บทบาทของโมเลกุล HLA CLASS I และเซลล์ CD8+ ทีลิมโฟไซต์ ที่ตอบสนองอย่างจำเพาะต่อโปรตีนเอชไอวีและสร้างไซโตไคน์ชนิดอินเตอเฟอรอนแกมมา ต่อการควบคุมหรือการหลบหลีกของไวรัสเอชไอวี ในอาสาสมัครคนไทยที่ติดเชื้อเอชไอวี

นางสาวสุปราณี บูรณประดิษฐ์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE ROLES OF HIV-1 SPECIFIC INTERFERON GAMMA (IFN-gamma) PRODUCING CD8+ T LYMPHOCYTE RESPONSES AND HLA CLASS I ALLELES ON VIRAL ESCAPE AND VIRAL CONTROL IN HIV-1 INFECTED THAI INDIVIDUALS

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สุปราณี บูรณประดิษฐ์กุล: บทบาทของโมเลกุล HLA CLASS I และเซลล์ CD8+ ทีลิมโฟไซต์ ที่ตอบสนองอย่างจำเพาะต่อโปรตีนเอชไอวี และสร้างไซโตไคน์ชนิดอินเตอเฟอรอนแกมมา ต่อการควบคุมหรือการหลบหลีกของไวรัสเอชไอวี ในอาสาสมัครคนไทยที่ติดเชื้อเอชไอวี. (THE ROLES OF HIV-1 SPECIFIC INTERFERON GAMMA (IFN-gamma) PRODUCING CD8+ T LYMPHOCYTE RESPONSES AND HLA CLASS I ALLELES ON VIRAL ESCAPE AND VIRAL CONTROL IN HIV-1 INFECTED THAI INDIVIDUALS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.เกียรติ รักษ์รุ่งธรรม, 127 หน้า.

เนื่องจากยังไม่มีข้อมูลด้านบทบาทของ CD8+ T cell ที่เกี่ยวข้องกับการหลบหลีกภูมิคุ้มกันของไวรัสเอชไอวี และการควบคุมปริมาณไวรัสในสายพันธุ์ CRF01_AE ซึ่งเป็นสายพันธุ์ที่พบบ่อยที่สุดในคนไทยและในตะวันออกเฉียงใต้ อีกทั้งคนไทยยังมี HLA ที่แตกต่างจากกลุ่มชาวตะวันตก จึงทำการศึกษาผู้ติดเชื้อจำนวน 240 รายที่ยังไม่เคยได้รับการรักษาด้วยยาต้านไวรัส โดยการตรวจหาการตอบสนองทางภูมิคุ้มกันชนิด CD8+ T cells เมื่อกระตุ้นด้วยเปปไทด์ ขนาดความยาว 18 กรดอะมิโนที่เหลื่อมกัน 11 กรดอะมิโน สังเคราะห์จากลำดับเบสของเชื้อ HIV-1 สายพันธุ์ CRF01_AE จำนวนทั้งสิ้น 413 เส้น และตรวจวัดด้วยวิธี IFN-γ ELISpot และใช้ bioinformatic ในการหา epitope ใหม่ เพื่อทำการตรวจหาด้วยวิธี HLA restriction และ fine mapping ต่อไป รวมทั้งได้ทำการตรวจลำดับเบสของเชื้อ HIV ในอาสาสมัครส่วนใหญ่ด้วยสถิติในการวิเคราะห์หาความสัมพันธ์ของ epitope และ HLA allele ต่อการหลบหลีกภูมิคุ้มกันของไวรัสและการควบคุมปริมาณไวรัส ผลการศึกษาพบว่า HLA-B*1302 และ HLA-A*2410 (ในอาสาสมัครที่ไม่มี protective allele ที่เคยมีรายงาน) มีความสัมพันธ์กับการมีปริมาณไวรัสในเลือดต่ำ อย่างมีนัยสำคัญทางสถิติ (p = 0.0024 และ p = 0.0062 ตามลำดับ) นอกจากนี้ HIV โปรตีนที่มีการตอบสนองสูงได้แก่ Pol 91%, Gag 87%, Env 83% และ Nef 78% ดังที่เคยมีรายงานในอาสาสมัครชาติพันธุ์อื่นและเชื้อสายพันธุ์อื่น ในการศึกษานี้ยังพบว่าการตอบสนองต่อ Gag มีความสัมพันธ์กับการมีปริมาณไวรัสในเลือดต่ำ ในขณะที่ การตอบสนองต่อ Env มีความสัมพันธ์กับการมีปริมาณไวรัสในเลือดสูง จากการวิเคราะห์ผลเฉพาะสายพันธุ์ CRF01_AE จำนวน 195 ราย พบ epitope ใหม่จำนวน 33 เส้น ตรวจพบ epitope ใหม่ที่เป็น immunodominant คือ YI9 (อยู่ใน p24 ของ Gag) มีการตอบสนองใน 29% ของอาสาสมัคร โดยการนำเสนอด้วย HLA-Cw*0102 จากผลวิเคราะห์ลำดับเบสทางพันธุกรรมของเชื้อในอาสาสมัครที่มี HLA นี้ พบว่ามีการหลบหลีกภูมิคุ้มกันของไวรัสเกิดขึ้น คือมีการกลายพันธุ์ที่ตำแหน่ง P2 (S278X), P4 (V280X) and P5 (S281G) ซึ่งมีผลทำให้สูญเสียการตอบสนองด้วยวิธี ELISpot ไป การกลายพันธุ์ที่ P2 พบมากที่สุด (p = 0.0002) นอกจากนี้ได้มีการค้นพบ epitope ที่เกี่ยวข้องกับการควบคุมไวรัส คือ RI10 (อยู่ใน HIV-protease) ผ่านทาง HLA-A*0203 ในคนไทย (ซึ่งเดิมมีรายงานในสายพันธุ์ B ว่านำเสนอโดย HLA-B*13) ที่น่าสนใจคือผู้ที่มี HLA-A*0203 และมีการตอบสนองต่อ Pol-OLP16 จะมีค่าปริมาณไวรัสในเลือดต่ำกว่าผู้ที่มี HLA-A*0203 แต่ไม่มีการตอบสนองต่อ Pol-OLP16 อย่างมีนัยสำคัญทางสถิติ (p = 0.0167) มีข้อสังเกตว่า การกลายพันธุ์ของไวรัสในบริเวณ RI10 นี้ แม้จะเกิดการหลบหลีกการตอบสนองต่อ CD8 T cells แต่อาสาสมัครเหล่านี้ยังคงมีค่าปริมาณไวรัสต่ำในเลือด สันนิษฐานว่าการกลายพันธุ์บริเวณ RI10 นี้อาจก่อให้เกิดการสูญเสียความสามารถในการแบ่งตัวของเชื้อได้ นอกจากนี้ AK11 epitope ซึ่งนำเสนอโดย HLA-A*1101 (epitope ที่เคยรายงานมาก่อน) ในการศึกษานี้ พบว่า AK11 มีความเกี่ยวข้องกับการควบคุมไวรัสและไม่พบการกลายพันธุ์ของไวรัสในบริเวณนี้

โดยสรุป การศึกษาในผู้ติดเชื้อคนไทยที่ส่วนใหญ่ติดเชื้อสายพันธุ์ CRF01_AE และมี HLA polymorphism ที่แตกต่างจากชาวตะวันตก ผลวิจัยที่ตรงกันคือ การตอบสนองของ CD8 T cells ที่จำเพาะต่อ HIV-1 Gag มีส่วนสำคัญในการควบคุมปริมาณเชื้อไวรัส สิ่งที่ค้นพบใหม่คือ คุณลักษณะของ epitope จำนวน 3 เส้น ได้แก่ YI9, RI10 และ AK11 ซึ่งนำเสนอโดย HLA-Cw*0102, -A*0203 และ -A*1101 ตามลำดับ ทั้งนี้ RI10 และ AK11 มีความสัมพันธ์กับปริมาณไวรัสในเลือดต่ำ และอาจช่วยในการควบคุมไวรัสได้ นอกจากนี้ยังมี epitope ใหม่อีกหลายเส้นที่จะมีการศึกษาวิจัยต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: HIV-1/ CD8+ T LYMPHOCYTE/ HLA/ VIRAL ESCAPE/ VIRAL CONTROL/ ELISPOT ASSAY

SUPRANEE BURANAPRADITKUN : THE ROLES OF HIV-1 SPECIFIC INTERFERON GAMMA (IFN-gamma) PRODUCING CD8+ T LYMPHOCYTE RESPONSES AND HLA CLASS I ALLELES ON VIRAL ESCAPE AND VIRAL CONTROL IN HIV-1 INFECTED THAI INDIVIDUALS. ADVISOR: PROF. KIAT RUXRUNGTHAM, M.D., 127 pp.

The role of CD8+ T cells and HLA class I in viral escape and viral control has not been well characterized in clade CRF01_AE and Asian ethnics. 240 naïve HIV-1 infected Thai individuals were screened by IFN-γ ELISpot assay for HIV-1 specific CD8+ T cell responses with a set of 413 overlapping peptides (OLPs) of HIV-1 proteome. Novel epitopes were identified by bioinformatics analysis and were further characterized by HLA restriction and fine epitope mapping. The association of epitopes and/or HLA alleles with low plasma HIV-RNA levels and/or viral escape was analyzed using non parametric statistical analysis. We found that HLA-B*1302 and HLA-A*2410 after exclude other known protective alleles were associated with low plasma HIV-RNA level (p = 0.0024 and p = 0.0062, respectively). The four most common CD8+ T cell responded HIV-1 targets included Pol (91%), Gag (87%), Env (83%) and Nef (78%). As reported in other ethnic and subtypes, Gag-specific CD8+ T cell response was found associated with low plasma HIV-RNA level whereas Env-specific CD8+ T cell response was associated with high plasma HIV-RNA level. From 195 HIV-1 CRF01_AE infected Thais, 33 OLPs as potential novel epitopes were identified. A novel immunodominant (29% response rate) restricted by HLA-Cw*0102: YI9 (in Gag-p24) which was associated with viral escape. Mutations at P2 (S278X), P4 (V280X) and P5 (S281G) impaired the ELISpot responses, however the P2 anchor S278K mutation had the highest negative impact (p = 0.0002). We also found two viral control epitopes, the first one is RI10 (HIV-protease, previously described in HIV-1 B clade as -B*13 restricted) was found restricted by HLA-A*0203 in Thais. Interestingly, HLA-A*0203+ve patients with RI10 responders had a significantly lower plasma HIV-RNA level than non-responders (p = 0.0167). Moreover, mutation in this RI10 had resulted to immune escape, but it was remained associated with low plasma HIV-RNA level, this may be due to compromising on their viral fitness. The other one is the known HLA-A*1101 epitope, AK11 (in Gag-p24), there was no significant mutation of viruses found in patients expressing A*1101.

In conclusions: HLA-B*1302 and HLA*A2410 were associated with low plasma HIV-RNA level. Pol, Gag, Env and Nef are four most common HIV targets of CD8 T cells, however; only HIV-1 Gag was found associated with low plasma HIV-RNA level. Three CD8 epitopes: YI9, RI10 and AK11 restricted by HLA-Cw*0102, -A*0203 and -A*1101, respectively; were characterized. RI10 and AK11, but not YI9, were associated with lower plasma HIV-RNA level that possibly to HIV viral control. Further characterization of the rest of possible novel epitopes is warranted.

Field of Study: Medical Microbiology Student's Signature _____
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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ARC	AIDS related complex
ARV	AIDS associated retrovirus or antiretroviral
APC	Antigen presenting cell or Allophycocyanin
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
β_2m	Beta-2 microglobulin
BFA	Brefeldin A
BLCL	B lymphoblastoid cell lines
bp	Base pair
°C	Degree celsius
CCR5	Chemokine receptor 5
CD	Cluster differentiation
CO_2	Carbon dioxide
cpm	Count per minute
cpz	Chimpanzee
^{51}Cr	Chromium-51
CRF	Circulating recombinant form
CSA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
Cu.mm.	Cubic millimeter
Da	Dalton unit
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EC	Elite controller
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
env	Envelope gene
ER	Endoplasmic reticulum
et al.	et alii
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram (s)
gag	Group antigen gene
GALT	Gut-associated lymphoid tissue
γδ T cell	Gamma-delta T cell
GM-CSF	Granulocyte macrophage colony-stimulating factor
gp	Glycoprotein
h	Hour
HEPS	Highly-exposed persistently seronegative person
HIV	Human immunodeficiency virus

HLA	Human leukocyte antigen
HTLV III	Human T-lymphotropic virus type III
I	Ionomycin
ICAM3	Intercellular adhesion molecule 3
ICCS	Intracellular cytokine staining
ICE	Interleukin-1β-converting enzyme
IFN	Interferon
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-2	Interleukin 2
IL-7	Interleukin 7
IN	Integrase
Ir	Immune response gene
IVDU	Intravenous drug user
kb	Kilobases
kD	Kilodaltons
LAV	Lymphadenopathy-associated virus
LC	Langerhans cell
LT	Lymphotoxin
LTNP	long term nonprogressor
LTR	Long terminal repeat
mAb	Monoclonal antibody
mDC	Myeloid dendritic cell
Mg2+	Magnesium
MHC	Major histocompatibility complex
min	Minute (s)
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
ml	Mililitre (s)
mm^3	Milimetre (s)
µl	Microlitre (s)
µg	Microgram (s)
mRNA	Messenger ribonucleic acid
MSM	Men sex with men
MTCT	Mother to child transmission
M-tropic	Monocytotropic
nef	Negative factor gene
NIH	National Institute of Health
NK cell	Natural killer cell
nm	Nanometer
OLP	Overlapping peptide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PHI	Primary HIV infection
PMA	Phorbolmyristate acetate
pol	Polymerase gene
PR	Protease
PVDF	Polyvinylidine fluoride
pDC	Plasmacytoid dendritic cell
PHI	Primary HIV Infection
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
Rev	Regulator protein of expression of viral proteins
RNA	Ribonucleic acid
RPMI1640	Rosewell Park Memorial Institute formular 1640
RRE	Rev-response element
rpm	Round per minute
RT	Reverse transcriptase
s	Second (s)
SBT	Sequenced Based Typing
SFU	IFN-γ spot-forming unit
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SR	Spontaneous release
TAP	Transporter associated with processing
TAR	transactivating response element
tat	Transactivator of transcription gene
TCR	T cell receptor
Th	T helper cell
TLR	Toll like receptors
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TR	Total release
TX-100	Triton X-100
vif	Viral infectivity factor gene
Vpr	Viral protein R
Vpu	Viral protein U
WBC	White blood cell
WHO	World Health Organization
WT	Wild type

CHAPTER I

INTRODUCTION

Human Immunodeficiency Virus (HIV) is a retrovirus causing acquired immunodeficiency syndrome (AIDS). Although as many as 40 million people as estimated by WHO in 2008 were infected with HIV worldwide[1], there is still no any effective HIV vaccine available. Prevalence of HIV is geographically different. The highest prevalence is found in Sub-Saharan Africa (5.2%) and the rest which lower than 1% is found in Eastern Europe and Central Asia (0.7%), North America (0.6%) and South and South East Asia (0.3%). In Thailand, HIV prevalence is approximately 1.4% of Thai population. The majority is sexually transmitted, of which approximately 40% are heterosexual, 33% are men who have sex with men (MSM). Ten percent are intravenous drug users (IVDUs), 0.85% are mother to child transmission (MTCT) and 0.1% are blood donors[2].

HIV-1, a diploid single-stranded 9.2 kb RNA virus, is in the family *Retroviridae*. HIV has reverse transcriptase (RT) that can synthesize DNA from its RNA template. The genome of HIV-1 contains 3 structural genes (*gag, pol,* and *env*), and 6 accessory genes (*nef, rev, tat, vif, vpu* and *vpr*)[3]. According to the high mutation rate of HIV genome as resulted from poor proof-reading capacity of HIV-1 RT, 9 clades (A through K, excluding E and I) and more than 30 circulating recombinant forms (CRFs) have been classified based on HIV-1 genetic diversity[4]. The most common epidemic HIV-1 strain which can be found in most developed countries is Clade B[5]. In contrast, CRF01_AE is the major clade (more than 90%) found in Thailand and in South East Asia whereas clade B' (Thai B, V3 motif GQGP) is the much less common (5-10%).

After infection, HIV as free viral particles or within infected CD4+ T cells can stimulate the cellular and humoral immune responses necessary to combat with viral infection. In addition, the pathogenesis of the disease can be described in 3 phases of HIV-1 infection, acute, chronic and AIDS[6]. The acute infection phase (~100 days) consists of eclipse phase and fiebig stage (I-V). The eclipse phase takes approximately 10 days started from infection until the first plasma HIV-RNA level detection. The fiebig stage I-V defined by the time of detecting the HIV-1 antigen and antibody in different techniques such as p24 antigen, anti HIV antibodies by ELISA or western blot. The level of CD4+ T cells is generally high in acute infection phase. During 20 days of fiebig stage I-V, plasma HIV-RNA level shows an exponential increasing approximately 10^6 copies/ml and peaks at 21-28 days after infection.

Decreasing of plasma HIV-RNA level is found in fiebig stage V and begins to plateau (viral set point) before the early chronic infection phase[7].

In the period of acute infection phase, the immune responses to HIV-1 can be detected after the first viral replication occurs. Within 2 to 4 weeks, an increasing number of HIV-specific CD8+ cytotoxic T lymphocytes (CTL) can be detected prior to peak viremia. The plasma HIV-RNA level decreases along with the peak of the CD8+ T cell response detection. Then, the incomplete recovery of CD4+ T cells level is observed. HIV-specific antibodies are usually presented in the second and the third week of infection. The wild type (WT) virus can be detected during infection before HIV-1 specific CD8+ T cell expansion. However, the viral escape is occurred within 10 days after T cell expansion indicating that HIV-1 is selected by the pressure of HIV-1 specific CTLs. Even though the viral escape occurs, the HIV-1 specific CD8+ T cells and later on with HIV-1 specific neutralizing antibody can maintain the virus to viral set point[7,8]. In terms of HIV-1 antigen targeting, CD8+ T cell responses specific against Env and Nef can be earlier detected at the acute infection phase while specific against Gag and Pol can be detected during the period of viral steady stage[7].

Several research groups have found high levels of specific cytotoxic T cell activity directed to most HIV proteins in patients during or even before seroconversion. CTLs play an essential role in protection and control of HIV infection[9]. CTL are capable of killing target cells expressing viral specific antigens with approximately 9-12 amino acids in length. CTLs use their T cell receptor (TCR) to interact with peptide antigens derived from internally processed protein presented in the groove of human leukocyte antigen (HLA) class I on HIV-1 infected cells[10-12]. The level of HIV-1 specific CTL response is strongly correlated to disease progression[13-14]. CTL responses have been found in conversely correlated to low amount of plasma HIV-RNA levels. Highly-exposed persistently seronegative persons (HEPS)[15] and long term nonprogressors (LTNPs) have found to maintain strong[16] and diverse anti-HIV CTL responses. In macaques acutely-infected with Simian Immunodeficiency Virus (SIV), CD8+ T cell depletion is correlated to high plasma SIV-RNA level and rapid progression to AIDS[17-19].

Gag-specific CTL responses have shown to play a role in controlling HIV-1 replication[20]**.** In addition, there are some studies of HIV-1 specific CTL response in regulatory proteins. Tat and Rev are involved with viral replication and earlier expressed viral protein before Nef that can down-regulate HLA class I molecules on infected cell surface[21]. HIV-1 specific CTL response against Tat and Rev were detected 19% and 37% of HIV-1 infection, respectively[22].

HLA class I molecules have been shown that they are associated with either slower or faster rates of HIV disease progression[23]. The HLA-B alleles, in particular seem affect the disease progression more than other alleles[27]. The reported protective HLA alleles include HLA-B*27, -B*57 and –B*58 have been found associated with slow disease progression[24-27]. Recently, HLA-B*63 is found associated with a decreased plasma HIV-RNA level[28]. In contrast, HLA-B*35 is associated with a fast disease progression[29]. In addition, HLA-B*15 is found higher plasma HIV-RNA level in HIV-1 clade C[30-32] and in CRF01_AE such as Thailand[33] but not in clade B[30].

HLA is highly polymorphic and locates on the short arm of chromosome 6 in humans. HLA is divided into 2 classes which are HLA class I and II. HLA class I has 3 isotypes such as HLA-A, -B and -C present peptide antigens which is 9-12 amino acids in length that derived from endogenous pathway to CD8+ T cells or CTLs whereas HLA class II has 3 isotypes such as HLA-DP, -DQ and -DR present peptide antigens derived from exogenous pathway to CD4+ T cells or helper T cells. HLA allele frequencies vary greatly among different ethnic groups. In Thailand, the common HLA-A is HLA-A*1101 (26%), HLA-A*0203 (13%), HLA-B*4601 (17%), HLA-B*1301 (9%), HLA-C*0102 (17%) and HLA-C*0702 (19%)[34].

In response to viral antigen, differences of HLA can affect CTL responses. While there is extensive literature on CTL responses in Caucasians infected by clade B, HLA typing among HIV-1 infected Thai population is still not widely and appropriately studied. In addition, there are very much less data to focus on HIV-1 specific CTL responses against the most common HIV-1 circulating subtype “CRF01_AE” including in Southeast Asia and Thailand.

To guide our future regional specific HIV vaccine design, in this study, a total of 240 HIV-infected Thais are investigated for their HIV-1 specific CD8+ T cell responses, their HLAs typing, viral sequences to explore the patterns of CTL responses and its impact on viral escape or viral control. In addition, immunodominant and new CD8 epitopes will be characterized and studied in depth.

