the V1 (A) and V2 (B) regions of gp120 on 35 CRF01_AE Env derived from chronically infected individuals (Genbank accession numbers EU743757-EU743759 and EU743763-EU743794) (181) (Chronic), as well as on 14 early CRF01_AE Env derived from recently infected Thai individuals (Recent), were manually counted and plotted. Horizontal solid lines show median values, and the number of samples studied (n) is shown below the panels. Differences among groups were analyzed with the unpaired t test, and p values (*p*) are shown in the panels.

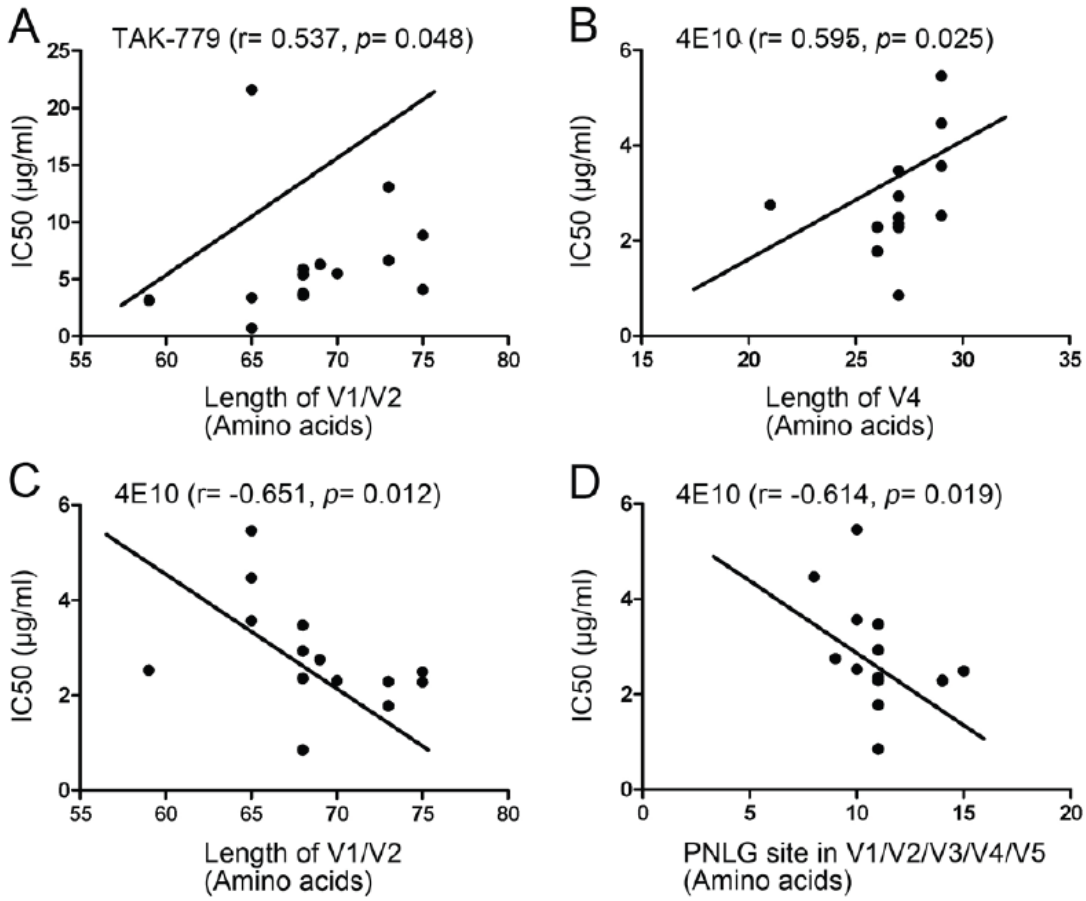


Figure 5.18 Correlations between the neutralization susceptibility of T/F CRF01_AE Env-recombinant virus and the length of gp120 variable regions or the number of PNLG sites. Neutralization susceptibility of CRF01_AE Env-recombinant virus to a CCR5 antagonist, TAK-779 (A) and to a NHMAb, 4E10 (B-D) was determined, as described in Materials and methods. The correlations were examined between the neutralization susceptibility of the early CRF01_AE Env-recombinant virus to TAK-779 or 4E10 and the length of indicated variable regions of gp120 (A-C) or the number of PNLG sites in the gp120 V1/V2/V3/V4/V5 regions (D). Correlations were evaluated using Spearman's rank correlation test, and the correlation coefficient (r) and p values (*p*) are shown above the panels.