CHAPTER II

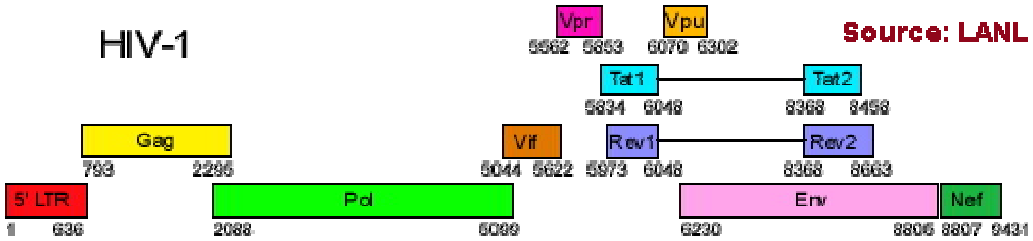
LITERATURE REVIEW

HIV is the cause of AIDS. The disease was first described by Gottlieb MS, *et al.*, and Masur *et al*. in 1981 by an unusual clustering of diseases with Kaposi's sarcoma and pneumocystis pneumonia in the young homosexual men[35-37]. In 1982, AIDS were also reported in other patient populations such as IVDUs and hemophiliacs[38-40]. In 1983, Montagnier et al. isolated the virus from patients who suffered from lymphadenopathy syndrome and named as lymphadenopathy-associated virus (LAV)[41]. One year later, Montagnier et al., and Gallo *et al*., did fully characterized the virus and named it as human T-lymphotropic virus type III (HTLV-III)[42]. Simultaneously, Levy et al., also name this virus as AIDS-associated retrovirus (ARV)[43]. This virus was later classified as HIV-1. In 1986, Montagnier et al., identified HIV-2 that isolated from West African patients with AIDS or AIDS related complex (ARC)[44]. In this study, we focused on HIV-1 that is the major cause of AIDS in worldwide including Thailand.

2.1 HIV-1 Biology[45-48]

HIV-1 is classified in family *Retroviridae*, subfamily *Lentivirinae* and genus *Lentivirus*. HIV-1 has a diploid single-stranded plus-sense RNA genome, about 9.2 kilo bases (kb) in length, within an icosahedral sphere with a diameter of approximately 100 nm. The outer phospholipids bilayer of HIV-1 is derived from host. HIV-1 contains 9 genes, structural 3 genes which are *gag, pol* and *env* and regulatory 6 genes which are *tat, rev, nef, vif, vpu and vpr* (Figure 1).

Figure 1. Diagrammatic represent HIV-1 genome which are composed of 3 structural genes (*gag, pol* and *env*) and 6 regulatory genes (t*at, rev, nef, vif, vpu and vpr*).
http://bioquest.org/bedrock/problem_spaces/hiv/background.php
Date of retrieval: 23/3/2011



The long terminal repeat (LTR) is located at the end of both sites of HIV genome. Integrase proteins used LTR to insert HIV genome into host DNA. LTR acts as switches to control viral replication. They can be activated by either HIV or host proteins.

2.1.1 Structural genes

1. ***Gag* (group antigen gene)**

The *gag* gene encodes 53 kDa protein precursor or p53. This protein undergoes proteolytically cleavage by HIV protease during viral assembly to produced four small products including matrix protein (p17), major capsid protein (p24) and nucleocapsid protein (p7/p9). These proteins are components of core protein and also serve as a nucleic acid and lipid membrane binding.

2. ***Pol* (polymerase gene)**

The *Pol* gene encodes a large polypeptide precursor from the same genomic RNA message of gag proteins but different open reading frame. The precursor is processed into three proteins including protease (PR), reverse transcriptase (RT) and integrase (IN). HIV-1 PR plays a critical role in virus biology, it cleave gag and pol polypeptide precursors into functional active proteins. The RT of HIV-1 is a magnesium 2+ (Mg2+) requiring RNA-dependent DNA polymerase responsible for replicating of viral RNA genome. The integrase is an important in proviral integration.

3. ***Env* (Envelope gene)**

The *env* gene encodes 160 kDa glycosylated polypeptide precursor or gp160. The protein was then processed into 120 kDa exterior glycoprotein (gp120) and 41 kDa transmembrane glycoprotein (gp41). Both of these proteins constitute the viral envelope possesses the domains accountable for CD4 binding and fusion. It also the epitopes primarily involved in viral-host immune interaction.

2.1.2 Regulatory genes[47-48]

1. ***Tat* (transactivator of transcription)**

Tat is a transactivator protein to up-regulate transcription process by binding to the trans-activating response element (TAR) on a LTR

2. ***Rev* (regulator protein of expression of viral proteins)**

Rev protein promotes export of unspliced and singly spliced viral messenger RNAs (mRNAs) from nucleus by binding at the RRE (rev-responsive element). *Rev* can function as a switch between the early synthesis of highly spliced mRNAs (*tat,*

rev and *nef*), the later synthesis of unspliced (*gag* and *pol*) and singly-spliced (*env, vpu, vif* and *vpr*) mRNAs.

3. ***Nef* (negative effector)**

Nef protein is required for efficient replication *in vivo* and down-regulates the surface expression of CD4 and HLA class I molecules on HIV-1 infected cell. In addition, Nef protein also interfere the T cell signaling pathways of T-cell activation.

4. ***Vpu* (viral protein U)**

The Vpu protein is located on the viral membrane. It is an essential for virion release and CD4 molecule down-regulation[43].

5. ***Vpr* (viral protein R)**

The protein product in association with matrix protein (MA) brings a pre-integration complex (proviral DNA) into nucleus and arrest cellular growth in G2 phase.

6. ***Vif* (viral infectivity factor)**

The Vif protein is an important factor for viral replication. The protein product is required for infectivity of virion, especially in peripheral blood mononuclear cells (PBMCs). Moreover, Vif can block the endogenous inhibitory activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) by interfere the function of RT.

2.2 Genetic Diversity of HIV-1

HIV-1 had been interested in the genetic, antigenic and biological variation regarding to the relevance of variability to HIV vaccine development [49]. Several factors are known to be contributed to the generation of new viral variants. With the lack of proofreading activity of the RT, this error-prone nature could generate a mutated virus by nucleotide substitution at rate of approximately 3×10^{-5} nucleotide changes per site per replication cycle [50]. High rate of viral production (up to 10^{10} virions per day) and large numbers of replication cycles (approximately 300 per day) could sustain HIV-1 infection [51]. Upon exposure to anti-retroviral agent, the virus could rapidly escape to be the drug resistant mutants under selection pressure from the host. Taken together, these mechanisms generate extraordinary HIV-1 viral variants and drive HIV-1 to evolution in the infected populations worldwide [51]. However, recent evidence suggests that there is the genetic recombination among highly diverge HIV-1 strains occur in frequently.

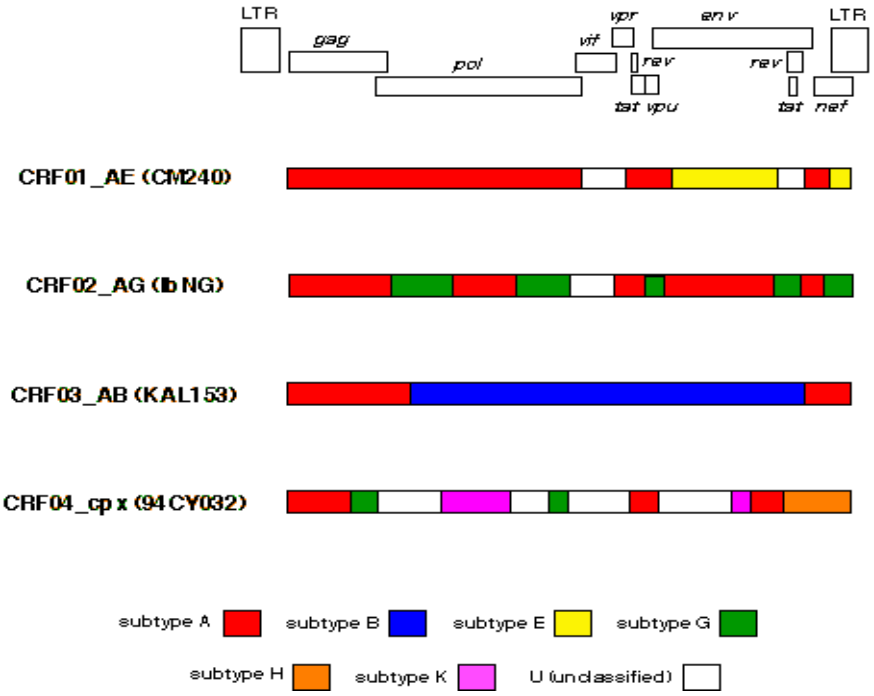
High mutation rates of HIV genome made HIV-1 to be a "Quasispecies". There are four broad groups of HIV-1, designated by M, O, N[52] and P[53], which are defined as distinct clusters on phylogenetic trees. Group M (Major) comprises the great majority of HIV-1 which is sub-classified into subtype 1 to 9 or sometimes called genotypes or clades, designated by A to D, F to H and J to K [52]. The member of subtype I which was earlier classified as a recombinant A/G (Cyprus isolate) and putative new subtype I (Greece isolate) [54] were recently removed from the list when a genome sequence of virus was reanalyzed. The result revealed that these isolates were assorted with genomic regions identical to A, G, K, H and unclassified regions (**Figure 2**). This strain was later named as unclassified. Group O (Outlier) is discovered more recently and the number is very small. These viruses were mostly derived from Cameroon and Gabon [55]. Both groups are usually characterized based on amino acid sequence identity. Sequence analysis of env amino acid sequences revealed that variation of group M was ranging from 3 to 23% identity among member of the same subtypes and between 25 to 35% identity for different subtypes. Group O viruses exhibit a similar level of diversity but have not yet been classified into subtypes due to the small number of representatives [56]. The two groups, M and O were 47% identity in their env protein sequences. Recently, a new subgroup named N (non-M and non-O) has been identified from Cameroon [57]. It is a recombinant between SIV chimpanzees (SIV cpz) and HIV-1. Current studies suggest that the distribution of HIV-1 genetic subtypes can be varied significantly from country to country, associated with mode of transmission and also changed over time. New group P (Pending) is just found in 2009. These viruses were discovered in wild gorillas (SIV gor) that similarly to SIV than SIV cpz. They were isolated from Cameroonian woman.

The branching order of phylogenetic trees based on *gag* and *env* sequences in 1993 were very similar for major isolates except for HIV-1_{MAL}, an African isolate from Zaire. This virus has *env* and *gag* sequences derived from subtype D and A, respectively [58]. Likewise, Thai isolate, CM240, contains *env* and *gag* sequences arisen from subtype E and A, respectively even if the parental E subtype has never been identified [59].

With an advance technology of full-length genomic DNA sequencing, HIV-1 subtypes in group M currently classified into 9 subtypes including A, B, C, D, F, G, H, J, K and more than 30 CRFs such as CRF01_AE (CM240) from southeast Asia, CRF01_AG (IbNG) from west and central Africa, and CRF01_AB (KAL153) from Russia and CRF01_AGI (CY032) Cyprus and Greece. CRFs are recombinant viruses which classified based on the following criteria including; (1) at least two isolates should be sequenced in their entire genome, (2) they should be resembled to each

other but no other existing CRF in their subtype structure and (3) they should be found in at least two epidemiological unlike individuals[60].

Figure 2. Diagrammatic represents the CRF01_AE, CRF02_AG, CRF03_AB, and CRF04_cpx which are mosaic genome structures of the four currently recognized circulating recombinant forms.
http://www.hiv.lanl.gov/content/sequence/HIV/REVIEWS/nomenclature/Nomen.html
Date of retrieval: 23/3/2011



2.3 Viral and host factors in HIV-1 infection[61,62]

Good clinical outcome is an essential in viral infection prevention. Some HIV-1 infection can control viral replication which shows stable CD4+ T cell counts and undetectable of plasma HIV-RNA level. There were two main factors; viral factors (such as viral genetics) and host factors (such as host genetics and host immune responses). These factors will help in predicting the progression of HIV-1 disease and viral replication of HIV-1 infection. The viral factor was examined by high mutation rate of HIV-1 genome as results of different subtypes based on HIV-1 genetic diversity. Many studies indicated that quantity of plasma HIV-RNA level can predict the HIV-1 disease outcome. Low plasma HIV-RNA level was associated with slow disease progression whereas high plasma HIV-RNA level was associated with rapid

disease progression. Some studies showed undetectable plasma HIV-RNA level in LTNP or elite controller (EC) who is infected with defective virus. On the other hand, the host factor is determined by host genetic variation and host immune responses in order to control of HIV-1 infection. Previous studies showed R5-tropic virus cannot infect patients with homozygous deletion of 32-base pair (bp) in chemokine receptor 5 gene (CCR5-Δ32). In addition, some studies found that HLA-B allele such as HLA-B*27, -B*57 and –B*58, play and importance role in control of HIV-1 infection.

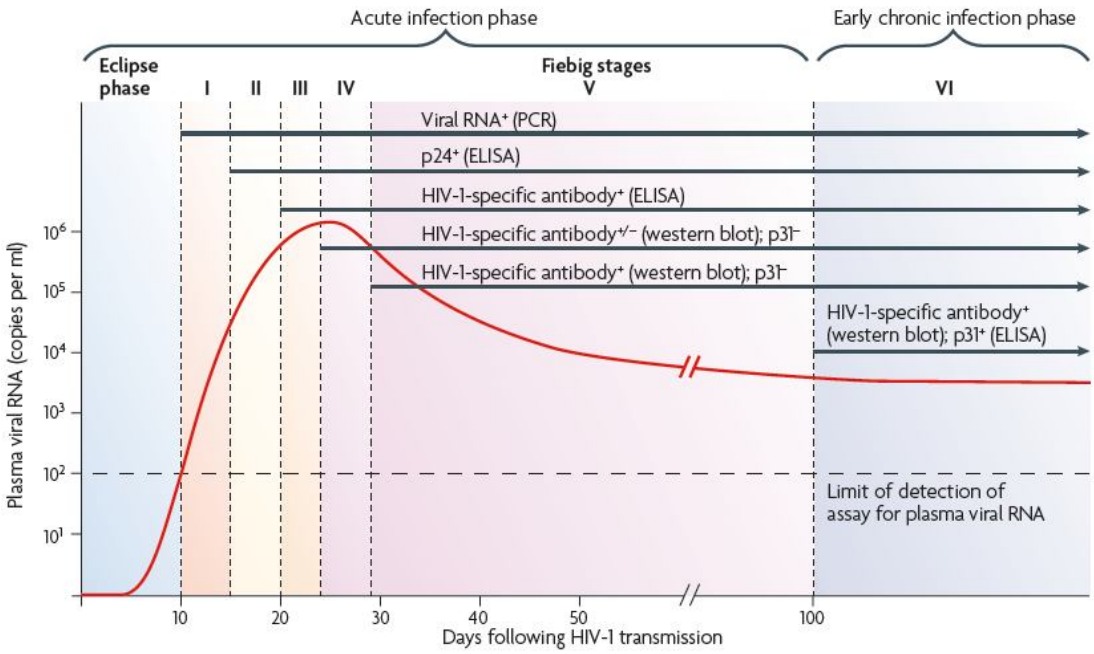
2.4 HIV-1 specific immune responses of HIV infection[7, 63-65]

After HIV-1 infection, both innate and adaptive immune responses can against the virus. Pathogenesis of the disease can be presented in three phases of infection including (1) primary or acute HIV infection (PHI), (2) chronic asymptomatic HIV-1 infection, and (3) AIDS-defining illness.

2.4.1 Acute or Primary HIV Infection (PHI)

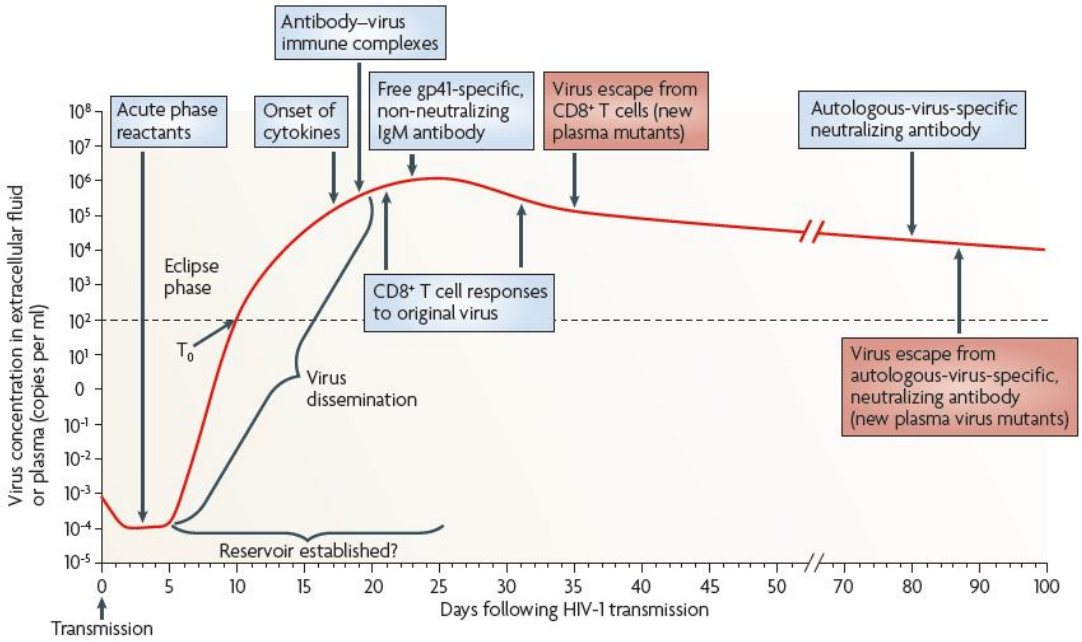
In the PHI phase (~100 days), about 50-70% of HIV-infected individuals develop an acute mononucleosis-like syndrome. This can be subdivided into 2 phases including eclipse phase (~10 days) and fiebig stage (I-V) (~90 days) (Figure 3). Eclipse phase is defined after HIV-1 enters to the genital tract or rectal mucosa of patients and initiated by single virus. At this stage, CD4+ T cells are more susceptible to be infected by virus than monocytes or macrophages. Phenotypic analysis of T cells revealed that CD4+CCR5+ T cells in mucosal tissue are primary target of virus to be infected. Upon viral infection, some virus binds to dendritic cells (DCs) via DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and some undergo the cycle of antigen processing and presentation. After process antigens, mature DCs present the virus antigens to T cells in lymph node and the T cells become activated. Many activated CD4+CCR5+ T cells are enriched in the lymph node therefore cell bound HIV on DC-SIGN can easily infect activated T cells and undergone productive infection this region. The virus or virus infected cells subsequently moved to fiebig stage. Moreover, virus can disseminate to another lymphoid tissue, especially gut-associated lymphoid tissue (GALT), infect activated CD4+CCR5+ memory T cells and produce massive amount of the viruses. Experiment showed that 80% of CD4+ T cells in GALT loss within 3 weeks after HIV-1 infection. The immune responses to HIV-1 infection can be found after the first viral replication occurs and high HIV-RNA level is detected. Viremia peak can be detected on days 21-28 which plasma HIV-RNA level is approximately 10^6 copies/ml.

Figure 3. Acute infection phase of HIV-1[7]
http://www.nature.com/nri/journal/v10/n1/fig_tab/nri2674_F1.html
Date of retrieval: 23/3/2011



Within 2 to 4 weeks, an increasing number of HIV-specific CD8+ CTL can be detected prior to viremia peak. The plasma HIV-RNA level decreases along with the peak of CTL response which can detect within 1-2 weeks. The WT virus is detected at the time of viremia peak before HIV-specific CD8+ CTL detected. It is indicated that no immune pressure at this stage. Within 10 days, the viral escape can be detected when plasma HIV-RNA level decrease to the viral set point. Viral escape is usually pressured by HIV-specific CTL. The CTL can maintain the virus to plateau or viral set point for 12 – 20 weeks before entering to the chronic infection phase at the end of fiebig stage V. This is the reason to support that CTL can help to control of viral replication. However, the CD4+ T cells are not high as before infection. Also, HIV-specific antibodies are usually present in the second and third week of infection. The WT virus is detected during infection before HIV-1 specific CD8+ T cell expansion. However, the viral escape is occurred within 10 days after T cell expansion indicating that HIV-1 is selected by the pressure of HIV-1 specific CTLs. Even though the viral escape is occur, the HIV-1 specific CD8+ T cells and HIV-specific neutralizing antibody can maintain the virus to viral set point[7,8] (Figure 4). The events described above may be no symptoms in all PHI or acute infection patients

Figure 4. The earliest innate and adaptive immune responses detected after HIV-1 transmission[7]
http://www.nature.com/nri/journal/v10/n1/fig_tab/nri2674_F1.html
Date of retrieval: 23/3/2011



2.4.2 Chronic Asymptomatic HIV-1 infection

About 70-80% HIV infection belongs to the group of typical progressors. This stage took about eight to ten years. Most of patients have a period of "chronic asymptomatic" that lasts for years after PHI. The plasma HIV-RNA level is persistent replication during this period and all patients have a gradual deterioration of the immune system due to the progressive depletion of CD4+ T cells. Most of patients are usually asymptomatic at this stage and CD4+ T cell are higher than 350 cells/cu.mm. Reduction of CD4+ T cells happens within 6-10 years.

2.4.3 AIDS-defining Illness

This stage is defined when CD4+ T cell below 200 cells/cu.mm. The HIV cannot be suppressed because of loss of these immune regulatory cells. An AIDS-defining illness or clinically apparent disease is the inevitable outcome of the progressive deterioration of the immune system that occurs in most patients with HIV infection. Exceptions to the direct correlation between deteriorating immune function and clinically apparent disease are progressive generalized lymphadenopathy; Kaposi's sarcoma, which can occur before the onset of severe immunosuppression; and neurologic disease that may reflect direct or indirect effects of the virus or its

products on neurons. The profound immunosuppression that occurs during this phase of HIV infection is the end stage of the immunopathogenic events that began at the time of primary infection, and continued for years through the clinically latent with microbiologically active stages of infection.