CHAPTER VI
DISCUSSIONS

Importantly, immunogenic HIV-1 knowledge accounts for Env protein functions and properties against specific anti-HIV-1 immune responses. Viral genetic diversity limits the understanding of viral and host immune response interactions. In this regard, transmitted or founder virus is recommended to be used because it is relatively similar to viral vaccine strains for avoiding genetic diversities, as well as the viral panels may be revised for accurate, precise and reproductive measurements in vaccine candidates for standardized vaccine trails. Subsequently, early Env-recombinant viruses were constructed to clearly elicit the genotypic and phenotypic characterizations on *env* genes and also allow testing the breath, potentially specific anti-HIV-1 neutralizing antibodies and viral entry in infected HIV-1 individuals compared to the characteristics of *env* genes (188). At present, transmitted viruses have been the main interest in AIDS vaccine trials; in particular, characterized molecular clones on subtype B has been completed (189,190), whereas the number of HIV-1 strains non-subtype B has been partially limited for allowing the characterization of early *env* genes. Therefore, fourteen early Env-recombinant viruses subtype CRF01_AE HIV-1 circulating in Thailand were constructed to identify the *env* gene characterizations and to provide the useful tool for neutralization susceptibility evaluation in this study.

Results in this study revealed that transmitted or founder virus was established to properly determine the functional properties and understand the interactions between the characteristic *env* genes of transmitted virus and host immune response in acute HIV-1 infection. Importantly, Parrish et al., reported that transmitted or founder viruses carried 1.7-fold more infections than chronic viral variants, containing 1.9-fold viral particle comparing with chronically viral variants (191). Moreover, of the fourteen early CRF01_AE recombinant viruses, one was X4R5-tropic and thirteen were R5 viral variants. Parker et al., suggested that R5-troprism

was the most efficient transmissions in early or acute HIV-1 infection (192), which could mean that cell lines showed the high level CCR5 co-receptor expressions, especially the sexually mucosal transmission region (193). In parallel, the selective R5 transmitted HIV-1 viral variants were also over X4 viral variants in early primary stage as a consequence of the effective barrier, including intra-vaginal route, penile transmission, gastro-intestinal mucosal transmission and post-mucosal gatekeepers containing importantly biological properties such as CXCR4-binding chemokine (SDF-1, Stromal Cell-Derived Factor 1) for reducing X4 variants, and some restrictive defensins constrained against X4 viral variants (194). The mucosal gatekeeper efficiently selected R5 over X4 viral variants; however, X4 can transmit in early phase but they were the refractory replications (193,194). Moreover, the cationic gp120 in X4 bind to low affinity to heparin sulphate proteoglycans with difficulty, which is located on mucosal surfaces, leading to reduction in the number of these viruses than R5 viruses (195). This indicates that the selective mucosal tissue site pressures of transmitted or founder virus can be defined as R5 viral tropism in early phase. Additionally, T cell tropic R5 virus is the predominant phenotype in semen whereas macrophage tropic virus was rare in lymph nodes, blood and semen, demonstrating that a consistent pattern of $CD4^{hi}$ T cell R5 dependence predominantly occurs throughout acute HIV-1 infection (196,197).

Studies on the functional properties on *env* gene characterizations have shown that full length *env* genes cloned into Env-deficiency backbone allows for accurate characterizations and easy manipulations *in vitro* system. To date, two systems have been importantly utilized for the study of the interested *env* genes for testing potential neutralization susceptibility, including pseudotype-base (PV) assay and infectious molecular clones-base (IMC) assay. The HIV-1 long terminal repeat was utilized for prompt gene expression on IMC system, whereas an exogenous constitutive promoter was operated for PV system, such as cytomegalovirus immediate-early 1 gene (198). Provine et al., demonstrated that two systems differed from one in the context of Env incorporation and efficient cleavages; in contrast, they were similar to the ratio of proviral genomes and *env* gene expression plasmids. Furthermore, PV system may contain the strong promoter and appropriated on biochemical analysis (199). Besides, *env* plasmid DNA on IMC system showed low