2.5 Innate Immunity[76-68]

Innate immunity plays a crucial role to control HIV-1 infection. Various innate immune cells such as natural killer cells (NK), macrophage and dendritic cells (DC) secrete the humoral components including complement, interferon (IFN) and chemokine that may inhibit HIV replication and transmission.

Complement-mediated lysis and its product could activate adaptive immune response by priming CD8+ naïve T cells. The complement-mediated lysis is not efficient to kill the HIV virus but it enhances the virus to bind to DC and facilitate T cell infection.

Interferon type I can inhibit viral replication in HIV-1 infected cells. It is compose of IFN-alpha (IFN-α) derived from white blood cell (WBC) and IFN-beta (IFN-β) derived from fibroblast. IFNs type I can induce DC maturation marker (CD83), co-stimulatory molecules and cytokines such as IFN-α/β, tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). In acute HIV-1 infection, plasmacytoid DCs (pDCs) were activated with HIV-1 via toll like receptors 7 (TLR7), TLR8 or TLR9 leading to IFN-α secretion. The decline of pDCs is correlated to disease progression however LTNP shows high pDCs. In addition, IFN-α induce APOBEC3G to abrogate HIV-vif neutralization of APOBEC3 proteins.

The CCR5, an HIV co-receptor in function together with CD4 are required for macrophage viral entry of HIV-1 and the virus is called monocytotropic (M-tropic). The homozygosity deletion of a 32-base pair *CCR5* gene (CCR5-Δ32) resulting in loss of CCR5 expression on cell surface. HIV-infection individual who have CCR5-Δ32 will be completed protection from HIV-1 infection. Another group of chemokine called β-chemokine which is including macrophage inflammatory protein-1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β) and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) can also suppress of HIV-1 infection. When binding to its cognate receptor, CCR5, it blocks HIV to enter to the cells. β-chemokine is commonly produced from activation CD8+ T cells, macrophages, DCs and NK cells.

Dendritic cells (DCs) are the professional antigen presenting cells. Two types of DCs are found in the body. One is conventional myeloid DCs (mDC; CD11c+/HLA-DR+) such as langerhans cells (LCs), dermal DCs and blood DCs. Another is plasmacytoid DCs (pDCs; CD123+/HLA-DR+). It was reported that loss of both mDCs and pDCs from blood circulating happened in an early phase of HIV-1 infection. This may imply that recovery the circulating DCs may help to improve immune response against HIV. In addition, Env protein can bind CD4+ expressed on pDCs and secrete the IFN-α to enhance the adaptive immunity.

Natural killer cells (NK cells) derived from bone marrow precursor are lymphocytes that not express TCR and CD3+. They were activated by type I IFN to produced type II IFN, β-chemokine, TNF-α and GM-CSF. NK cells act as effector cells in innate immunity. They can direct killing by balance the signal of activating and inhibitory NK cells receptors. As HIV down regulates the HLA class I molecules on the surface of viral infected cell, NK cells can kill HIV-infected cells by direct lysis or by ADCC (antibody-dependent cell-mediated cytotoxicity).

At mucosa layer, resident macrophages are the first cells that the virus to interact with. They bridge between innate and adaptive immunity. They process and present HIV peptide antigens via HLA class II. However, HIV can destroy phagocytosis of apoptosis of macrophage.

Gamma-Delta (γδ) T cells found at the gastrointestinal mucosa play an important role in HIV control. Experiment showed that γδT cells could induce cytotoxicity after co-culture with HIV-1 infected cells. They can produce HIV-1 suppressive mediators such as β-chemokines to prevent HIV-1 infection.

2.6 Adaptive immunity[66,69]

Upon HIV-1 infection, the adaptive immunity both humoral and cellular immune responses play an important role to combat HIV-1 infection.

B lymphocytes are the cells in humoral immune system. The major function is the production of antibodies. Antibody production and binding to a foreign substance or antigens often are critical as a means of signaling other cells to engulf, kill or remove that substance or antigen from the body. Antibodies are produced swiftly when the same antigen is encountered.

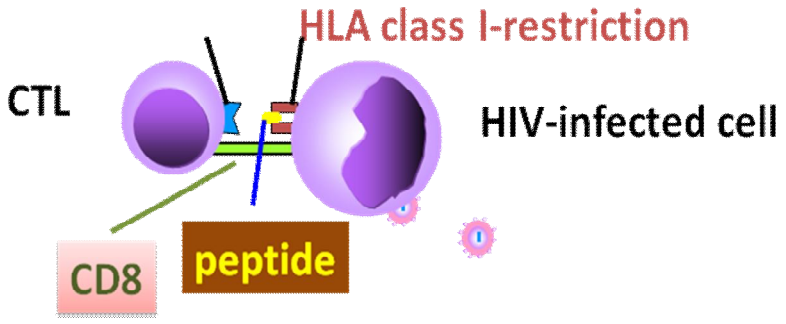
T lymphocytes are divided into two major subsets that are functionally and phenotypically different. The T helper (Th) subset called as the CD4+ T cells is a

coordinator of immune regulation. Their function is to secrete cytokines such as IL-2 to activate other cells such as B lymphocytes, CD8+ T cells and macrophages. Another is CD8+ T cells or suppressor or killer T cell. These cells are an important to kill tumor cells or viral infected cells including HIV-1.

2.7 HIV-1 specific CD8+ or Cytotoxic T lymphocytes (CTLs)

CTLs are essential role in defense against virally-infected cells[9] including HIV infection, in rejection of foreign tissue grafts and possibly also in immune responses to certain tumor types. CTLs are capable of killing target cells expressing specific antigens. The CTLs use the TCR which interacts with peptide antigen (9-12 amino acids) derived from internally synthesized proteins (the endogenous pathway of antigen presentation). The proteins will be presented in the groove of an major histocompatibility complex (MHC) or HLA class I molecule on target cells or HIV-infected cells[10-12] (Figure 5).

Figure 5. CTL recognize peptide on HIV-infected cell



2.7.1 The endogenous pathway of HIV-1 infected cells[68]

The endogenous pathway is the de novo pathway that synthesizes of complex molecules within antigen presenting cell (APC) from simple molecule such as amino acids. Formation of these complexes begins with newly translated proteins being cleaved into small peptides at C-termini by proteasomal processing. The resulting peptides (suitable the peptide binding groove) are transported by the transporter associated with processing (TAP) complex into the endoplasmic reticulum (ER), where they encounter nascent HLA class I molecules with β_2 microglobulin (β_2m). In the ER, peptides will be trimmed at the N-terminal to optimal peptide. Peptides that fit a certain motif (epitopes) then bind to the HLA. Epitopes are usually approximately 9-12 amino acids in length and express certain residues that serve as anchors, allowing each peptide to sit in a cleft in the HLA. Anchor residues vary according to the HLA molecule, each of which has its own motif (there are three HLA class I loci in

humans, and thus up to six different alleles). After the peptide-HLA complex is formed, it is presented on the cell surface to TCR of CTL.

The HLA-peptide complex can bind restricted by TCR. The outer amino acids of the peptide bind to HLA; the central amino acids protrude from the cleft of the HLA molecule and contact the TCR during engagement. Even single amino acid alterations in these residues potentially can completely abrogate binding. Engagement of the TCR triggers a signal to lyse the target cell via release of proteolytic enzymes, as well as to induce cell death (apoptosis).

2.7.2 T cell receptor (TCR)

TCR is a heterodimer on lymphocyte. It consists of two polypeptide chains, majority is α-chain on chromosome 14 and β-chain on chromosome 6 and minority is γ chain on chromosome 13 and δ on chromosome 14. Alpha (α)-chain is the same as light chain of immunoglobulin (*Ig*) that formed by the rearrangement of continuous Variable (*V*) genes, Joining (*J*) genes and Constant (*C*) genes. Beta (β)-chain is the same as heavy chain of *Ig* that formed by the rearrangement of continuous Variable (*V*), Diversity (*D*), Joining (*J*) and Constant (*C*). The *TCR* gene has not somatic mutation that is different from *Ig* gene.

2.7.3 The induction of CTL response

The induction of a CTL response is dependent upon CD4 T+ lymphocytes to provide help, in the form of activation and expansion. In addition, the CD4+ T cells can be generated, required for clonal expansion of cytotoxic CD8+ T-cell responses and primed CD8+ T cells to memory cells. CD8+ T cell activation and proliferation are derived from CD40-CD154 cell-cell interaction and IL-2 producing from activated CD4+ T cells to long-lived CD8+ memory T cells[70]. Moreover, DCs can activate CD8+ T cell by cross presenting antigens and lead to cytokine production and costimulatory expression. CD8+ T lymphocytes with the potential to become CTLs exit the thymus expressing their specific TCR but cannot lyse target cells at this stage. In the periphery, when a cytotoxic response is required, the pre-CTLs are activated by CD4+ T lymphocytes, which are involved in an immune reaction complementary to that of the CTL. For example, a CD4+ Th lymphocyte is primed to response to viral peptides after viral particles have been ingested and presented by macrophages at the site of infection. In turn, the activated Th cell activates virus-specific CTLs. The main cytokine signal is interleukin 2 (IL-2), but IFN-γ and IL-6 are also stimulators of CTLs. Activation of CTLs requires the typical two signals. The first signal is TCR on T cell activating by the peptide/MHC complex on viral infected cells to provide specific response. The second signal is CD28 on T cells activating by co-stimulatory signal such as CD80 (B7.1) to provide a regulating response mechanism. The CTL

response can be divided in to four phases[71,72]. The first phase is activation or initiation phase that prime the naive CTL precursors to killing infected cells. The second phase is clonal expansion phase that expand the specific T cells. The third phase is contraction phase that effectors CTL mostly die and only 5-10% generated to long-lived memory CTL. The last phase is memory phase that provides protection to the host.

2.7.4 CD8+ T cells killing process[73]

The killing by CD8+ T cells will be MHC class I restricted and the killing requires cell-cell contact. Only those cells to which a CTL becomes attached are killed. There is no "bystander death" because the lytic molecules are released only at the site of TCR contact. The major mechanism of cytotoxicity of CTL is Fas-FasL and granzyme induced apoptosis in the target cell. The membrane bound granules appear in the cytoplasm. There is cytokine production and release. Cytotoxic granules contain perforin which is a pore forming protein and granzymes which is a group of serine proteases. Perforin causes osmotic damage due to its binding of phosphorylcholine headgroups, polymerization, and subsequent pore formation in the lipid bilayer of the target cell. CTL-mediated target cell death generally involves changes such as chromatin condensation, extensive membrane blebbing and ultimately nuclear DNA fragmentation (apoptosis). These events clearly occur sometime before appreciable perforin-mediated cell lysis and purified perforin alone is incapable of causing DNA fragmentation. Indeed, it is likely that a supplementary role is played by granzymes in target cell killing and this postulate is supported by much *in vitro* and *in vivo* experimental evidence. Granzymes are the major protein complements of CTL and NK cell granules and they synergize with perforin to trigger an internal disintegration pathway in the target cell. Most notably, the killer cell serine protease granzyme B shares Asp-ase (peptide cleavage after aspartic acid) specificity with an increasingly large family of cell death cysteine proteases (termed caspases), including the IL-1β-converting enzyme (ICE)-like family members. Granzyme B appears to trigger an endogenous cell death cascade by activating key target cell caspases. Collectively, these data indicate that granzyme B can greatly amplify the activation of key signaling components shared by other forms of cell death (including the Fas/TNFR pathways) and directly contribute to apoptotic nuclear morphology.

2.8 Techniques for measurement of CD8+ T cell or CTL[74-77]

At least 4 techniques use to detect CTL activities have been described, i.e., the classical chromium-51 (^{51}Cr) release assay, enzyme-linked immunospot (ELISPOT) assays, intracellular cellular cytokine staining (ICCS; IFN-γ) assays and HLA-peptide tetrameric complex assay (Table 1).

2.8.1 ^{51}Cr release Assay

This is the standard assay for cytotoxic function. It was first described by Burnner and co-workers. The assay involves the radiolabelling of target cells with sodium chromate ($Na_2{}^{51}CrO_4$). The target cells are then incubated with the test effector cells population for a short period (4 hours). The amount of ^{51}Cr released into the supernatant is then quantified, to provide a measure of target cells lysis.

2.8.2 ELISpot Assay

In the ELISPOT method, cells that have been stimulated with antigen *in vitro* were incubated in nitrocellulose-lined microtitre wells, which have been pre-coated with anti-cytokine antibody. The cytokine secreted from an activating T cell is directly captured with anti-cytokine antibody during the incubation time before diluted in the supernatant. After incubation (for several hours or days), the local production of cytokines around "producing cells" can be visualized by adding a second antibody that is labeled with enzyme alkaline phosphatase or horseradish peroxidase, and then adding a substrate that is enzymatically converted into an insoluble colored product. Cytokine-producing cells can then be visualized as "spots". The spot are quantified by under steromicroscope or ELISpot reader.

2.8.3 Intracellular IFN-γ Assays

This assay was measured and analyzed by Flow cytometry. The single CD8+ T cell can secrete IFN-γ cytokine after stimulated *in vitro* with Ag or mitogen such as phorbolmyristate acetate (PMA) and ionomycin (I). They were blocked the transport of cytokines through the golgi apparatus by adding monensin or brefeldin A (BFA). The T cells are then fixed, permeabilised (to allow cytokine-specific antibodies to enter the cell), and stained for the presence of intracellular cytokines (IFN-γ) using directly conjugated anti-cytokine antibodies.

2.8.4 HLA-peptide Tetrameric Complex (Tetramer) Assay

This assay based on the basis of the binding specific to the HLA-peptide complex. HLA tetameric complexes are able to directly quantitate antigen specific T cells by flow cytometry. The tetrameric complex contains monomeric complex of HLA heavy chain, β_2m, and peptide. HLA heavy chain is expressed in Escherichia coli (*E. coli*) with an engineered COOH-terminal signal sequence containing a biotinylation site for the enzyme BirA. After refolding, the monomeric complex is biotinylated. Because each streptavidin molecule has four biotin-binding sites, a tetrameric complex is produced, which contains four MHC class I molecules. This complex has a greater affinity for T cells. The presence of the fluorochrome allows T cells that have bound the tetramer to be detected by flow cytometry.

Table 1. Comparison of each technique for detect of CTL activity

Measurement of CTL	Cell type measurement (function)	Time (day)	HLA typing required	assay type	Radio-labeled	sensitivity
1. Cr-51 assay	Target (lysis)	14	No / Yes	Qualitative	Yes	$1/5x10^4$
2. ELISpot	Effector (IFN-γ secretion)	2	No / Yes	Quantitative	No	$1/1x10^5$
3. Tetramer	Effector (binding)	1	Yes	Quantitative	No	$1/5x10^4$
4. Intracellular IFN-γ assay	Effector (IFN-γ expression)	1	No / Yes	Quantitative	No	$1/5x10^4$

In Our study, we used the IFN-γ ELISpot assay to measure the frequencies of HIV-1 antigen-specific T cell because of this assay is high sensitivity and specificity. The ELISpot assay is an adaptation of the enzyme-linked immunosorbent assay (ELISA), which measures the local concentration of cytokines (i.e., IFN-γ). However, this assay cannot define the phenotypic analysis such as CD4+ or CD8+ T cells but the depleted CD4+ or CD8+ T cells can be used an alternative method. Moreover, we can measure specific CD8+ T cell by using 9-12 amino acid as short peptide antigen that can bind only HLA class I alleles.

CD8+ T cells secrete many cytokines after stimulated with Ag. Some studies have shown that activating CD8+ cells can secrete up to five different of cytokines such as IFN-γ, IL-2, TNF-α, MIP-1β and CD107a. The good quality of effector CD8+ T cells are able to secrete more cytokines called as "polyfunctional" than poor quality of effector CD8+ T cells that can secrete one or two cytokines called as "monofunctional". We select IFN-γ cytokine to do ELISpot assay because this cytokine is majority secreted from Ag-specific T cell[78]. Moreover, it was almost the last cytokine production to disappear and high amount of production.

2.9 Evidence support the CD8+ T cells or CTL in the Control of HIV infection[23]

CTLs play an important role to control viral replication including HIV. There are many researchers that studied the evidence supported CTL control HIV-1 infection and disease outcome. There are three additional lines of evidence support the central role of CD8+ T cells in the control of HIV infection. Firstly, specific HLA class I molecules are associated with HIV disease outcomes. Secondly, more rapid disease progression can be observed in patients with HLA class I homozygosity.

Thirdly, viral escape CD8+ T-cell recognition from selection pressure is temporally associated with loss of immune control of infection.

In 1999, Jin X and Schmitz JE et al., studied the SIV-infected macaques[17-18]. The results showed that after CD8+ T cells depletion, the plasma SIV-RNA level was increasing and too rapid progression. It can imply that CD8+ T cell plays a role in controlling SIV infection in macaque. In addition, in the same year, Ogg GS et al., studied the longitudinal CD8+ T-cell responses evolution by CD8+ T cells staining with HLA-peptide tetrameric complexes[13]. They found a significant association between high frequencies of HLA-A*0201-restricted p17Gag/Pol tetramer binding cells and slower disease progression ($p < 0.01$). In 2006, Zuniga R et al., studied 45 HIV clade B-infected individuals in Lima and Peru. They tested by *ex vivo* IFN-γ ELISpot assays to assess total HIV-1 and individual protein-specific[20]. They found that the Gag-specific immunity in effective control of HIV infection.

In addition, Gag-specific CTL responses and more breadth were associated with low plasma HIV-RNA level whereas Env-specific CTL responses and more breadth were associated with high plasma HIV-RNA level in HIV-1 infected individuals from South Africa. Recent studies in HIV-1 clade B had shown the individual protein, Gag- and Rev-specific repertoires showed more epitopes restricted by "good" HLA alleles than by "bad" ones, whereas Nef, Env, Pol, Tat, Vif, Vpu and Vpr showed more epitopes restricted by "bad" HLA alleles than by "good" ones[79]. Moreover, CTL epitopes in p24-Gag-specific CTL responses and increasing breadth were associated with low plasma HIV-RNA level and delayed disease progression as found in HIV-1 CRF01_AE[80].

2.10 HIV-1 immune evasion and escape mechanism to CTL killing[81-85]

Under CTL pressure, surviving of HIV-1 will be observed. There are many mechanisms to evade the CTL recognition and killing such as (1) down regulation of HLA class I molecule (2) inhibition of Ag processing and (3) up regulation of FasL. For instant, Nef protein can promote immune evasion. It can eliminate the apoptosis pathway, increased viral infectivity and activating T cell. In addition, it is able to down regulate CD4 and HLA class I molecule such as HLA-A and HLA-B but not HLA-C and HLA-E that is recognized with inhibitory NK cell receptors that CTL and NK cell known as self antigen and no destroy the HIV-1 infected cell. Nef is no effect of HLA class I synthesis and transport to Golgi apparatus. But, Nef can block HLA class I transportation to cell surface. Finally, CTL cannot recognize HLA class I on HIV-1 infected cell surface. In addition, the changing of amino acid sequence within epitope may affect Ag processing, presentation and recognition. The possible

mechanisms are involved with the sequence variants such as (1) the flanking amino acids are changed and then epitope is not cleavage (2) the anchor residual is changed and then the Ag processing may occur but cannot bind to HLA class I molecule (3) the nonanchor residual is changed and then the Ag processing is occur and bind to HLA class I molecule. However, TCR cannot recognize or it occur sub optimal recognition (antagonism). Moreover, Nef can up regulate FasL on HIV-1 infected cell that may activate apoptosis in CTLs.

Even though, viral escape occurs under CTL pressure. However, CD8+ T cell or CTL is associated with control of viral replication and disease progression. In addition, there are many researchers found that HLA class I molecule is also associated with control of viral replication and disease progression.

2.11 Human Leukocyte Antigen (HLA) [86]

Major Histocompatibility Complex (MHC) called in human as Human Leukocyte Antigen (HLA). HLA is highly polymorphic. HLA is located on the short arm of chromosome 6 and expressed on the surfaces of a variety of cells. β_2m is encoded by a gene on chromosome 15. The HLA gene is very large (about 3,500 kb) and organized as follows: (1) class II genes (HLA-DP, HLA-DQ, HLA-DR) (2) complement genes (3) heat shock protein and cytokine (TNF, lymphotoxin (LT) and LT-β) genes, and (4) class I genes (HLA-B, HLA-C, HLA-A).

The genes that controlled such immune responses were called immune response (*Ir*) genes. It was later shown that *Ir* genes controlled the activation of Th lymphocytes, which were necessary for antibody responses to protein antigens. The central role of HLA genes in immune responses to protein antigens was explained in last 1970s, with the demonstration that antigen-specific T lymphocytes do not recognize antigens in free or soluble form but recognize portions of protein antigens (i.e., peptides) that are non-covalently bound to HLA gene products. In other words, HLA molecules provide a system for displaying antigenic peptides to T cells. This allows T cells to survey the body for the presence of peptides derived from foreign proteins.

HLA is divided into three classes which are class I and II HLA molecules. HLA class I have 3 isotypes such as HLA-A, -B and -C present peptide antigens which is 9-12 amino acids in length that derived from endogenous pathway to CD8+ T cells whereas HLA class II have 3 isotypes such as HLA-DP, -DQ and -DR present peptide antigens derived from exogenous pathway to CD4+ T cells (Table 2). HLA class III is related the complement system.

Table 2. Comparison of HLA class I and class II molecules

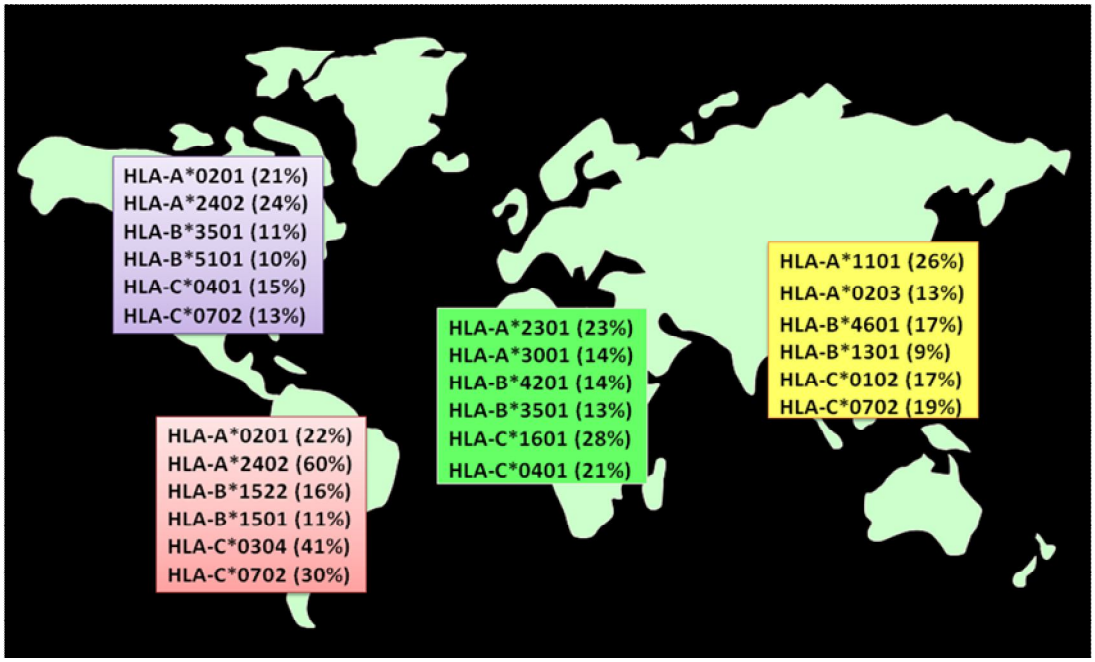
	HLA class I molecule	HLA class II molecule
Isotype	A, B, C	DP, DQ, DR
Heterodimer form	α-chain binds with β2m	α-chain binds with β-chain
Heterodimer binding	non-covalent bond	non-covalent bond
Peptide binding cleft form	α1 and α2 domains	α1 and β1 domain
Antigen processing pathway	endogenous	exogenous
Antigen length	9-12 amino acids	9-30 amino acids
Present antigen to	CD8+ T cell	CD4+ T cell

2.11.1 Expression of HLA Molecules[87]

The expression of HLA molecules on different cell types determines whether or not T lymphocytes can interact with foreign antigens present on the surface of these cells. CD8+ CTL recognize foreign antigens such as viral polypeptides when they are bound to class I HLA molecules. The ability of CTLs to lyse a virally infected cell is a direct function of the quantity of class I molecules expressed. In contrast, CD4+ Th lymphocytes recognize antigens bound to class II MHC molecules.

The peptide-binding region of HLA class I molecules is formed by the α1 and the α2 segments of the heavy chain. It consists of a cleft measuring approximately $25A^{o}x10A^{o}x11A^{o}$ with α helical sides and an eight-strand β-plated sheet floor. The cleft can accommodate a peptide of about 9 to 12 amino acid residues. Peptides bind to class I molecules largely through allele-specific motifs, which fit into complementary pockets formed by polymorphic residues of the class I molecule. The analogous cleft of class II molecules is formed by the α1 and β1 domains of the two chains. The cleft in class II molecules is open at the ends so that class II molecules can accommodate larger peptides of 10 to 30 or more amino acid residues. In both class I and class II molecules, the polymorphic amino acid residues located in the peptide-binding region determine the specificity of peptide binding and T cell antigen recognition. Currently, HLA allele frequencies vary greatly among different ethnic groups. The common HLA class I molecules around the world shows in Figure 6.

Figure 6. Common HLA class I molecules reported in different regions of the world[34]



Infection of HIV-1 is associated with HLA class I restricted by CTL response. By analogy to a number of animal models of viral infection, these CTLs are likely to play an important role as a host defense in infected persons and are likely to be an important component of an effective AIDS vaccine. HIV-1 CTL epitope, 8-12 amino acids in length are the optimal peptide to bind into specific pockets of HLA class I molecule with hydrogen bonding. These pockets, designated A, B, C, D, E and F, are located in the antigen binding site on the class I molecule formed by two α-helicles, with the floor of the groove formed by a β-sheet structure[88]. As the pockets are located at the edge of the peptide binding groove, the anchor positions in the peptides are often in positions 1, 2 and 9 of the processed peptide[89]. The characterization of the antigenic epitopes involved in CTL induction is an important not only for a better understanding of disease pathogenesis, but also for possible vaccine development. There were many laboratories identification the optimal CTL epitopes of HIV-1 infected individuals[90-91]. The Los Alamos HIV Molecular Immunology Database contains all reported sequences with CTL activity, regardless of their definition as optimal epitopes. A summary of those sequences is accessible in HIV database http://www.hiv.lanl.gov/content/immunology/tables/optimal_ctl_summary.html. It contains more than 1,300 epitopes, which contain putative HLA class I restricted epitopes.

2.12 HLA typing[92]

The typing of HLA molecule is an essential in organ transplant and immune function. In HIV-1 infection, HLA class I alleles are associated with HIV-1 disease outcome. In currently, there are at least 4 assays for HLA typing such as the serotyping (gold standard), cellular typing, gene sequencing (SBT) and phenotyping (SSP-PCR). In Our study, we got the high resolution (4 digits) HLA Class I typing result based on DNA Sequenced Based Typing (SBT) at The University of Oklahoma Health Sciences Center CLIA/ASHI certified high resolution HLA tying laboratory.

2.13 Impact of HLA class I molecules to HIV-1 disease outcome

CTL is associated with control of viral replication and disease progression. Previous studies found that HLA class I allele is also associated with control of HIV-1 replication and HIV-1 disease progression[26]. Moreover, HLA is related to autoimmune and infectious disease including AIDS. Currently, HLA-B is the most polymorphic with 817 differences. HLA-A and HLA-C molecules described in 486 and 263 differences, respectively[23]. In 2004, Kiepiela et al., reported that HLA-B alleles appear to impact HIV disease progression more than other alleles[27]. There are many researchers reported the HLA class I allele associated with HIV disease progression such as HLA-B*5701, -B*5702, -B*5703, -B*5801, -B*63, -B*1503 and -B*1302 associated with HIV delay progression or HLA-B*3502, -B*3503, -B*5802 and -B*1503 associated with HIV rapid progression.

2.14 Viral control associated with HLA class I molecules

Previous studies reported that protective HLA alleles (HLA-B*5701, -B*5702, -B*5703, -B*5801, -B*63, -B*1503 and -B*1302) were associated with HIV delay progression. In 2000, Migueles SA et al., studied 13 LTNPs compared with 200 progressors[24]. They found 85% of HLA-B*5701 in LTNPs and 9.5% in progressors. Therefore, they concluded that the HLA-B*5701 was related to delay progression. In 2006, Lazaryan A et al., studied the HLA-B*58 supertype with an infected native Africa[93]. There were 423 of Zambian clade C and 202 of Rwanda clade A patients. They found that HLA-B*5703 and HLA-B*5801 were associated with low plasma HIV-RNA level. In 2005, Frahm N et al., studied 21 of patient with clade B and 7 of patients with clade C in term of HLA-B*63[28]. HLA-B*63 is a serological marker related to HLA-B*1516 and HLA-B*1517. It was the same epitope associated with HLA-B*57 and HLA-B*58 that was associated with delay progression. The results showed that novel epitope of HLA-B*63 was related to delay progression. In 2006, Frahm N et al., studied the HLA-B*1503 in 248 of clade B and 169 of clade C

patients[30]. They found that HLA-B*1503 showed 9.7% in patients with clade B less than 14.2% in patients with clade C. Also, HLA-B*1503 in clade B was related to low plasma HIV-RNA level. In 2007, Honeyborne I et al., studied the HLA-B*1302, the allele associated with low viremia[94]. In the results of 578 of clade C patients in South Africa found that three of six epitopes were gag-specific CD8+ T cells. So, they implied that the gag-specific CD8+ T cells would be associated with suppression of viremia.

2.15 Viral escape associated with HLA class I molecules

There are several data of HLA alleles associated with rapid progression such as HLA-B*3502, -B*3503, -B*5802 and -B*1503. In 1999, Carrington M et al., studied HLA typing and disease progression in 498 HIV-1 infected Caucasian. They found that HLA-B*35 was associated with rapid progressor to AIDS[29]. In 2001, Gao X et al., studied the effects of single amino acids change to the HLA class I allele affects the rate of disease to AIDS[95]. Studied by HLA-B*35 subtype was found that HLA-B*3502 and HLA-B*3503 was positively related to the disease to AIDS faster than the HLA-B*3501. In 2004, Nguyen L et al., studied the relationship of HLA typing and plasma HIV-RNA level in 128 IVDUs HIV-infected Thai patients[33]. They found that 20% of patients with HLA-B*15 were associated with high plasma HIV-RNA level. In 2006, Lazaryan A et al., studied the HLA-B*58 supertype with an infected native Africa[93]. There were 423 of Zambian clade C patients and 202 of Rwanda clade A patients. They found that the patients with HLA-B*5802 were associated with high plasma HIV-RNA level. In 2006, Frahm N et al., studied the HLA-B*1503 in 248 clade B and 169 clade C patients and found that patients with HLA-B*1503 in the clade B showed 9.7% less than in the clade C that showed 14.2%[30]. Also, they concluded that HLA-B*1503 in clade C was related to high plasma HIV-RNA level.

2.16 HIV-1 specific CD8+ T cell response in Thailand and Asian population

Some researchers were studied the CD8+ T cell responses in Thailand and Asian population (Table 3). Most of studies were clade B and focused on characterize the HLA-specific CTL epitopes. Only two studies were clade CRF01_AE and focused on gag (p24) specific CTL responses (Table 3).

Table 3. HIV-specific CD8+ T cell responses in Thailand and Asian population

	Sample		Ethnics	Inference	References
	N	Clade			
1	54	B'	Chinese	To identify CD8+ T cell responses, they focused on HLA-A*2 and HLA-A*11 that majority in the Chinese population. They found that HLA-A*2 showed higher frequency and magnitude response than HLA-A*11.	Gong X et al. (2006)
2	35	C	Indian	To identify CD8+ T cell responses, they tested the CD8+ T cell response by ELISpot and ICCS assay. They found that p24-Gag specific CD8+ T cell response was the most frequency and magnitude. They also observed the magnitude of IFN-γ higher than IL-2 response.	Kaushik S et al. (2005)
3	30	C	Indian	To characterize Gag and Nef-specific CD8+ T cell responses, they found 73.3% responses in Gag and 90% responses in Nef. The magnitude of Gag-specific CTL responses was associated with low plasma HIV-RNA level whereas Nef-specific CTL responses was associated with high plasma HIV-RNA level.	Mendiratta S et al. (2009)
4	10	B	Japanese	To identify and characterize HLA-A*3303-restricted CTL epitope, they found 3 epitopes from Pol and one epitope from Gag-restricted CTL responses can kill target cell infected with HIV-1 recombinant vaccinia.	Hossain MS et al. (2003)
5	4	B	Japanese	To identify and characterize HLA-A*2603-restricted CTL epitope, they found one Gag peptide (169-177) can bind both HLA-A*2601 and -A*2603 while one Env peptide (63-72) can bind HLA-A*2603 stronger than HLA-A*2601.	Kawashima Y et al. (2005)
6	8	B	Japanese	To identify and characterize HLA-B*5401-restricted CTL epitope, they found 3 epitopes from Pol and two epitope from Nef-restricted CTL responses can kill target cell infected with HIV-1 recombinant vaccinia.	Kitano M et al. (2008)
7	7	B	Japanese	To identify and characterize HLA-B*4801-restricted Gag-specific CTL epitope, their finding confirmed gag epitopes process by endogenous pathways.	Murakoshi H et al. (2009)

	Sample		Ethnics	Inference	References
	N	Clade			
8	219	CRF01 _AE	Thai	p24 Gag associated with low plasma HIV-RNA level and high CD4+ T cell counts	Gesprasert G et al. (2010)
9	137	CRF01 _AE	Thai	Both breadth and magnitude of p24-Gag CTL responses were associated with low plasma HIV-RNA level and high CD4+ T cell counst and delayed disease outcome.	Mori M et al. (2011)

As mentioned above, CTL and HLA are associated with control viral replication and viral disease progression. In addition, there are different clades and HLA molecules in other countries. HIV-1 CRF01_AE is the most common subtype epidemic in Thailand. HLA-A*1101, -B*4601 and -C*0702 are the common alleles in Thai population. Most publication about the CD8+ or CTL responses are clade B in Caucasians infected individuals. Moreover, there are little data to focus on novel epitope associated with low plasma HIV-RNA level or high plasma HIV-RNA level of HIV-1 specific CD8+ or CTL responses in Thailand. The identification of HIV-1 whole proteins specific CTL responses in clade CRF01_AE with theirs HLA typing will be important for the development of HIV-1 vaccines in Thailand.

CHAPTER III

HYPOTHESIS, OBJECTIVES AND CONCEPTUAL FRAMEWORK

Research questions

1. Is/are there any novel HIV-1 CD8 epitope restricted by different HLA class I alleles associated with viral control or viral escape among HIV-1 infected Thai population?
2. Are any of the known epitopes and HLA class I alleles from other ethnic population and HIV-1 clade relevant to Thai population?

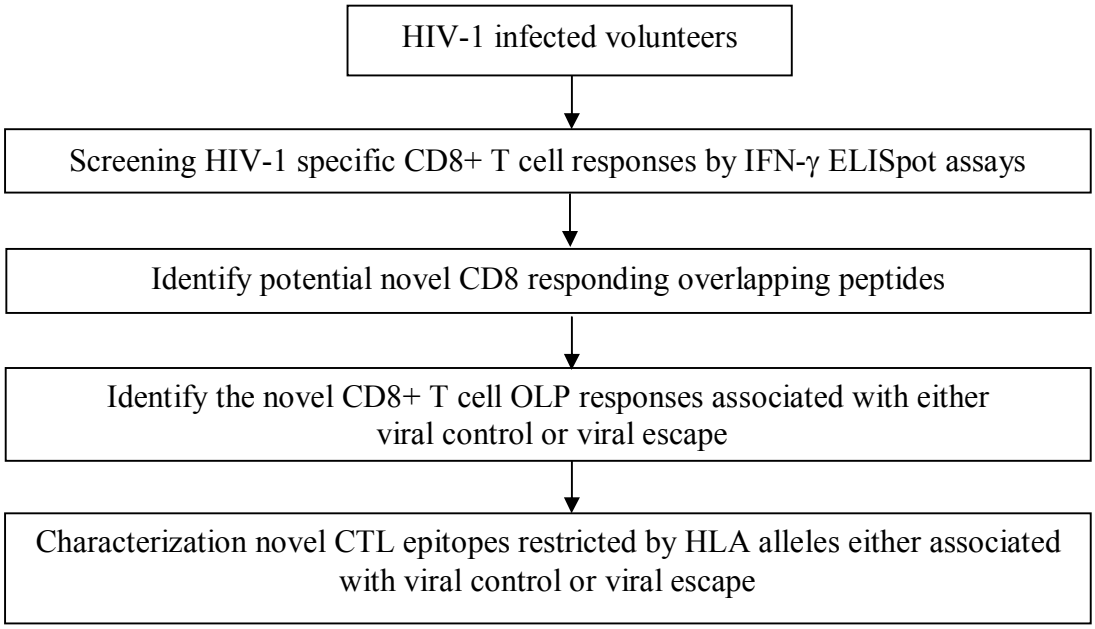
Hypothesis

Viral control or viral escape in Thai patients infected with HIV-1 is associated mainly with different CD8 epitopes and HLA-allele-restriction compared to the findings found in clade B infected Caucasians.

Objectives

1. To determine the frequency, breadth and magnitude of HIV-1 specific CD8+ T cell responses to various types of HIV-1 antigens in HIV-1 infected Thai individuals
2. To identify novel CD8+ T cell epitopes in HIV-1 infected Thai individuals
3. To identify HIV-1 known and novel CD8+ T cell epitopes that is/ are associated with either viral control or viral escape

Conceptual Framework



Definition of "a novel epitope" in this study was based on HIV immunology database updated 2010 version and literature search updated to December 2011 and found one of the following criteria

1. A new sequence of optimal peptide with a new HLA-restriction alleles
2. A new sequence of optimal peptide with a known reported HLA-restriction alleles
3. A known-reported sequence of optimal peptide with a new HLA-restriction alleles

Definition of "viral control" and "viral escape" in this study

1. **Viral control** is defined by when patients have either HIV-specific CD8+ T cells response or HLA class I found to be associated with low plasma HIV-RNA level (or viral load)
2. **Viral escape** is identified by when at least one mutation within an epitope is shown to be associated with immune escape or with a loss or a significant decrease of HIV-specific CD8+ T cell ELISpot response

Expected Outcome and Applications

1. Identify frequency, breadth and magnitude of HIV-1 specific CD8+ T cell responses against all HIV-1 proteins in Thai population
2. Identify which HIV-protein recognized by CD8 T cells that associated with either viral control or viral escape
3. Identify HLA alleles that were associated with either viral control or viral escape
4. Identify novel HIV-1 CRF01_AE in Thai patients CD8+ T cell OLPs
5. Identify novel CD8+ T cell epitopes that are associated with either viral control or viral escape

Research methodology

1. PBMC preparation collected from HIV-1 infected treatment-naïve Thai individuals according to the inclusion and exclusion criteria
2. Screening for HIV-1 specific CD8+ T cell response against various HIV-1 OLPs (413 OLPs) by using IFN-γ ELISpot assay.
3. Identification of immunodominant CD8 epitopes and novel CD8 epitopes that associated with either viral control or viral escape
4. Further characterize the epitope(s) in regard to HLA restriction and peptide titration by IFN-γ ELISpot assay
5. Data analysis was examined using the Spearman's rank correlation coefficient test and unpaired *t*-test for qualitative data and using the Mann–Whitney U test or Chi-square (Fisher's exact) test for Quantitative data.

CHAPTER IV

METHODOLOGY

This study is a part of the contract NIH-NIAID-DAIDS-03-13 "HLA Typing and Epitope Mapping Relative to HIV Vaccine Design".

4.1 Study population

4.1.1 HIV-1 infected population

240 frozen PBMCs from HIV-1 infected treatment-naïve Thai individuals were enrolled in this study. They were taken from HIV-1 infected individuals attended the Anonymous Clinic, Thai Red Cross AIDS Research Centre and Chualongkorn Hospital. The inclusion criteria of HIV-infected patients were (1) HIV-1 seropositive adults and at least 18 years old (2) No antiretroviral (ARV) treatment (3) CD4+ T cell, Plasma HIV-RNA levels, HLA-typing and HIV-1 sequencing data were collected (4) Ability and willingness to given written informed consent. The exclusion criteria of HIV-infected patients were active concurrent infection or opportunistic infection or on immunosuppressive treatment.

Sample size

(1) Percentage of reported protective HLA alleles: HLA-B*13, -B*27 and –B*58 (that, had been found associated with delay progression) in Thailand were 10.6%, 2.9% and 8.6%, respectively. Percentage of HIV-1 gag epitopes CTL response in HIV-1-infected Thais population was approximately 80%.
(P = (0.106 + 0.029 + 0.086) x 0.8 = 0.1768)[34]

(2) Q = 1-P (Q = 1 – 0.1768 = 0.8232)

(3) Definition of an acceptable error in this study was less than 5% and a confidence value was 95% (d = an acceptable error = 0.05).

(4) $Z_{\alpha/2} = Z_{0.05/2} = 1.96$ (two-tails)
Formula $N = (Z_{\alpha/2})^2 PQ/d^2$

$N = (1.96)^2 (0.1768)(0.8232)/(0.05)^2 = 224$

(5) Unevaluable data 10%

According to the criteria, we selected the patients who have all data of CD4+ T cell, plasma HIV-RNA level and HLA-typing. For the HIV-1 sequencing data, we had only data from patients who had plasma HIV-RNA level more than 50 copies/ml.

Therefore, total 240 frozen PBMCs from HIV-1 infected Thai individuals were tested in this study.

4.1.2 HIV-1 uninfected population (control population)

148 frozen PBMCs from healthy adults not infected with HIV as a control. They were taken from National Blood Centre, Thai Red Cross Society. They had HLA-typing data. All donors signed an informed consent.

4.2 Peripheral Blood Mononuclear Cell (PBMC) Preparation

Frozen PBMCs having been stored in liquid nitrogen tank were thawed at 37^{o}C, washed with RPMI1640 (GIBCO) and resuspended in RPMI1640 supplemented with 10,000 Units/ml Penicillin G, 10,000 μg/ml Streptomycin and 10% fetal bovine serum (FBS; Biowhitteker, Walkersville, MD, USA) as complete medium or R10. PBMCs were counted and adjusted at 1x10^{6} cell/ml.

4.3 Peptides

Peptides were designed based on sequences of HIV-1 CRF01_AE, available at HIV immunology database (http://www.hiv.lanl.gov). Each peptide was 18 amino acids in length, with 10 amino acids overlap between sequential peptides. Total 413 overlapping peptides (OLPs) were consisted of structural proteins which were 67 peptides of Gag, 133 peptides of Pol and 114 peptides of Env and regulatory proteins which were 27 peptides of Nef, 15 peptides of Rev, 13 peptides of Tat, 24 peptides of Vif, 9 peptides of Vpu and 11 peptides of Vpr. They were synthesized on an automated peptide synthesizer from Massachusetts General Hospital. The truncated peptides of Gag-OLP38 were MI10 (MYSPVSILDI), MD9 (MYSPVSILD), YI9 (YSPVSILDI), YD8 (YSPVSILD) and SI8 (SPVSILDI) and Pol-OLP16 were DI13 (DQILIEICGKKAI), QK12 (QYDQILIEICGK), QI9 (QYDQILIEI), QK9 (QILIEICGK), IL9 (ILIEICGKK), VI11 (VRQYDQILIEI) and RI10 (RQYDQILIEI). The truncated peptides of Gag-OLP38 were provided by Prof. Sarah L. Rowland-Jones, Oxford, UK.