infectivity as results of low amounts of plasmid *env* DNA incorporation and higher *env* cleavage on viral productions. Interestingly, the highest one priority of newly *env* genes not only proved to utilize and also considered to protect the unusually sensitive and resistant neutralizing antibodies mediated viral variants (200). Early CRF01_AE Env-recombinant viruses showed low infectivity, indicating that Env-recombinant viruses may defect on early *env* genes which were a consistent with previous report in chronically CRF01_AE infected HIV-1 by Utachee et al., (181,182). Although, p24 gag protein was properly presented in supernatant on HIV-1 receptor expression cell lines, triggering the immature viral particles into mature viral particles (data not shown). However, two systems showed different neutralization susceptibility to NHMAbs and viral entry inhibitions. The early Env infectious molecular clones showed various levels of relative infectivity on infected target cell lines, compared with luciferase activity on pNL4-3-infected U87.X4 (15).

Genetic diversities are important mechanisms of HIV-1 infection avoiding host immune responses and impeding developed effective vaccines (109). The standard pattern of transmitted virus was observed on mucosal tissue of viral infection by low-dose inoculation, selective homogenous sequences and phenotypic characterizations enhancing to single viral variant penetration (201). Nevertheless, the transmissions can establish few or more variants related to primary isolations. Due to initiating mucosal transmissions, transmitted virus importantly required of the greatest selective pressure on gp120 proteins than the others; in particular, Zhu et al., reported that Env proteins differed from each other and it was estimated 10% compared with those proteins (168). V3 loop is relatively conserved with 35 amino acid residues and low sequence variability on early subtype CRF01_AE transmitted variants. This is the predominantly characteristic *env* gene of transmitted or founder virus that is shown less genetic diversity than chronically CRF01_AE viral variants (181). The full length gp160 was observed in early CRF01_AE Env-recombinant viruses, amino acid residues of interclones were differed from chronic recombinant viruses, including gp160, gp120, gp41, C2-V5 and V3 (181). However, transmitted and founder viruses may be elicited both genetically homogenous together with heterogenous variants in early phase, but almost all viral variants were relatively show the genetic homogeneities comparing with chronic infections, demonstrating that transmitted or

founder viruses were predominantly obtained from the genetically homogenous variants as well as being closely related to parental isolations (33) in acute or early phase, providing the opportunistic window for eliciting viral and host immune response interactions, leading to focus in biological viral properties and explanation for effective development of AIDS vaccines (158,163,201).

Fourteen early CRF01_AE *env* genes were obtained, the length V1/V2 of amino acids similar to subtype B, the most extensively studied HIV-1 subtype. The length of V4 amino acid residues were different among early *env* genes in subtype E, B and C and also the number of PNLG sites on gp160 of early CRF01_AE was different (202,203) (data not shown). V1/V2 amino acid residues on early *env* genes subtype A were shorter than subtype B and no significant difference on PNLG sites. (204,205). Additionally, the lengths of V1 and V2 transmitted HIV-1 individuals are significantly different compared with chronically CRF01_AE infected HIV-1 individuals (Fig5.17)(182) which was consistent with previous reports (204,206), demonstrating that shorter and fewer glycosylation sites on gp120 variable region of recently infected HIV-1 may enhance Nabs accessibility for generating neutralizing antibodies and providing vaccine-elicited broad neutralizing antibodies. Moreover, we can deduce that T/F viruses change the number of envelope lengths and added glycosylation sites on *env* genes are expanded throughout the course of HIV-1 infection compared with chronically HIV-1 infection (181,182). Taken together, the different subtypes of HIV-1 infection lead to different biological properties as subsequent of HIV-1 pathogenesis, transmission and AIDS progression.