4.4 Interferon Gamma Enzyme Linked Immunospot (IFN-γ ELISpot) assay

To detect the IFN-γ secreting cells by ELISpot assay, briefly, 96-well filtration plates (Millipore Corp, Bedford, MA, USA) were coated with 100 μL/well of 2 μg/ml of anti-human IFN-γ monoclonal antibody (mAb) 1-D1k (Mabtech, Nacka, Sweden) in phosphate buffered saline (PBS) and incubated at 37^{o}C in 5% CO_2 for 3 h. The

plates were washed with six times with 200 μl PBS/well and blocked with 200 μl R10/well for 1 h at room temperature. PBMC ($1x10^5$ cells/well) were incubated for 16 h either with 413 OLPs individuals at a final concentration of 2 μg/ml, complete medium as control and phytohemagglutinin (PHA) (10 μg/ml) as positive control. After culture, the plates were washed and incubated for 3 h with biotinylated anti-mouse IFN-γ (clone 7-B6-1; Mabtech) and then 1 hour with streptavidin-alkaline phosphatase (Mabtech) and developed with alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA) for 5 min. IFN-γ spot-forming units (SFUs) were quantified by ELISpot reader (Carl Zeiss, Germany). The number of cytokine-secreting cells was calculated by subtracting the average negative control value and expressed as SFU per 10^6 input cells. A response was considered positive if it was greater than (a) 3 x the mean background, (b) the mean background + 3 standard deviations or (c) 50 SFU per 10^6 input cells, whichever was higher.

4.5 B Lymphoblastoid Cell Lines (BLCL) Preparation

$5x10^6$ PBMC of each subject were incubated with supernatant of Epstein-Barr virus (EBV) for 1 h at 37°C in 5%CO_2. After wash once with RPMI1640, EBV-infected PBMCs were resuspensed in culture medium R20 with 1 μg/ml cyclosporin A (CSA) at $1x10^6$ cells/ml and culture in 24-well flat-bottom tissue culture plate. After 1-2 weeks of culture, proliferating foci of B lymphocytes will be observed under microscope. Once immortalized, BLCL are transferred to tissue culture flasks and cultured at the concentration of $0.5x10^6$ cells/ml in culture median R20. The B95-8 cell line was kindly provided by The National Institute of Health (NIH) Thailand.

4.6 Generation of CTL lines by *in vitro* stimulation

$10x10^6$ PBMC were cultured with relevant peptide at 10 μg/ml for 1 h. After incubation, the cells were washed and placed in 24-well plates at amount $5x10^6$ cells/ml in R10 containing 330 U/ml of rhIL-7 (Genzyme). On days 3, 7 and 10, 100 U/ml of rhIL-2 (kindly provided by NIH, USA) was added to cultures and CTL activity assessed on day 14.

4.7 CTL activity by classical ^{51}Cr release assay

5 x 10^5 BLCL were put into a 15 ml centrifuge tube (one tube should be set up for each target cell). After centrifugation, the supernatant was discarded and 100 μl of FBS was added. Then, BLCLs were labeled with ^{51}Cr and pulsed with 25 μl of peptide (200 μg/ml). After incubated at 37°C with a humid atmosphere of 5% CO_2 in

air for 1 h, target cells were washed twice with 5 ml of RPMI. Finally, target cells were resuspended with 10 ml of culture media R20 (final dilution 5 x 10^4 cells/ml).

During the time, the effector cells were adjusted at the concentration of 5 x 10^6 cells/ml with culture media R10. Effector and target cells are plated in a 96 well, U-bottomed microtiter plate, 200 µl final volume of each 100 µl of effector cells concentration in triplicate and 100 µl of target cells. The ratios of effector cells to target cells were at 30:1 and 15:1. For the total release (TR) or maximum release 5% triton X-100 (TX-100) was added to the target cells. The ^{51}Cr release from the wells with culture media R10 only added is used to calculate the spontaneuos release (SR) or minimum release. Plates were centrifuged at 500 rpm for 5 min and incubated at 37^oC with a humid atmosphere of 5% CO_2 in air for 4 h.

After 4 h, the plate was removed from incubator and centrifuged at 500 rpm for 5 min. 100 µl of supernatant was put into a microtube which have 100 µl of 5% TX 100 and gamma ray was counted with a gamma counter.

4.8 Specific killing calculation

The level of CTL activity as denoted by the percent specific lysis (% specific lysis) of labeled BLCL targets is determined by the following formula.

$$\% \text{ spontaneous lysis} = \frac{\text{cpm. Spontaneous Release (SR)}}{\text{cpm. Total Release (TR)}} \times 100$$

$$\% \text{ specific lysis} = \frac{\text{cpm. Test} - \text{cpm. SR}}{\text{cpm. TR} - \text{cpm. SR}} \times 100$$

The criteria of positive CTL activity are following
(1) % Spontaneous release is less than or equal to 30%
(2) % specific cytotoxicity of the test is more than 10% lysis of the no peptide pulsed BLCL at the both effector to target ratio.
(3) % specific cytotoxicity of the test is value subtracted with the result of the no peptide pulsed BLCL

4.9 HLA-restriction and fine mapping

HLA restriction was performed with autologous and partially HLA-matched target cells pulsed with each OLP and no pulsed peptide as the control in ELISpot assay. Fine mapping of the optimal epitope was performed using single amino acid truncations and extensions of the predicted optimal epitope using ELISpot at effector to target cell ratios of 30:1 and 15:1.

4.10 Statistics

Qualitative differences data between samples were examined using the Spearman's rank correlation coefficient test and unpaired t test. Quantitative differences between samples are compared using the Mann–Whitney U test or Chi-square (Fisher's exact) test as appropriate.

4.11 Remarks

These were materials and methods that support for this study.

1. The data of CD4+ and CD8+ T cells were assessed the immunophenotypic at Vaccine and Cellular Immunology Laboratory, Allergy and Clinical Immunology unit, Faculty of Medicine, Chulalongkorn University.
2. The plasma HIV-RNA level was measured by the Chiron b-DNA monitor test (Quantiplex v3.0, Bayer) with a sensitivity of 50 copies/ml at Anonymous Clinic, Thai Red Cross AIDS Research Centre, Chulalongkorn Hospital.
3. The HLA typing was performed by DNA Sequenced Based Typing (SBT) at The University of Oklahoma Health Sciences Center CLIA/ASHI certified high resolution HLA typing laboratory.
4. The HIV sequencing was sequenced the autologous virus from plasma RNA using population sequencing and bi-directionally on an ABI 3130 automated sequencer at Ragon Institute of MGH, MIT and Harvard, Boston, Massachusetts, USA.

4.11.1 The immunophenotypic CD4+ T cell staining

EDTA blood was incubated with monoclonal antibodies CD4-FITC/CD8-PE/CD3-PerCP (BD Biosciences 340298, San Diego, CA, USA) for 20 min at room temperature and erythrocyte was lysed with 1x FACS lysing solution (BD Biosciences 349202). The centrifuged cells were washed by 1x PBS (Sigma-Aldrich, P-3813), and then fixed by 1% paraformaldehyde (PFA; Sigma-Aldrich, P-1648). The staining cells were analyzed on FACS Calibur flow cytometer with CellQuestPro software (Becton Dickinson, San Jose, CA, USA). Absolute counts were determined

by multiplying flow cytometry percentages by the lymphocyte counts obtained from the automated hematology machine (Sysmex model SF3000, Gobe, Japan).

*** FITC : fluorescein isothiocyanate
PE : phycoerythrin
PerCP : peridinin chlorophyll protein

4.11.2 Plasma HIV-RNA level measurements

Plasma HIV-RNA level was measured by the Chiron b-DNA Monitor test (Quantiplex v3.0, Bayer) with a sensitivity of 50 copies/mL (according to the manufacturer's instructions).

4.11.3 HLA typing

High resolution HLA Class I typing was performed by DNA Sequenced Based Typing (SBT) at The University of Oklahoma Health Sciences Center CLIA/ASHI certified high resolution HLA tying laboratory. Briefly, exons 2 and 3 of class I gene were amplified and DNA sequenced. Ambiguous types are resolved to 4 digits with either group specific amplification followed by DNA sequencing of the specifically amplified allele and/or by PEL-FREEZ/Invitrogen UNITRAY SSP kits.

4.11.4 HIV Sequencing

Autologous virus was sequenced from plasma HIV-RNA using population sequencing as described previously[30]. HIV-RNA was extracted from plasma using QIAmp RNA Viral Mini kit (QIAGEN, Germany) and nested PCR was performed using primers specific for clades B and CRF01_AE. First round PCR cycling conditions were as follows: 95°C for 15 min followed by 35 cycles of 30 seconds (s) at 94°C, 30 s at 54°C, 2.25 min at 72°C and a final extension of 68°C for 20 min. Nested PCR reactions were 95°C for 2 min followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 1.75 min at 72°C and a final extension of 68°C for 20 min. PCR fragments were then sequenced bi-directionally on an ABI 3130 automated sequencer using previously published primers. Sequencher (Gene Codes Corp., Ann Arbor, MI) and MacVector 4.1 (Oxford Molecular) were used to edit and align the sequences.

CHAPTER V

RESULTS

5.1 Demographic and clinical data

In this study, 240 HIV-1 infected patients were asymptomatic and ARV naïve. More than a half (57%) were male. The age ranged from 19 to 66 years old with the mean of 34 years old. The median duration of HIV-1 infection after the first diagnostic was 3 years (ranged from 0 to 18). The median CD4+ T cell was 318 cells/cu.mm. (ranged from 1 to 1,292 cells/cu.mm.). Median plasma HIV-RNA level was 20,994 copies/ml. (ranged from <50 to >500,000 copies/ml.). Seventy four percentages of patients were heterosexuals, 21% were homosexuals or bisexuals, 2% were IVDUs and 3% were blood transfusion (Table 4). There were no significant differences in age, sero-diagnosis, CD4+ T cells and plasma HIV-RNA level between female and male.

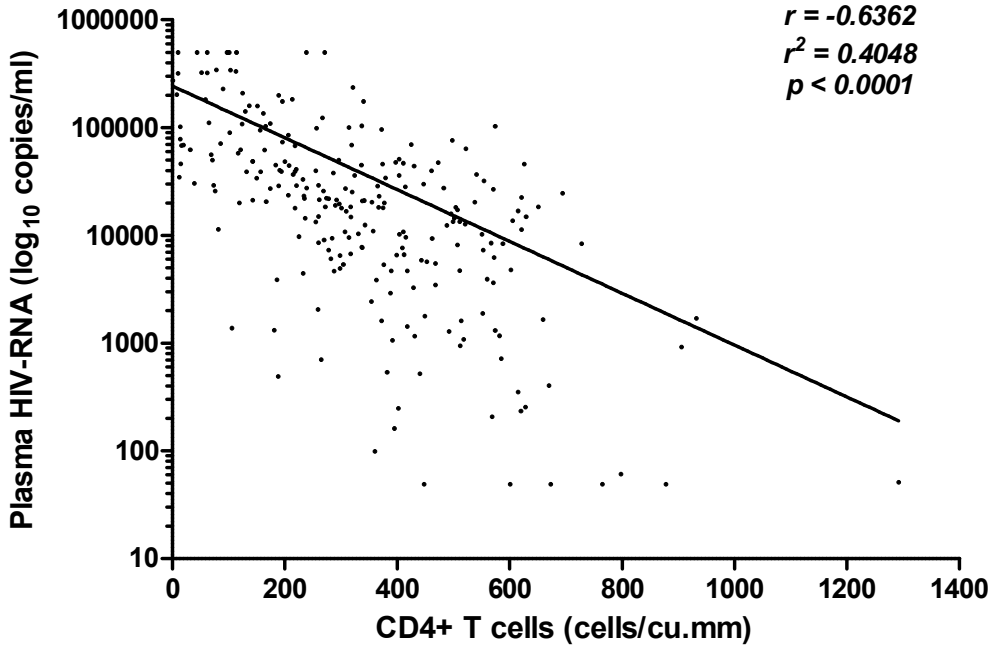
Table 4. Demographic and clinical data of 240 HIV-1 infected patients.

	Total	**Female**	**Male**
Number of patients	240	103	137
Mean age (years, range)	34 (19-66)	35 (21-66)	33 (19-60)
Median sero-diagnosis (years, range)	3 (0-18)	4 (0-16)	3 (0-18)
Median CD4+ T cells (cells/cu.mm., range)	318 (1-1,292)	317 (8-1,292)	319 (1-878)
Median plasma HIV- RNA (copies/ml, range)	20,994 (<50->500,000)	18,286 (<50->500,000)	24,709 (<50->500,000)
Risk of transmission (n, %)			
- Heterosexual intercourse	179 (74%)	99 (96%)	80 (58%)
- MSM and/or bisexual intercourse	50 (21%)	1 (1%)	49 (36%)
- IVDUs	4 (2%)	1 (1%)	3 (2%)
- Blood transfusion/Tattoo	7 (3%)	2 (2%)	5 (4%)

5.2 Correlation between CD4+ T cells and plasma HIV-RNA level

In this cohort, we analyzed the correlation between CD4+ T cells and plasma HIV-RNA level. The results showed significant inverse correlation between CD4+ T cells and plasma HIV-RNA level (p <0.0001) (Figure 7).

Figure 7. The correlation between CD4+ T cells (cells/cu.mm.) and plasma HIV-RNA level (copies/ml) in 240 HIV-1 infected patients (p < 0.0001).



5.3 HIV-1 clade distribution

To determine the HIV-1 clade distribution, we used 231 of Gag, Nef and full-length sequencing data. Gag and Nef sequences were done in VCI laboratory, Faculty of Medicine, Chulalongkorn University and full-length sequences were done in Ragon Institute of MGH, MIT and Harvard, Boston, Massachusetts, USA. Based on HIV-1 genotyping, 85% of patients were infected with clade CRF01_AE, 12% clade B, 2% clade CRF01_AE_B and 0.5% clade C and F (Figure 8). There is no significant difference in plasma HIV-RNA level between different subtypes (Figure 9).

Figure 8. The distribution of HIV-1 clade among 231 sequencing data based on Gag, Nef and full-length sequences.

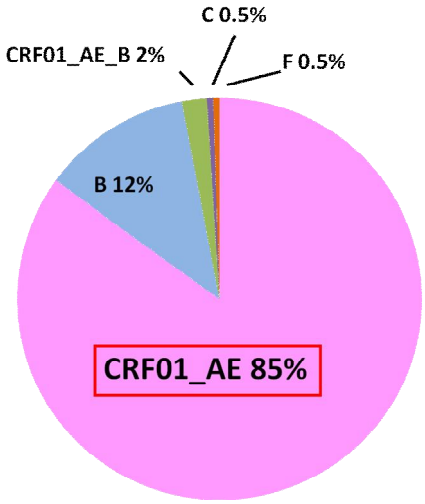
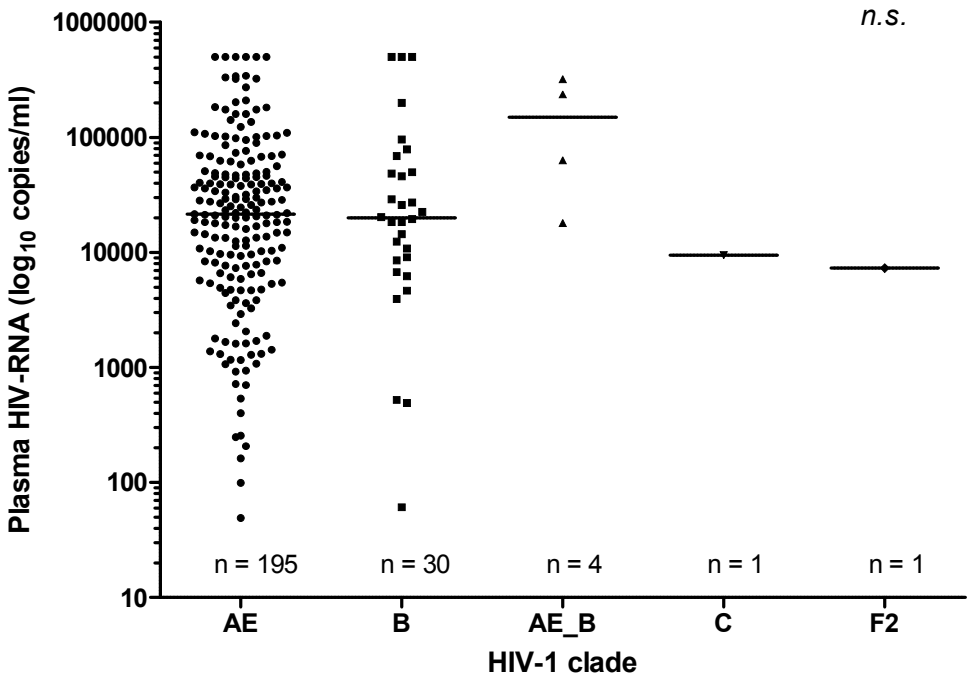


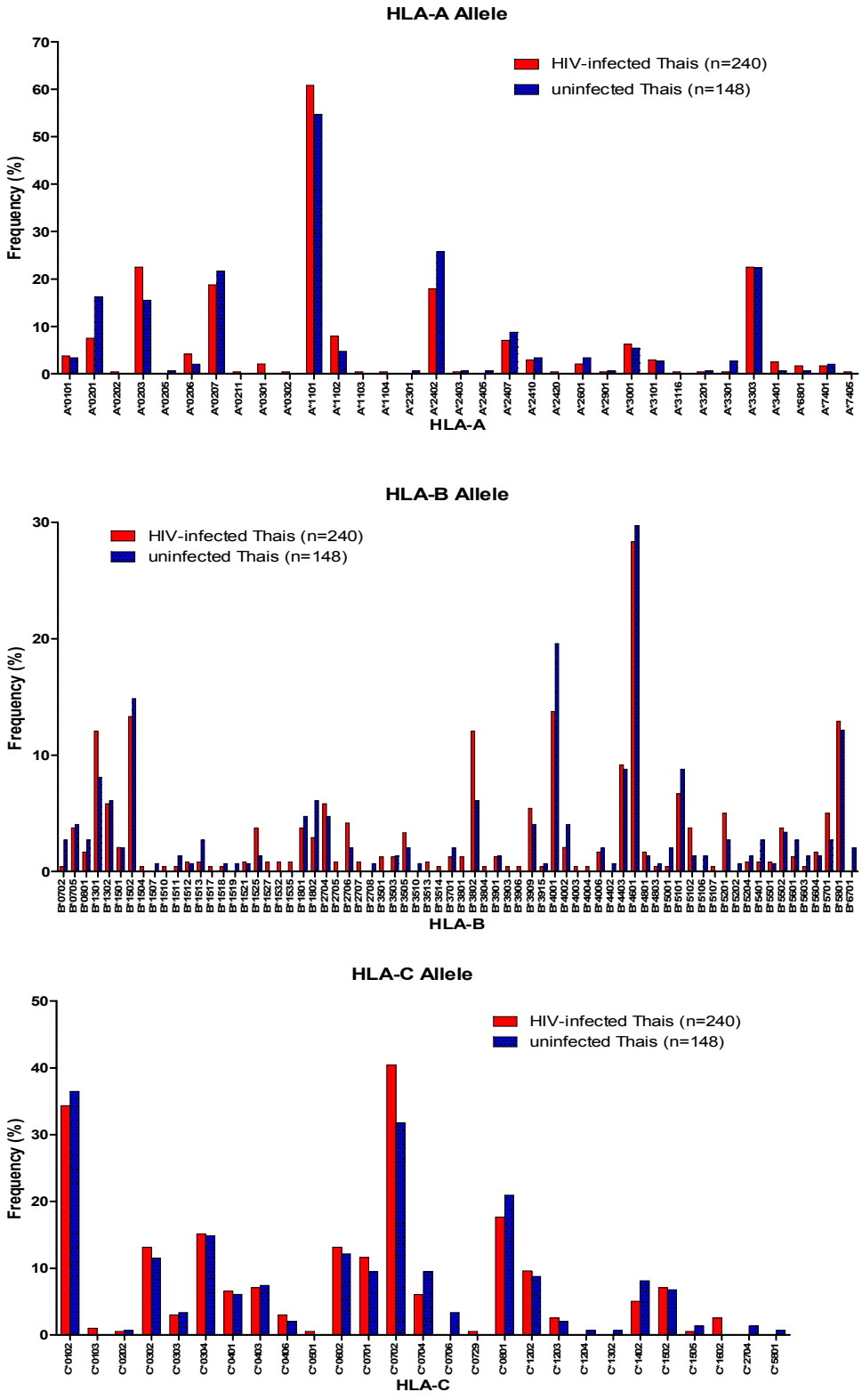
Figure 9. The correlation between HIV-1 clade and plasma HIV-RNA level (log_{10} copies/ml) in 231 HIV-1 infected patients.



5.4 HLA class I allele's distribution in individuals with and without HIV infection.

To identify HLA class I alleles distribution in Thai individuals, four digit alleles of HLA-A, HLA-B and HLA-C were analyzed in 240 HIV-1 infected and 148 uninfected Thai individuals. The HLA class I alleles frequencies data were shown in Figure 10. HLA allele distributions were similar between the groups. The frequencies of common HLA class I alleles in both groups include HLA-A*1101 (61% vs. 55%), -A*0203 (23% vs. 16%), -A*3303 (23% vs. 22%), -A*2402 (18% vs. 26%), -B*4601 (28% vs. 30%), -B*4001 (14% vs. 29%), -B*1502 (13% vs.15%), -C*0702 (40% vs. 32%), -C*0102 (34% vs. 36%), and -C*0801 (18% vs. 21%). There were no significant different in HLA class I alleles frequencies between HIV-1 infected and uninfected individuals. In addition, we found 6% HLA-B*2704, 7% HLA-B*5101, 3% HLA-B*5701 and 7% HLA-B*5801. These alleles have been reported as protective HLA alleles in patients infected with HIV-1 clade B[(22)].

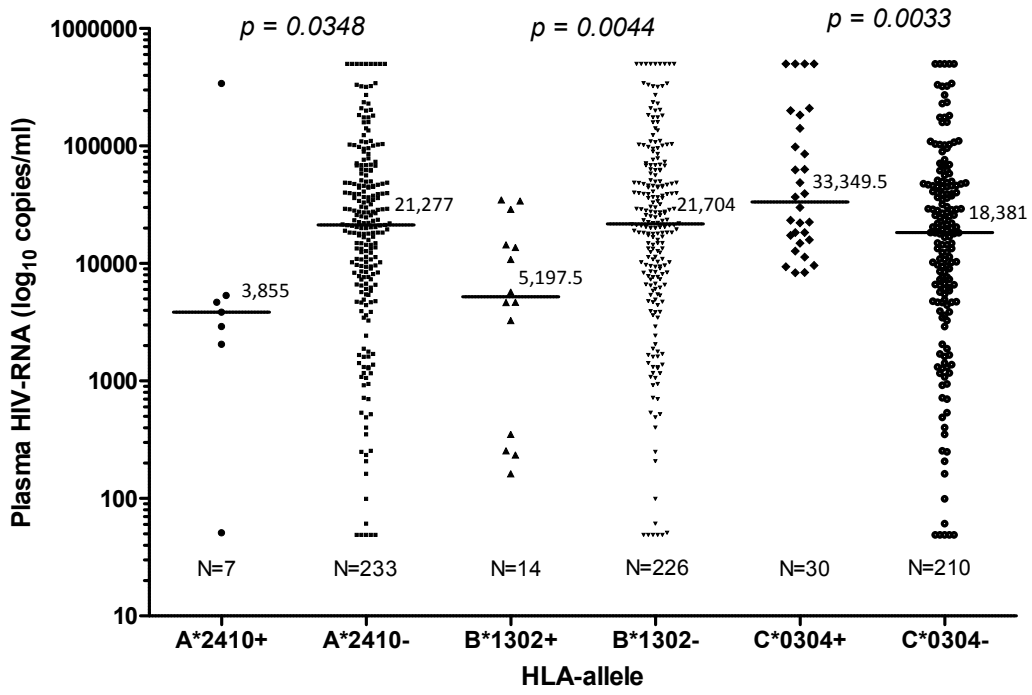
Figure 10. HLA class I alleles frequency of HLA-A, -B and -C in HIV-1 infected and uninfected Thai individuals (240 HIV-1 infected and 148 uninfected individuals).



5.5 Correlation between HLA class I alleles and plasma HIV-RNA level

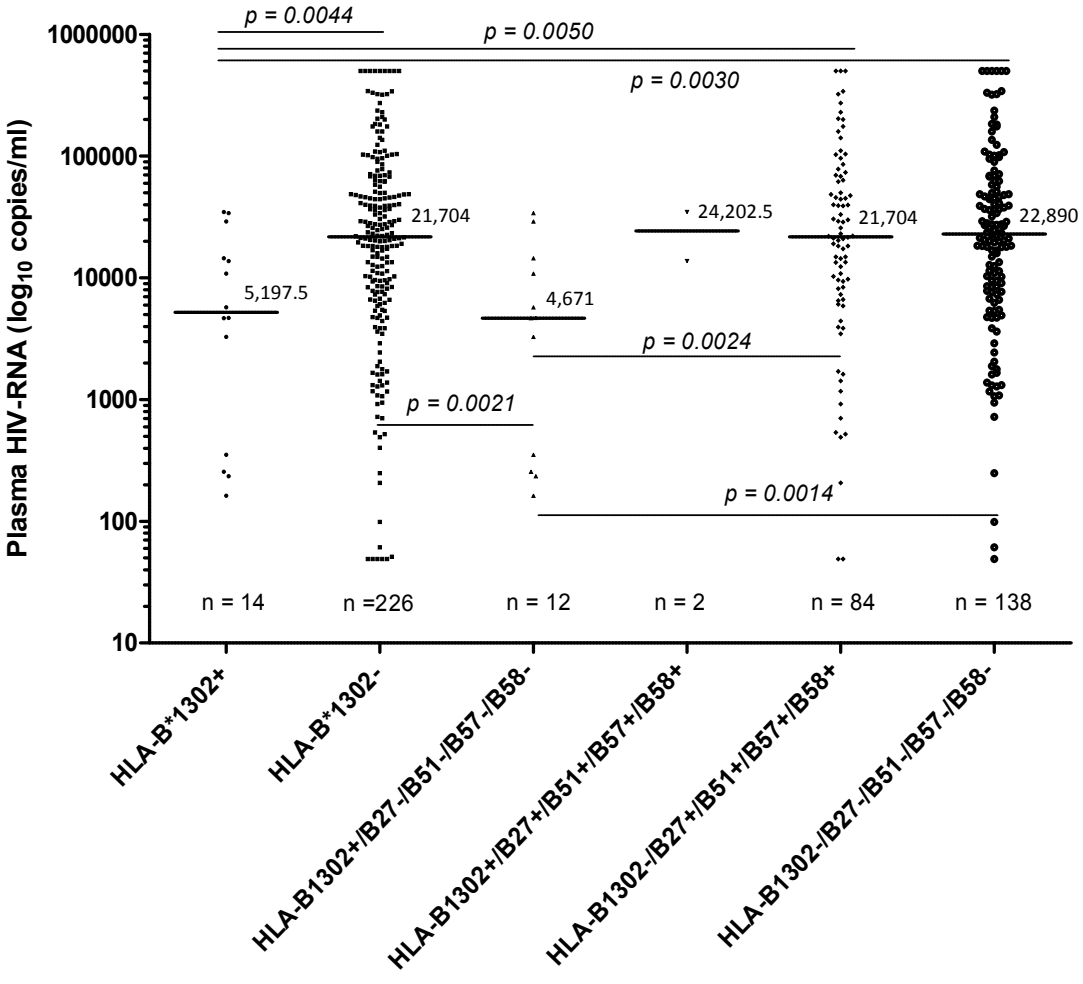
To assess the correlation between HLA class I alleles and plasma HIV-RNA level, each genotype of HLA-A, -B and -C allele (HLA-A 33 genotypes, HLA-B 70 genotypes and HLA-C 27 genotypes) was analyzed its association with the corresponding plasma HIV-RNA level. The results showed that patients with HLA-A*2410 or -B*1302 was significantly associated with lower plasma HIV-RNA level than patients without HLA-A*2410 or -B*1302 (p = 0.0348 and 0.0044, respectively) whereas patients with HLA-C*0304 was significantly associated with higher plasma HIV-RNA level than patients without HLA-C*0304 (p = 0.0033) (Figure 11).

Figure 11. The correlation between HLA class I alleles with plasma HIV-RNA level (log_{10} copies/ml) in 240 HIV-1 infected individuals.



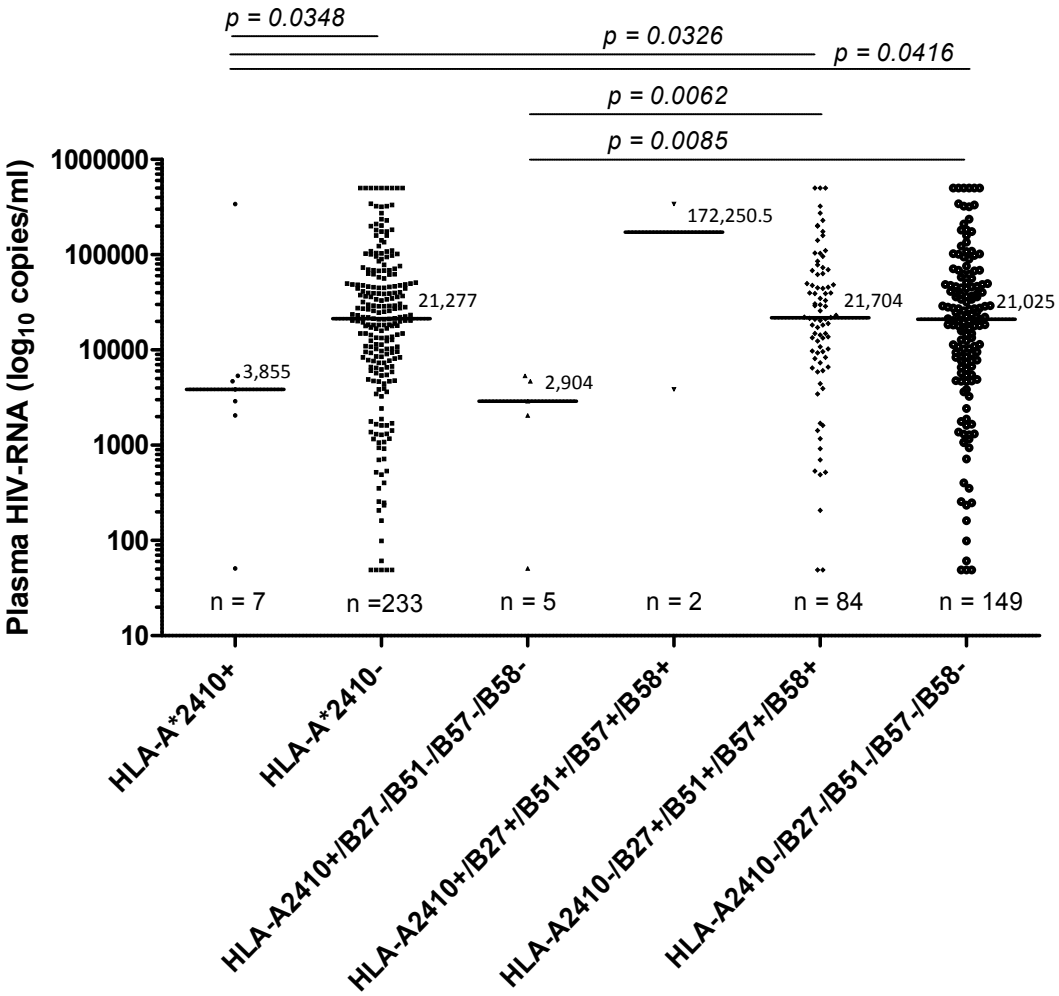
To confirm that the association of HLA-B*1302 allele with low plasma HIV-RNA level was independent from other known protective alleles, we investigated by excluded HLA-B*27, -B*51, -B*57 and -B*58 alleles. We found patients who have HLA-B*1302 allele without any of the protective alleles was strongly associated with lower plasma HIV-RNA level than the patients without HLA-B*1302 allele together with or without protective alleles (p = 0.0024 and 0.0014, respectively) (Figure 12).

Figure 12. The correlation between patients with HLA-B*1302 allele with and without protective alleles (HLA-B*27, -B*51, -B*57 and -B*58) and plasma HIV-RNA level (log_{10} copies/ml) in 240 HIV-1 infected individuals.



We also found that patients with HLA-A*2410 allele but have no any of the protective alleles was strongly significant associated with lower plasma HIV-RNA level than patients without HLA-A*2410 allele with and without protective alleles (p = 0.0062 and 0.0085, respectively) (Figure 13).

Figure 13. The correlation between patients who have HLA-A*2410 allele with and without other protective alleles (HLA-B*27, -B*51, -B*57 and -B*58) and plasma HIV-RNA level (log_{10} copies/ml) in 240 HIV-1 infected individuals.



5.6 Frequency, breadth and magnitude of HIV-1 targeted proteins responses

5.6.1 IFN-γ ELISpot responses against whole HIV-1 CRF01_AE Proteome

5.6.1.1 Frequency of responses

Among 240 HIV-1 infected individuals, HIV-1 specific CD8+ T cell responses were detected against all viral proteins (Figure 14). Almost every individual (97%) responded to at least one OLP. The results showed that Pol was the most common response (91%), followed by Gag (87%) and Env (83%) in structural proteins whereas 78% respond to Nef (the most common among regulatory proteins) (Figure 15).

Figure 14. The CD8+ T cell responses against different HIV-1 CRF01_AE proteins in 240 HIV-1 infected individuals (tested by IFN-γ ELISpot assay). The X-axis represents OLPs of HIV-1 CRF01_AE proteins and Y-axis represents CD8+ T cell responses (SFU/1,000,000 PBMCs). Each bar represents one ELISpot response against one OLP.

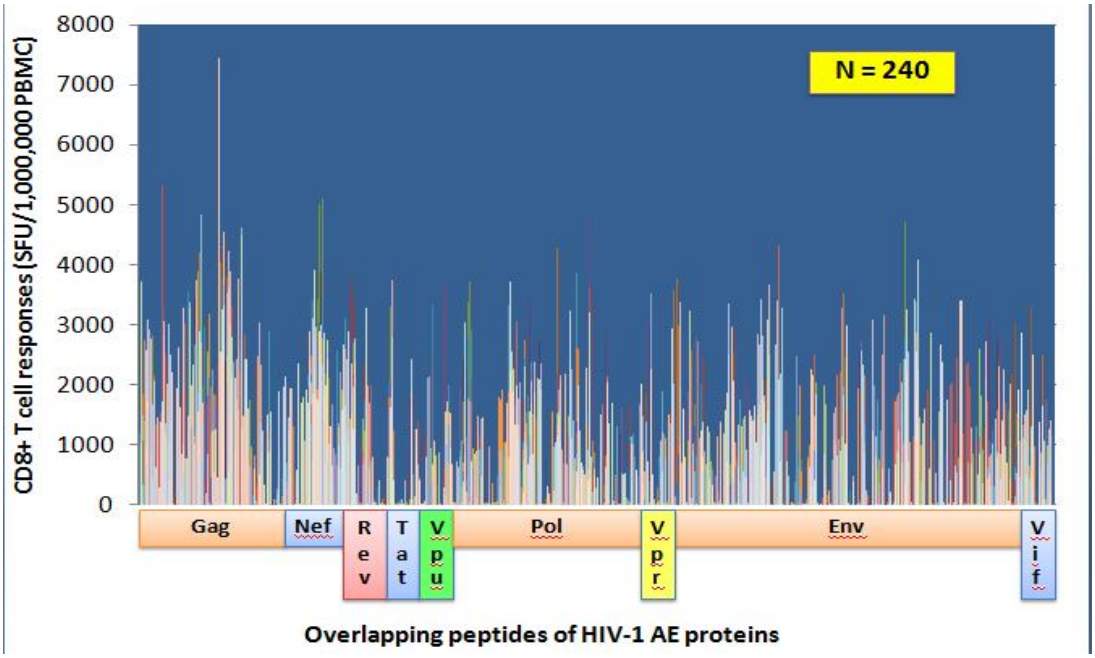
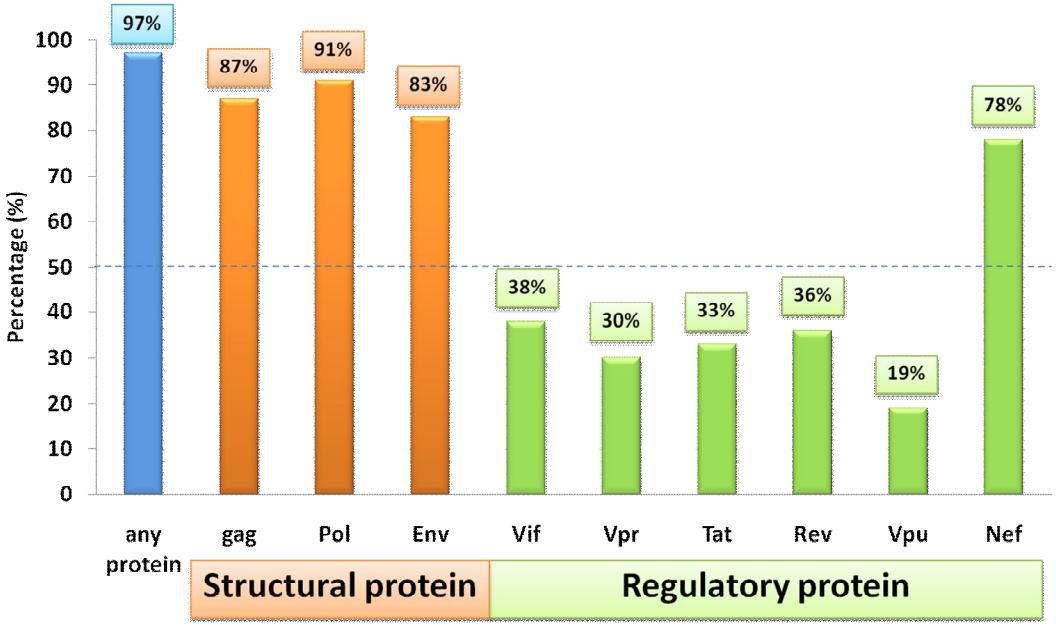


Figure 15. The frequency of CD8+ T cell IFN-γ ELISpot responses to each HIV-1 CRF01_AE protein among 240 HIV-1 infected patients. The X-axis represents HIV-proteins and Y-axis represents percentage (%).



Correlation between patients with and without structural proteins responses and plasma HIV-RNA levels were analyzed, and showed that patients with Gag responses were significantly associated with low plasma HIV-RNA level (p = 0.0054). In contrast, patients with Env responses were significantly associated with high plasma HIV-RNA level (p = 0.0013) (Figure 16). No significant correlation between regulatory proteins responses and plasma HIV-RNA level was observed (Figure 17).

Figure 16. The correlation between HIV-1 specific structural protein CD8+ T cell responses and plasma HIV-RNA levels (log_{10} copies/ml) in 240 HIV-1 infected individuals.

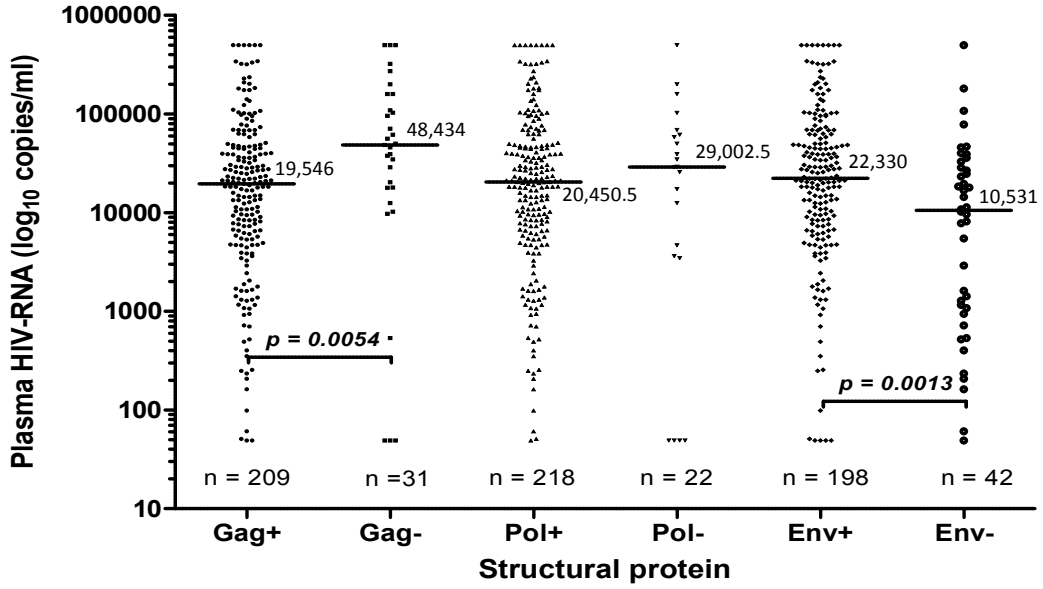
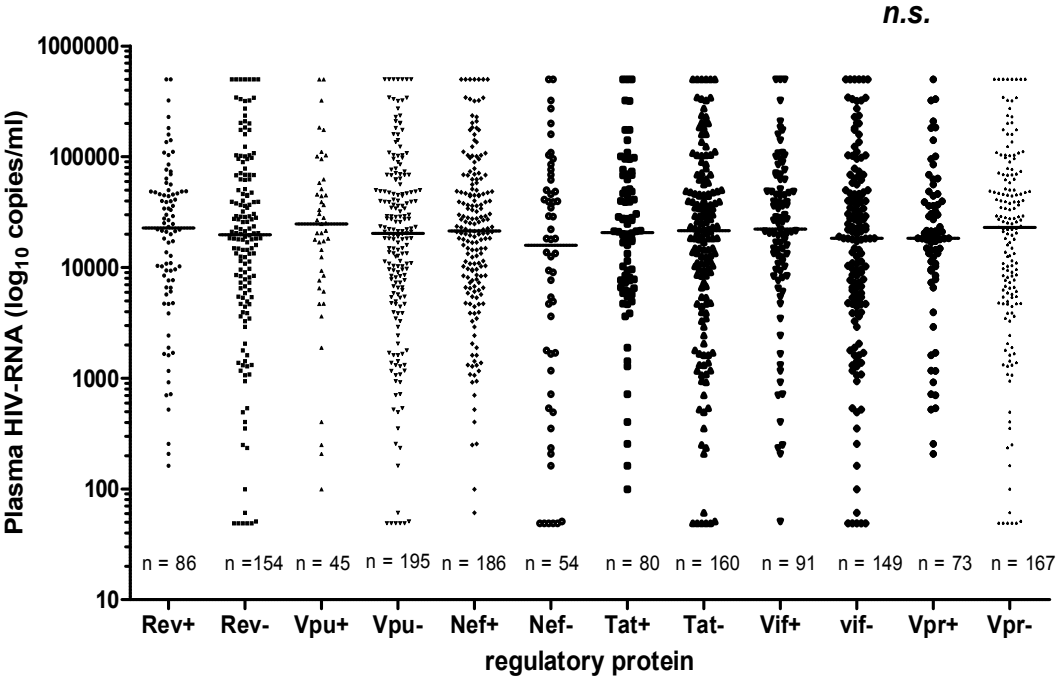


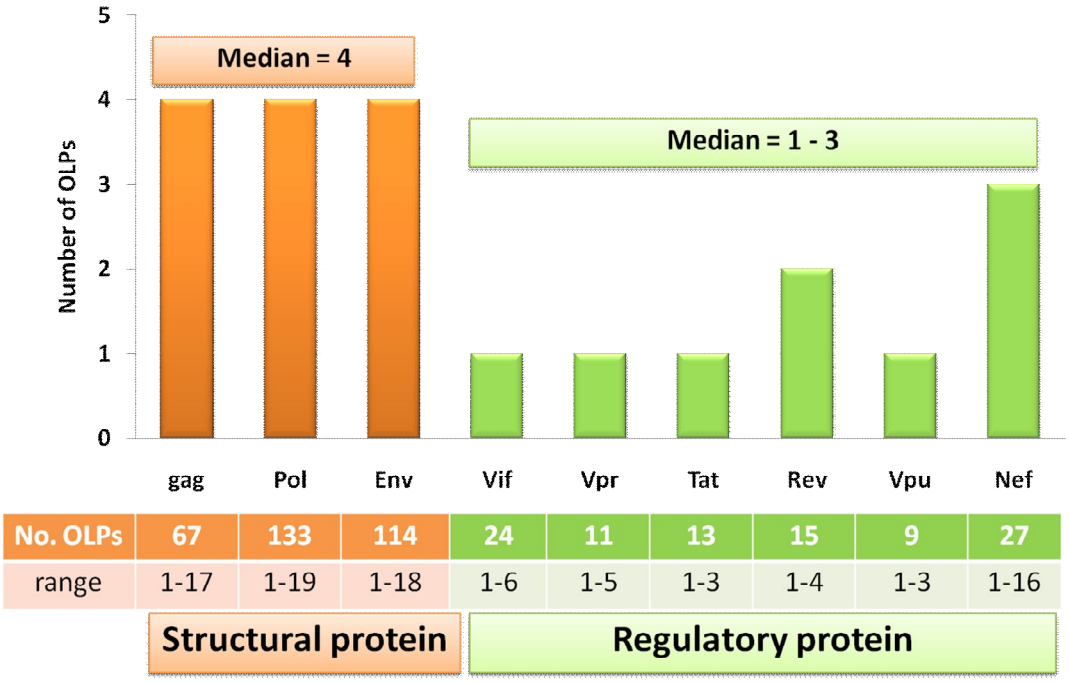
Figure 17. The insignificant correlation between HIV-1 regulatory protein specific CD8+ T cell responses and plasma HIV-RNA levels (log_{10} copies/ml).