Humoral immune responses are developed in the natural course of HIV-1 infections and also limited to cross neutralizing activities. The potential N-linked glycosylation sites and envelope lengths on gp120 initially determine neutralization susceptibility properties and functions (207). The conserved epitopes of broad neutralizing antibodies have been remained in the widely neutralization sensitivities over the course of HIV-1 infection. Disclosed V3 loop contributes to neutralizing antibodies (NAbs) accessibility, unfortunately, the cryptically nature V3 evolves in HIV-1 by masking important epitopes. The mechanisms of viral variant avoided the NAbs accessibility by both adding potential N-linked glycosylation sites and the conformational change in V2 loop (122). Gp120 was higher in N-linked glycosylated

sites than O-linked glycans and N-linked was importantly identified in details. These glycans also shielded the effective neutralizing antibody and decreasing immunogenicities (207) such as the monoclonal antibody 2G12 specifically recognized a mannose-dependent epitopes on gp120 (138). Transmitted viruses are less glycosylated on sites and not harbor mannose epitopes on this monoclonal antibody, leading to no susceptibility to 2G12 neutralizing monoclonal antibody, thus, early CRF01_AE Env-recombinant can be highly replicated on target cells as a representing 2G12 which was similar to non-clade B. Moreover, the pattern of PNLG sites were increased changing on gp120 over the time course of HIV-1 infection but they were not specific to 2G12 epitopes, leading to unsuccessful development of this monoclonal antibody in the therapeutic against HIV-1 infection. For instance, gp41 has been mostly obtained from conserved amino acids sequences and less diversity than gp120 (207). Newly *env* genes were highly susceptible to 4E10 and 2F5, which were recognized on contagiously conserved regions on the membrane proximal external regions (MPER) of gp41, as a correspondence to conserved region, 2F5 (ELDKWA) (143), 4E10 (NWFDIT) taken together with consistent chronic CRF01_AF and transmitted or founder virus non clade B (203,204). Neutralization susceptibility to 4E10 and 2F5 are highly effective to specific epitopes on gp41. A to T showed on *env* genes enhancing to resistant 2F5 but mutation on conserved regions of 4E10 were not affected, demonstrating that amino acid sequence mutation are recognized on 2F5 conserved region, leading to resistance neutralization susceptibility, whereas 4E10 susceptibility was not affected.

At present, the several monoclonal antibodies for recognized CD4bs have different abilities to induce conformational changes on gp120; such as, IgG1, b12 was the broadly active CD4bs antibody which bound on specific on bridging sheet. After CD4 binding, V1-V2 loop plays the important role to recruit CD4 and bridging sheet formation, leading to second co-receptor incorporations (208) together with V1-V2 loop residues located at the apex of the unliganded trimer. The length of V1/V2 amino acid residues in early CRF01_AE *env* gene was significantly different from chronic infection. Therefore, early *env* genes of HIV-1 infection are determined to susceptible neutralization to IgG1 b12 which is in concordance with previous studies on subtype C (203,209), demonstrating that transmitted or founder virus on subtype CRF01_AE *env*

genes may contain IgG1 b12 epitopes and the shorter length of V1/V2 amino acid residues than chronic HIV-1 infection.

T-20 is a compound, blocking the six-helix bundle formations on fusion mechanism of HIV-1 and is sensitized by mimic 36 amino acids of HR2, after binding between virus and CD4, T-20 highly inhibits fusion efficiency. Previous studies observed that the amino acid substitution on HR1 (N42S, G36D) resisted to T-20 phenotype which is in absence of the mutation on RTA24 which was T-20 resistance for suppressing viral replication. Additionally, Milchi et al., confirmed that carrying Δ32-CCR5 on host cell results in more favorably responsiveness to T-20 (210), demonstrating that R5-tropic can confer the high affinity binding affecting to reduce T-20 functional time on HR1 region enhancing faster fusion (211,212). In contrast, viral variants are reduced CCR5 affinity binding leading to increased T20 sensitiveness (213). Thus, viral strains may determine neutralization susceptibility to T-20 as a result of high level co-receptor CCR5 affinity (213). Taken together, recently HIV-1 subtype B variants were not sensitive to T-20 as a result of mutation at the position of codons from 36 to 45 amino acid residues (GIVQQQNNLL) on HR1 and changed N42D and G36D (31,32,210). For instance, X4 viruses are less resistant co-receptor antagonist than R5 viruses. However, the mutation of V1, V2, V3 and V4 on gp120 has been related to X4 viruses to resist CXCR4 antagonist, especially, a five amino acid residues deletion on V4 loop (position 364-368), FNSTW (194). Furthermore, viral variant isolations determine co-receptor density and different affinity HIV-1 *env* for CCR5 co-receptor binding leading to viral efficient fusion and entry target cell (207). During the course of infection, R5 viruses tend to increase CCR5 co-receptor affinity enhancing to reduce susceptibility with co-receptor antagonists and fusion inhibitors (212,214).