5.6.1.2 Breadth of CD8+ T cell responses

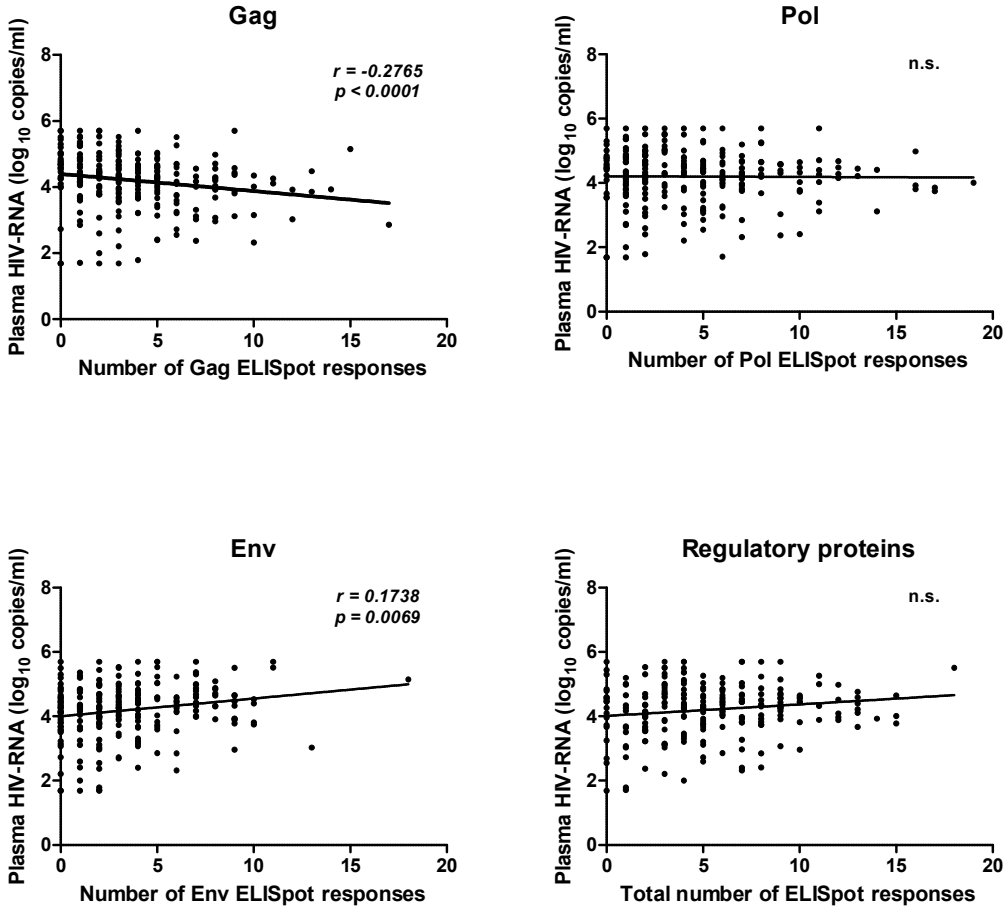
To determine the breadth of HIV-1 specific CD8+ T cell responses against HIV-1 CRF01_AE proteins, we analyzed the number of OLPs responses in each patient. We found that the median of breadth of structural proteins were 4 OLPs and the median of breadth of regulatory proteins were varied from 1 - 3 OLPs (the highest breadth was found in Nef) (Figure 18).

Figure 18. The median of breadth of CD8+ T cell responses in each HIV-1 CRF01_AE protein showed in HIV-1 patients by IFN-γ ELISpot assay. The X-axis represents the HIV-1 specific proteins and Y-axis represents a number of CD8+ T cell responded-OLPs.



The correlation between breadth of responses against these various HIV proteins and plasma HIV-RNA levels was further analyzed. The number of Gag OLP responses was shown associated significantly with low plasma HIV-RNA level ($p < 0.0001$) whereas the number of Env OLP responses was in contrast significantly associated with high plasma HIV-RNA level ($p = 0.0069$). No correlation between the breadth of responses and plasma HIV-RNA level was found in Pol and all regulatory proteins (Figure 19).

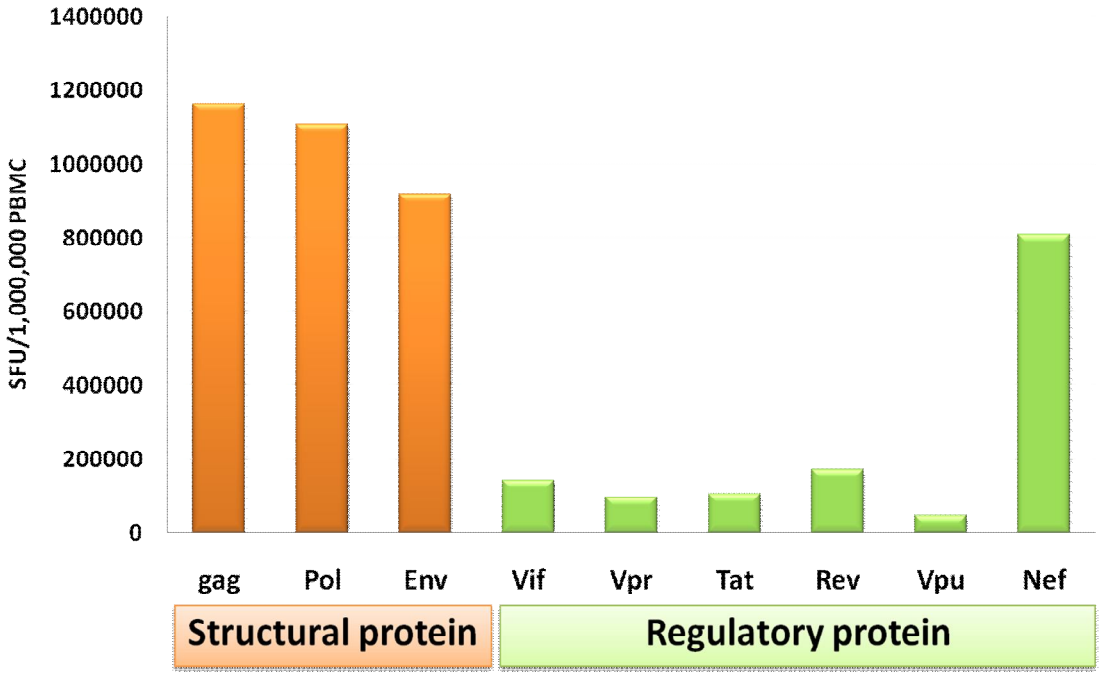
Figure 19. The correlation between the breadth of CD8+ T cell responses and plasma HIV-RNA levels (log_{10} copies/ml).



5.6.1.3 Magnitude of responses

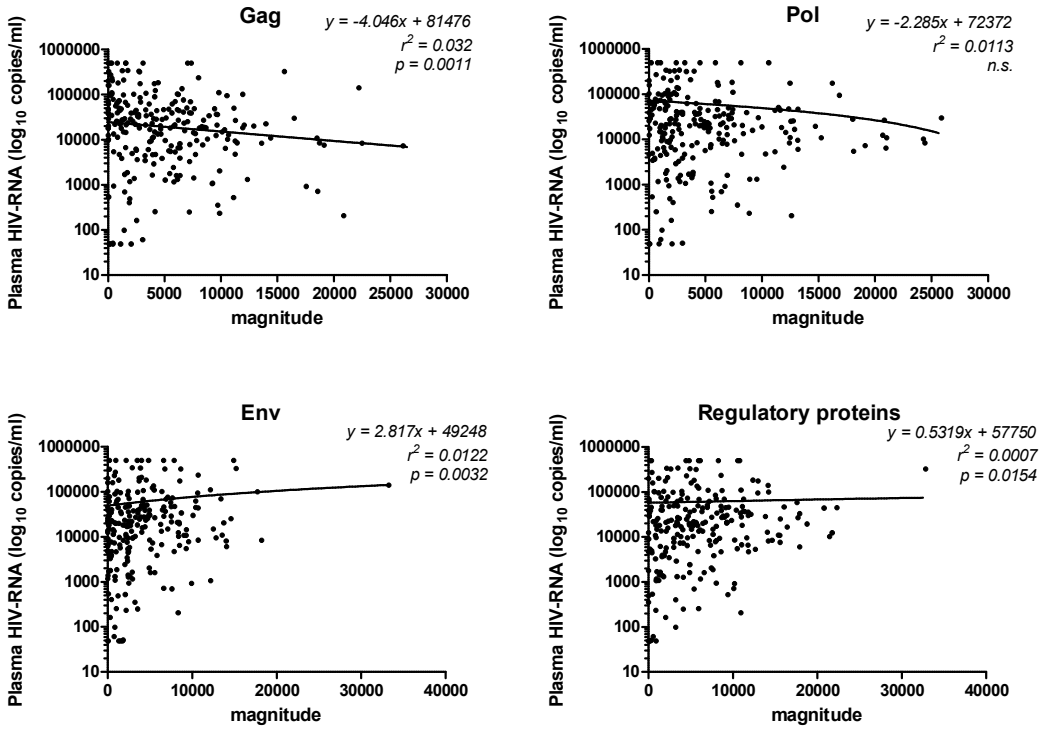
To determine the magnitude of HIV-1 specific CD8+ T cell responses against HIV-1 CRF01_AE proteins, we analyzed the summation of the ELISpot responses one of each patient. We found that cumulative magnitude of the responses against structural proteins were higher than the cumulative magnitude of responses to regulatory proteins (Figure 20).

Figure 20. The cumulative magnitude of CD8+ T cell responses against each HIV-1 CRF01_AE. The X-axis represents the HIV-1 specific proteins and the Y-axis represents CD8+ T cell responses (SFU/10^6 PBMCs).



The correlation between magnitude of HIV-1 specific CD8+ T cell responses against HIV-1 proteins and plasma HIV-RNA levels was analyzed. It is consistent that the magnitude of Gag responses was associated significantly with low plasma HIV-RNA level (p = 0.0011); in contrast, the magnitude of Env and regulatory proteins responses were associated significant with high plasma HIV-RNA level (p = 0.0032, 0.0154, respectively) (Figure 21).

Figure 21. The correlation between magnitudes of CD8+ T cell specific responses to various HIV proteins and plasma HIV-RNA levels (log_{10} copies/ml).

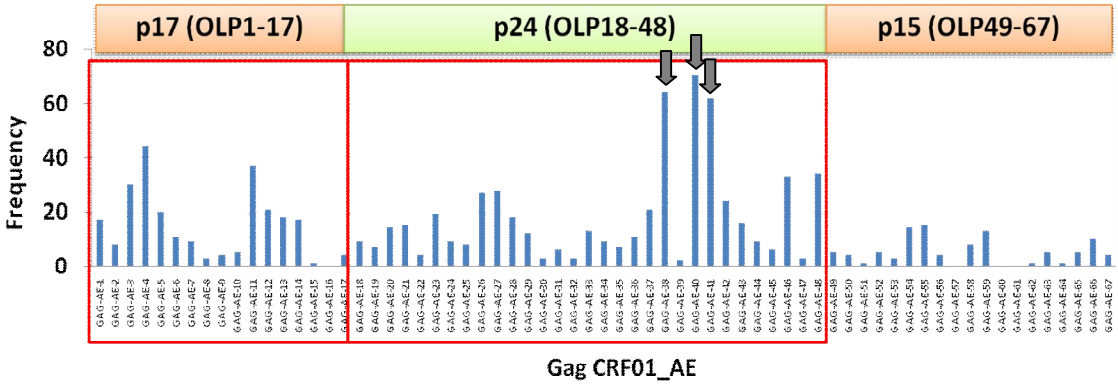


5.7 Patterns and common locations of CD8+ T cell epitopes in various HIV-1 target proteins

5.7.1 Gag OLPs recognition the mapping patterns

Of 67 Gag-OLPs, 63 (94%) were recognized at least one individual (Figure 22): 17 peptides in p17 (Gag-OLP1-17), 31 peptides in p24 (Gag-OLP18-48) and 19 peptides in p15 (Gag-OLP49-67). By ranking p24 (79%) was the most highly targeted protein region; p17 (53%) was the second most common, followed by p15 (24%). The most three frequently recognized peptides were located in the p24 region including Gag-OLP40 ($_{288}$GPKEPFRDYVDRFYKTLR$_{305}$) which was recognized in 30% of patients, Gag-OLP38 ($_{273}$IVRMYSPVSILDIRQGPK$_{290}$; with 27% recognition), and Gag-OLP41 ($_{296}$YVDRFYKTLRAEQATQEV$_{313}$; with 26% recognition). The mapping pattern of recognition was mainly located in N-terminal and middle region of Gag protein. Much fewer epitope was shown in C-terminal of Gag.

Figure 22. The frequency of Gag specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three most common responses were Gag-OLP40, -OLP38 and -OLP41 (with arrow). The recognition mapping pattern was mainly located in N-terminal and middle region of Gag protein.

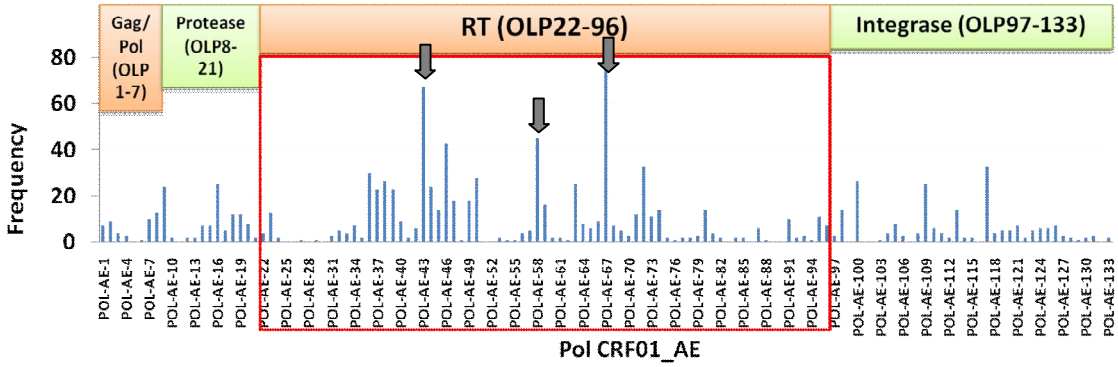


5.7.2 Pol OLPs recognition the mapping patterns

Of 133 Pol-OLPs, 115 (86%) were recognized at least one individual (Figure 23): 7 peptides in Gag/Pol (Pol-OLP1-7), 14 peptides in protease (PR) (Pol-OLP8-21), 74 peptides in reverse transcriptase (RT) (Pol-OLP22-96) and 37 peptides in integrase (IN) (Pol-OLP97-133). By ranking RT (81%) was the most highly targeted protein region; IN (55%) was the second most common, followed by PR (33%) and Gag/Pol (11%). The most three frequently recognized peptides were located in the RT region. There were Pol-OLP67 ($_{488}$GQDQWTYQIYQEPFKNLK$_{505}$) which was recognized in 32% of patients, Pol-OLP43 ($_{306}$QGWKGSPAIFQSSMTKIL$_{323}$; with

28% recognition) and Pol-OLP58 ($_{418}$KLNWASQIYAGIKVKQL$_{434}$; with 19% recognition). The mapping pattern of recognition was mainly located in middle region of Pol protein.

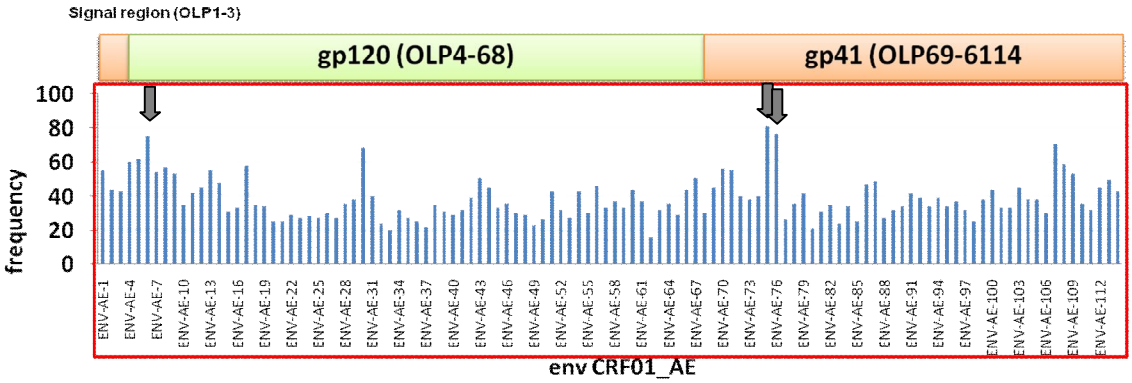
Figure 23. The frequency of Pol specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three most common responses were Pol-OLP67, -OLP43 and -OLP58 (with arrow). The recognition mapping pattern was mainly located in middle region of Pol protein.



5.7.3 Env OLP recognition the mapping patterns

Of 114 Env-OLPs, all OLPs (100%) were recognized at least one individual (Figure 24): 3 peptides in signal region (Env-OLP1-3), 65 peptides in gp120 (Env-OLP4-68) and 46 peptides in gp41 (Env-OLP69-114). By ranking gp41 (69%) was the most highly targeted protein region; gp120 (62.5%) was the second most common, followed by signal region (10%). The most two frequently recognized peptides were located in the gp41 region including Env-OLP75 ($_{548}$IVQQQSNLLRAIEAQQHL$_{565}$) which was recognized in 34% of patients and Env-OLP76 ($_{556}$LRAIEAQQHLLQLTVWGI$_{573}$; with 32% recognition). The third frequently recognized peptide was located in gp120 region which was Env-OLP6 ($_{37}$TVYYGVPVWRDADTTLF$_{53}$; 31% recognition). The mapping pattern of recognition was located in all regions of Env protein.

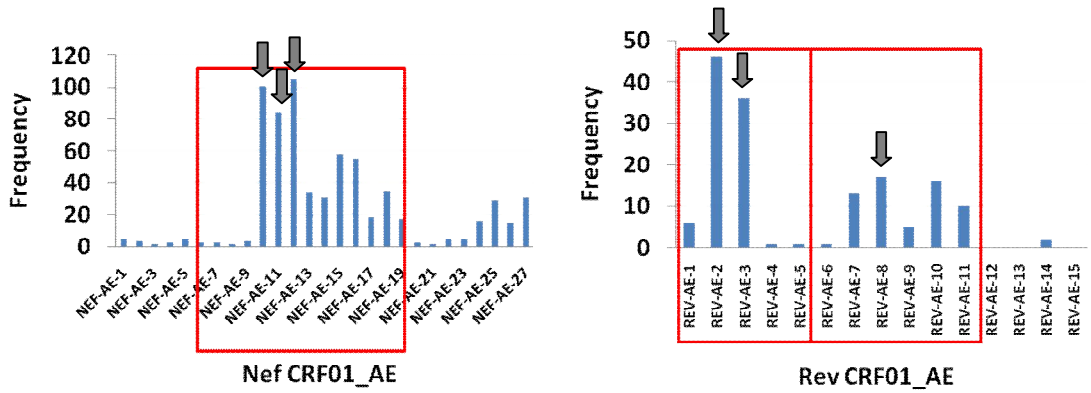
Figure 24. The frequency of Env specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three most common responses were Env-OLP75, -OLP76 and -OLP6 (with arrow). The recognition mapping pattern was located in all region of Env protein.