Interestingly, antigenic structure of envelope glycoproteins is predominantly determined for vaccine designs, but it is difficult to translate this message for generating broadly neutralizing antibodies. Utilizing statistic methods, they help study amino acid patterns on *env* gene which may share common immunogenic structures for vaccine fields (188). As a rule, broad neutralizing antibodies are required to inhibit viral entry either by direct recognition to the pre-receptor engaged native spike of viral surface or by receptor engaged requirement for

specific epitope regions and capable of mapping gp120 subunit (215). Env gene characterizations are examined in terms of the correlations of neutralization susceptibility with HNMAbs. Conversely significant correlations were observed among the characteristic newly *env* genes (the lengths of V1/V2, the number potential glycosylation sites on V1/V2 and V1/V2/V3/V4/V5) and 4E10. In contrast, no correlations were observed in chronic HIV-1 infections (182). Specific anti-MPER triggered their respective epitopes after receptor engagement and may bound viral spike directly which selected accessibility both V loop and gp41mutation (209,216). Moreover, Peachman et al., demonstrated that 2F5 and 4E10 bind to virions before CD4 ligation that is not determined to MPER epitopes binding (217). It can be acknowledged that the rearrangement quaternary MPER provided the selective antibody pressure on the variable loops or CD4 binding sites (209). Besides, correlation between the length of V1/V2 amino acid residues and the value IC50 TAK-779, is a small molecule to effectively inhibit CCR5 tropism for viral replication and also bound to the cavity loop of seven transmembrane receptors at 1, 2, 3 and 7 (32,186). It has been directly interacted with the extracellular loop as result of conformational change in second extracellular loop correspondence to monoclonal antibody activities. Furthermore, mutation on V3, C2, V2 and C4 elicited to resist CCR5 antagonist (218), acknowledging that CCR5 antagonist is associated with the characteristic *env* genes.

Early viral variants may also exhibit shorter envelope lengths and fewer potential N-linked glycosylation sites, which are the importantly *env* gene characterization lead to different neutralization susceptibilities comparing with chronic viral variants (181,182), demonstrating that transmitted or founder virus is different in terms of variable envelope lengths from chronic viral variants in subtype CRF01_AE HIV-1 infections. Enhancing newly recombinant viruses are useful tool to measure the susceptible neutralizing antibodies in the medical field researches. Unfortunately, this study is unable to obtain reciprocal dilution which is suppressed viral replication by pooled patient's serum. It can indicate that amount of antibodies in recent HIV-1 infection is few humoral antibody as a correspondence of my samples that were positive by BED ELISA at the low proportion of IgG antibody (OD cut off <0.8). Furthermore, pooled patient serum cannot inhibit viral replication at low

concentrations, demonstrating that generated Env-recombinant viruses against specific Nabs within weeks after infection are more effective responsive to autologous viruses on each individual than heterologous viruses and it is possible that serum samples were collected from the individuals in acute phase HIV-1infection prior the induction of anti-HIV-1 humoral immune responses. (122).

CHAPTER VII
CONCLUSIONS

This study is the first extensive report on newly *env* gene characterizations and functional properties on subtype CRF01_AE HIV-1 infected Thai individuals circulating in Thailand. Transmitted or founder virus shows genetic viral homogeneity, highly transmission fitness and strongly selective pressure on host immune responses, which are determined as an effective viral replication in different environments in the acute phase. Previous reports demonstrated that almost all early *env* genes of subtype CRF01_AE on Env-recombinant viruses are less diverse compared to chronic CRF01_AE HIV-1 infection. However, the highest amino acid diversity is observed from C2 to V5 of gp120 region. Furthermore, the quaternary V1/V2 amino acid residues play predominant roles in neutralization susceptibilities with neutralizing human monoclonal antibodies (NHMAbs) in acute HIV-1 infection and also changes amino acid residues throughout the course of HIV-1 infection. Besides, V4 amino acid residues lies on the outer domain of gp120 which is the first contact with CD4 receptor correlating with neutralizing accessibility and potential breadths. Thus, added PNLG sites on V4 amino acid residues has an effect on the length of V4 amino acids on gp120 region of subtype CRF01_AE, which also differs amino acid residues from other subtypes. Fourteen newly CRF01_AE Env-recombinant viruses reveal several degrees of neutralization susceptibility with neutralizing human monoclonal antibodies, co-receptor antagonists and a fusion inhibition of which functions and properties are able to evaluate molecular mechanisms as consequences of resistant phenotype to humoral immune responses or to viral entry.

Additionally, *env* genetic diversity is a predominant mechanism for both avoiding host immune responses and playing a pivotal role in immune damages. The important conserved epitopes are crucially pointed of vaccine designs against HIV-1 infection with effective antibody productions in AIDS vaccine trails. Therefore, newly CRF01_AE Env-recombinant viruses are constructed as a useful tool for studying the

characteristic newly *env* gene to neutralization susceptibility. Moreover, newly CRF01_AE *env* genes are also valuable for studying of the phenotypic properties of transmitted or founder viruses, including their antigenicities and mucosal tissue tropisms. The established early CRF01_AE Env-recombinant viruses are advantageous to evaluate of the efficacy of immune responses elicited by vaccine candidates, microbicides susceptible to HIV-1 treatments. In particular, Env-recombinant viruses can be continued to study in the immune response functions and properties on newly subtype CRE01_AE HIV-1 infection. However, pooled patient's serum showed no susceptibility to early Env-recombinant viruses in this study. This study will motivate us to successfully seek for the whole data in autologous and heterologous immune responses against transmitted or founder virus in early phase subtype CRF01_AE HIV type 1 infected individuals in further study.

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APPENDIX

Reagent for preparing competent culture in *E.coli*

1. Medium

1.1. SOB medium is the medium for preparing cells for chemical transformation and SOC is a special medium for incubating competent cells after transformation to permit the resistant gene expression before exposing cells to selective conditions (219)

Content	quantity
Bacto tryptone	2 gm
Bacto Yeast Extract	0.5 gm
5M NaCl	0.2 gm
2M KCl	0.125 gm
Deonized water	99 ml

Sterile by autoclave and add 1 ml of 2M Mg^{++}

Keep at $4^{\circ}C$

1.2 SOC Medium: Add 2M glucose in SOB (1ml/100ml of SOB)

1.3 LB broth: 20 gm add into 1000 ml of distill water

1.4 LB agar: 35 gm in 1000 ml of distill water

2. Transformation Buffer

Content	Quantity
- PIPES (piperazine-1,2-bis[2-ethanesulfonic acid])	1.5 gm
- $CaCl_2.2H_2O$	1.1 gm
- KCl	9.3 gm
- Deonized water	480 ml

2.1 Adjust pH to 6.7 by using 1M KOH solution and add $MnCl_2.4H_2O$ = 5.45 gm

2.2 Add distill water up to 500 ml sterile by using filtration (0.22 μm pore size)

3. DMEM cells culture

DMEM is modified from basal medium eagles and contain 1000 mg/L glucose, with L-glutamine and sodium biocarbonate.

3.1 T293 medium culture

Content	Quantity
DMEM	100 ml
10% FBS	10 ml

3.2 U87.CD4.CCR5 or U87.CD4.CXCR4

Content	Quantity
DMEM	80 ml
10% FBS	8 ml
Puromycin (1mg/ml)	80 μl
G418 (500mg/ml)	48 μl

4. TAE buffer

TAE buffer is used for separating nucleic acids including DNA and RNA in agarose gel. Typically, it is solution pH8 and EDTA sequesters divalent cations.

Content (50X)	Quantity
- Tris base	242 gm
- Glacial acetic acid	57.1 ml
- 500mM EDTA	100 gm
- Deonized water	1000 ml

5. Preparation Env-recombinant viruses

Content	Quantity
- Env-proviral	600 ng
- Fugene (Roche)	2 μl
- Optimem	100 μl
- 293T	60,000 cells

BIOGRAPHY

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