5.7.4 Nef OLP recognition the mapping patterns

Of 27 Nef-OLPs, all OLPs (100%) were recognized at least one individual (Figure 25 left). The most three frequently recognized peptides were Nef-OLP12 ($_{81}$YKGAFDLSFFLKEKGGL$_{97}$) which was recognized in 44% of patients, Nef-OLP10 ($_{65}$EVGFPVRPQVPLRPMTYK$_{82}$) which was recognized in 42% of patients and Nef-OLP11 ($_{73}$QVPLRPMTYKGAFDLSFF$_{90}$) which was recognized in 35% of patients. The mapping pattern of recognition was mainly located in middle region of Nef protein. No epitope was shown in N-terminal of Nef.

Figure 25. The frequency of Nef and Rev specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three most common responses in Nef were Nef-OLP12, -OLP10 and -OLP11 (with arrow, left figure). The most three frequently of responses in Rev were Rev-OLP2, -OLP3 and -OLP8 (with arrow, right figure). The recognition mapping pattern was located in middle region of Nef protein and was located in N-terminal and middle region of Rev protein.



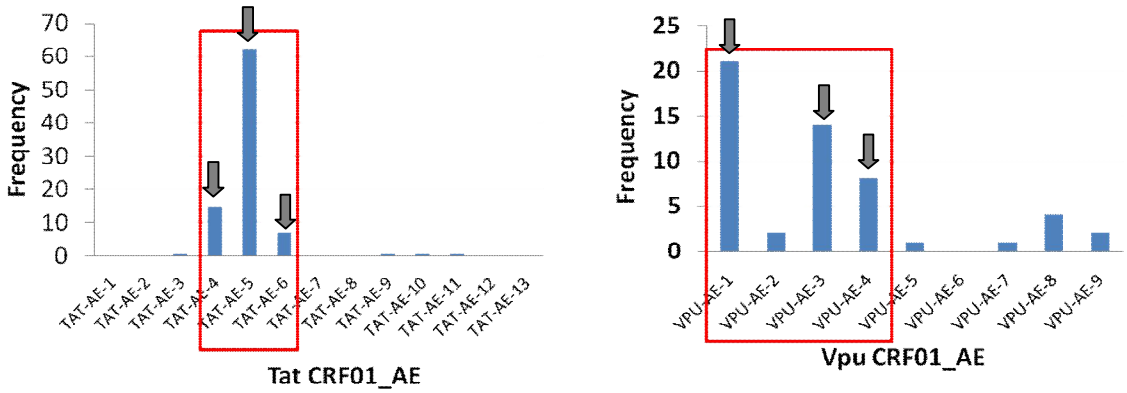
5.7.5 Rev OLP recognition the mapping patterns

Of 15 Rev-OLPs, 12 (80%) were recognized at least one individual (Figure 25 right). The most three frequently recognized peptides were Rev-OLP2 ($_{9}$DEELLRAVRIIKILY$_{23}$) which was recognized in 19% of patients, Rev-OLP3 ($_{14}$RAVRIIKILYQSNPY$_{28}$; with 15% recognition) and Rev-OLP8 ($_{49}$QRQIRAISERILSTCLGR$_{66}$; with 7% recognition). The mapping pattern of recognition was located in N-terminal and middle region of Rev protein.

5.7.6 Tat OLP recognition the mapping patterns

Of 13 Tat-OLPs, 7 (53.8%) were recognized at least one individual (Figure 26 left). The most three frequently recognized peptides were Tat-OLP5 ($_{32}$WHCQLCFLKKGLGISYGR$_{49}$) which was recognized in 26% of patients, Tat-OLP4 ($_{24}$KCYCKKCCWHCQLCFLKK$_{41}$) which recognized in 6% of patients and Tat-OLP6 ($_{40}$KKGLGISYGRKKRKHRRG$_{57}$) which was recognized in 3% of patients. The mapping pattern of recognition was located in middle region of Tat protein. No epitope was found in the N and C-terminal of Tat.

Figure 26. The frequency of Tat and Vpu specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three most common responses in Tat were Tat-OLP5, -OLP4 and -OLP6 (with arrow, left figure). Three common responses in Vpu were Vpu-OLP1, -OLP3 and -OLP4 (with arrow, right figure). The recognition mapping pattern was located in middle region of Tat protein and was located in N-terminal of Vpu protein.



5.7.7 Vpu OLP recognition the mapping patterns

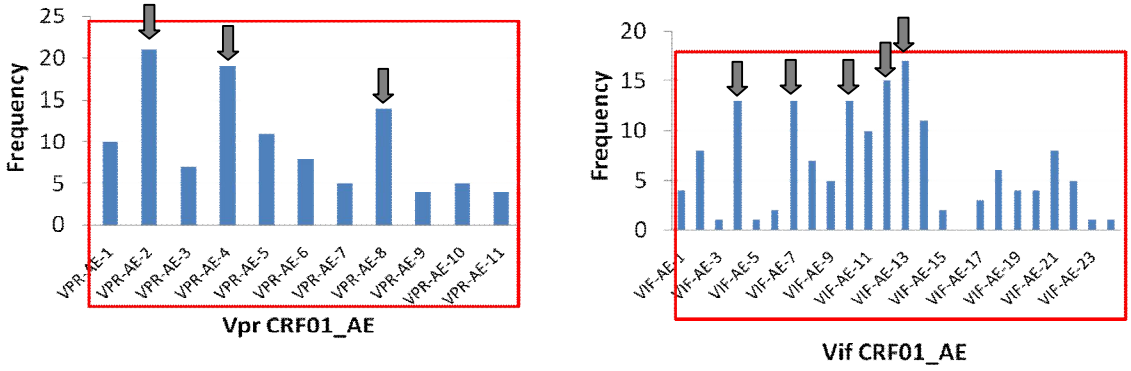
Of 9 Vpu-OLPs, 8 (88.9%) were recognized at least one individual (Figure 26 right). The most three frequently recognized peptides were Vpu-OLP1 ($_{1}$MTPLEISAIVGLIVALIL$_{18}$) which was recognized in 9% of patients, Vpu-OLP3 ($_{17}$ILAIVVWTIVAIEFKKIL$_{34}$; with 6% recognition) and Vpu-OLP4

($_{25}$IVAIEFKKILRQRKIDRL$_{42}$; with 3% recognition). The mapping pattern of recognition was located in N-terminal region of Vpu protein.

5.7.8 Vpr OLP recognition the mapping patterns

Of 11 Vpr-OLPs, all OLPs (100%) were recognized at least one individual (Figure 27 left). The most three frequently recognized peptides were Vpr-OLP2 ($_{9}$GPQREPYNEWTLELLEEL$_{26}$) which was recognized in 9% of patients, Vpr-OLP4 ($_{25}$ELKNEAVRHFPRPWLHGL$_{42}$) which was recognized in 8% of patients and Vpr-OLP8 ($_{55}$EGVEAIIRILQQLLFVHF$_{72}$) which was recognized in 6% of patients. The mapping pattern of recognition was located in all regions of Vpr protein.

Figure 27. The frequency of Vpr and Vif specific CD8+ T cell responses in each OLP among 240 HIV-1 patients by IFN-γ ELISpot assay. Three common responses in Vpr were Vpr-OLP2, -OLP4 and -OLP8 (with arrow, left figure). Three common responses in Vif were Vif-OLP13, -OLP12 and -OLP3, 7, 9 (with arrow, right figure). The recognition mapping pattern was located in all regions of Vpr and Vif protein.



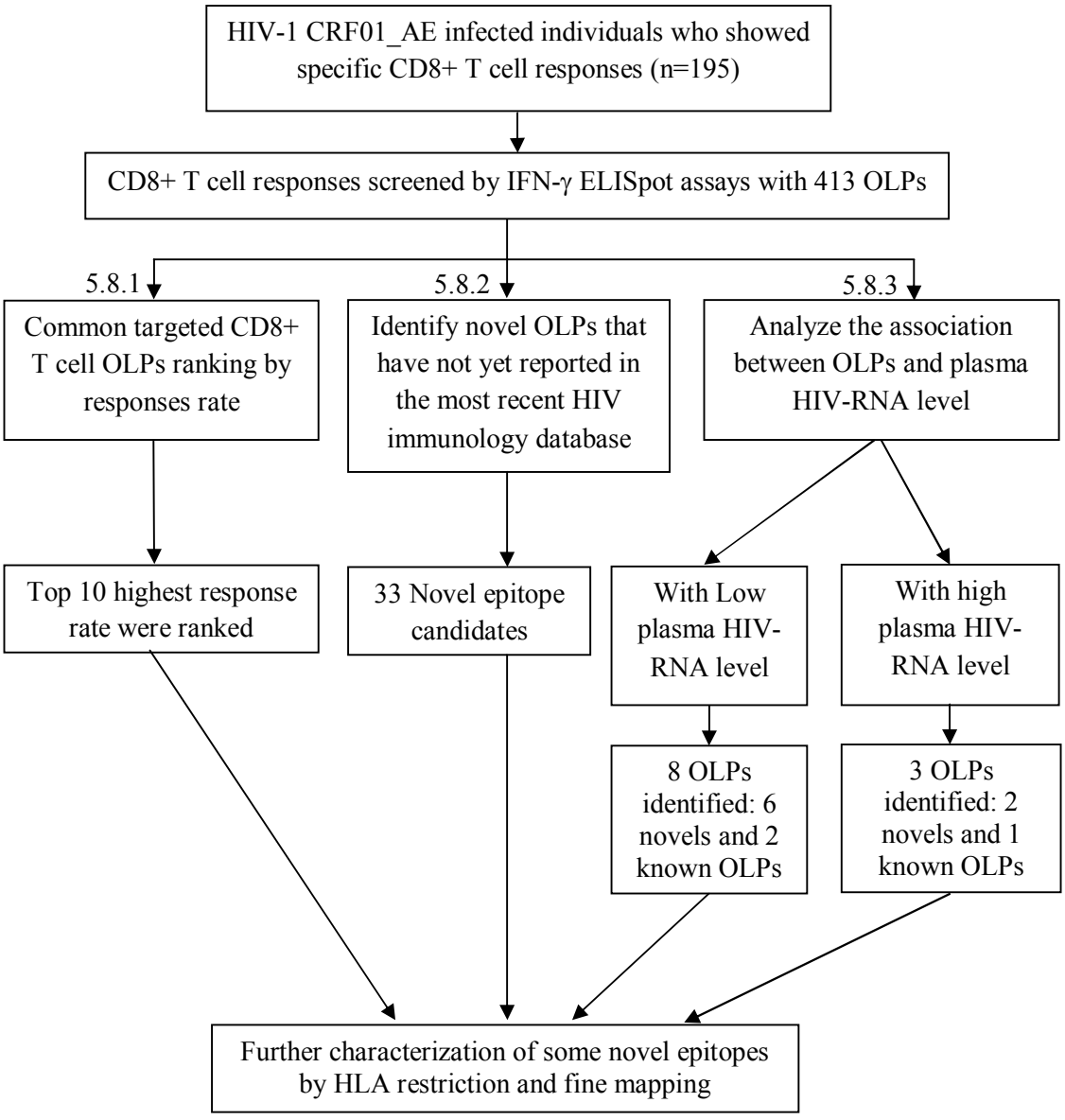
5.7.9 Vif OLP recognition the mapping patterns

Of 24 Vif-OLPs, 23 (95.8%) were recognized at least one individual (figure 27 right). The most three frequently recognized peptides were Vif-OLP13 ($_{93}$NYSTQIDPDLADQLIHLQ$_{110}$) which was recognized in 7% of patients, Vif-OLP12 ($_{85}$VSIEWRQRNYSTQIDPDL$_{102}$; with 6% recognition) and Vif-OLP4,7,10 ($_{25}$VKHHMYISKKAKKWFYRH$_{42}$, $_{48}$HPKVSSEVHIPLGEARLV$_{65}$ and $_{72}$LQTGEKDWQLGHGVSIEW$_{89}$, respectively), which were recognized by 5% of patients. The mapping pattern of recognition was located in all regions of Vif protein.

5.8 Potential novel CD8+ T cells epitope and the association with either viral escape or viral control: Analyses only patients who were infected with HIV-1 CRF01_AE

Of 240 patients, 195 were proven infected with CRF01_AE and had shown specific CD8+ T cell responses. Figure 28 shows the consorted flow chart of how novel candidate epitopes have been identified and characterized.

Figure 28. Consorted flow chart of how novel epitopes have been identified analyzed and characterized in this study.



5.8.1 Characterization of the most common targeted CD8+ T cell OLPs in the CRF01_AE infected cohort

To characterize the common targeted CD8+ T cell epitopes, we screened 195 HIV-1 CRF01_AE infected Thai individuals by IFN-γ ELISpot assay with a set of 413 OLPs. The results were then ranked by the IFN-γ ELISpot responding rate. The top 10 commonly targeted were 4 in Nef (Nef-OLP79, -OLP77, -OLP78 and -OLP82), 3 in Gag (Gag-OLP40, -OLP41 and -OLP38), 2 in Pol (Pol-OLP211 and -OLP187) and one in Tat (Tat-OLP115). The response rate of the top three targets (Nef-OLP79, -OLP77 and -OLP78) was 50.26%, 42.05% and 37.44%, respectively. As shown in Table 5, the average ELISpot response to these OLPs was 1,323, 1,132 and 1,120 SFU/10^6 PBMCs, respectively.

Table 5. The 10 most common targets of CD8+ T cells ranked by the IFN-γ ELISpot responding rate

Rank	Proteins	Peptide sequences	No. of responders	% responses	Range of responses (SFU/10^6 PBMCs)	Average responses (SFU/10^6 PBMCs)
1	NEF-AE-12	YKGAFDLSFFLKEKGGL	98	50.26	230 - 3,920	1,323
2	NEF-AE-10	EVGFPVRPQVPLRPMTYK	82	42.05	210 - 2,900	1,132
3	NEF-AE-11	QVPLRPMTYKGAFDLSFF	73	37.44	220 - 3,480	1,120
4	POL-AE-67	GQDQWTYQIYQEPFKNLK	65	33.33	270 - 3,860	1,100
5	GAG-AE-40	GPKEPFRDYVDRFYKTLR	58	29.74	250 - 4,240	1,381
6	POL-AE-43	QGWKGSPAIFQSSMTKIL	58	29.74	230 - 2,760	942
7	GAG-AE-38	IVRMYSPVSILDIRQGPK	57	29.23	230 - 4,550	1,746
8	GAG-AE-41	YVDRFYKTLRAEQATQEV	52	26.67	220 - 3,900	1,203
9	TAT-AE-5	WHCQLCFLKKGLGISYGR	52	26.67	380 - 3,760	1,331
10	NEF-AE-15	KKRQEILDLWVYNTQGFF	48	24.62	260 - 3,050	1,430

5.8.2 Identification of the novel targeted CD8+ T cell OLPs in the CRF01_AE infected cohort

Identification of the novel OLPs: the HIV immunology database analytical program (http://www.hiv.lanl.gov) was used to define probably epitopes within their OLPs based on HLA anchor motifs. We found 33 candidate novel OLPs; 5 of Gag, 3 of Pol, 16 of Env, 1 of Nef, 3 of Rev, 1 of Tat, 1 of Vif, 1 of Vpr and 2 of Vpu (Table 6).

Table 6. Thirty-three novel OLPs based on anchor motifs by HIV immunology database and ranked by the IFN-γ ELISpot responding rates.

No.	Proteins	Sequence	Responder (n)	% responses
1	Gag-OLP38 (p24)	IVRMYSPVSILDIRQGPK	57	29.23
2	Env-OLP75 (gp41)	IVQQQSNLLRAIEAQQHL	46	23.59
3	Rev-OLP2	DEELLRAVRIIKILY	45	23.08
4	Env-OLP76 (gp41)	LRAIEAQQHLLQLTVWGI	41	21.03
5	Nef-OLP14	GLDGLIYSKKRQEILDLW	28	14.36
6	Env-OLP30 (gp120)	IHYCTPAGYAILKCNDK	27	13.85
7	Env-OLP7 (gp120)	VWRDADTTLFCASDAKAH	24	12.31
8	Pol-OLP16 (protease)	KVRQYDQILIEICGKKAI	23	11.79
9	Vpu-OLP1	MTPLEISAIVGLIVALIL	20	10.26
10	Gag-OLP12 (p17)	SLFNTVATLWCVHQRIEV	18	9.23
11	Env-OLP71 (gp41)	AMIFGFLGAAGSTMGAA	17	8.72
12	Env-OLP113 (gp41)	WRAILHIPRRIRQGLERA	17	8.72
13	Env-OLP51 (gp120)	GDLEITMHHFNCRGEFFY	16	8.21
14	Env-OLP67 (gp120)	VVQIEPLGIAPTRAKRRV	16	8.21
15	Env-OLP70 (gp41)	RAVGIGAMIFGFLGAA	16	8.21
16	Vif-OLP13	NYSTQIDPDLADQLIHLQ	15	7.69
17	Gag-OLP43 (p24)	EVKNWMTETLLVQNA	14	7.18
18	Env-OLP69 (gp41)	RVVEREKRAVGIGAMIF	14	7.18
19	Vpu-OLP3	ILAIVVWTIVAIEFKKIL	14	7.18
20	Rev-OLP7	RRRRWRARQRQIRAISER	12	6.15
21	Env-OLP13 (gp120)	NVTENFNMWKNNMVEQMQ	11	5.64
22	Env-OLP14 (gp120)	WKNNMVEQMQEDVISLW	11	5.64
23	Rev-OLP1	MAGRSGSTDEELLRAVRI	6	3.08
24	Vpr-OLP4	ELKNEAVRHFPRPWLHGL	6	3.08
25	Gag-OLP52 (p15)	QRGNFKGQKRIKCF	5	2.56
26	Env-OLP66 (gp120)	ELYKYKVVQIEPLGIA	5	2.56
27	Pol-OLP125 (IN)	TKELQKQITKIQNFRVYY	5	2.56
28	Pol-OLP33 (RT)	ELNKRTQDFWEVQLGIPH	4	2.05
29	Pol-OLP31 (RT)	IKKKDSTKWRKLVDFREL	3	1.54
30	Env-OLP23 (gp120)	NMTTELRDKKQKVHALFY	3	1.54
31	Gag-OLP53 (p15)	KGQKRIKCFNCGKEGHL	3	1.54
32	Env-OLP60 (gp120)	SGRINCVSNITGILLTR	3	1.54
33	Tat-OLP9	SKDHQNPIPEQPLPIIR	1	0.51

5.8.3 Identification of targeted CD8+ T cell OLPs associated with low or high plasma HIV-RNA level in 195 HIV-1 CRF01_AE infected cohort

To identify which CD8+ T cell OLPs were associated with low or high plasma HIV-RNA level, we analyzed the correlation between CD8+ T cell recognized OLPs and plasma HIV-RNA level. We found 8 OLPs were associated with low plasma HIV-RNA level and 3 OLPs were associated with high plasma HIV-RNA level significantly ($p < 0.05$). After that, we defined the novel OLPs by using HIV immunology database. Among 8 OLPs associated with low plasma HIV-RNA level; 6 were novel OLPs and 2 were known reported OLPs. However, 3 OLPs were associated with high plasma HIV-RNA level; 2 were novel OLPs and one was known reported OLP (Table 7). The plasma HIV-RNA level was demonstrated in Table 8a and 8b.

Table 7. The CD8+ T cell OLPs associated with low or high plasma HIV-RNA level in 195 HIV-1 CRF01_AE infected cohort were ranked by the IFN-γ ELISpot responding rate ($p < 0.05$)

No.	Proteins	Sequence	Responder	% responses	Associated with plasma HIV-RNA level	
			(n)		Low	high
1	Env-OLP76 (gp41)	LRAIEAQQHLLQLTVWGI	41	21.03		yes
*2	Gag-OLP48 (p24)	ACQGVGGPSHKARVLAEA	28	14.36	yes	
3	Pol-OLP16 (protease)	KVRQYDQILIEICGKKAI	23	11.79	yes	
4	Vpu-OLP1	MTPLEISAIVGLIVALIL	20	10.26		yes
5	Gag-OLP12 (p17)	SLFNTVATLWCVHQRIEV	18	9.23	yes	
6	Gag-OLP43 (p24)	EVKNWMTETLLVQNA	14	7.18	yes	
*7	Env-OLP41 (gp120)	NCTRPSNNTRTSITI	6	3.08		yes
8	Gag-OLP52 (p15)	QRGNFKGQKRIKCF	5	2.56	yes	
9	Pol-OLP125 (integrase)	TKELQKQITKIQNFRVYY	5	2.56	yes	
10	Gag-OLP53 (p15)	KGQKRIKCFNCGKEGHL	3	1.54	yes	
11	Env-OLP60 (gp120)	SGRINCVSNITGILLTR	3	1.54	yes	

* Known reported OLPs

Table 8a. OLPs that showed a significant associated with low plasma HIV-RNA level (n = 195 of HIV-1 CRF01_AE infected patients)

No.	Proteins	Sequence	Responder (n)	Median VL (copies/ml)	Non responder (n)	Median VL (copies/ml)	*p* value
1	Gag-OLP48 (p24)	ACQGVGGPSHKARVLAEA	28	9,183	167	27,172	0.0004
2	Pol-OLP16 (protease)	KVRQYDQILIEICGKKAI	23	7,800	172	24,139.5	0.0155
3	Gag-OLP12 (p17)	SLFNTVATLWCVHQRIEV	18	12,536.5	177	23,570	0.0112
4	Gag-OLP43 (p24)	EVKNWMTETLLVQNA	14	5,673	181	23,289	0.0447
5	Gag-OLP52 (p15)	QRGNFKGQKRIKCF	5	1,310	190	22,130.5	0.0101
6	Pol-OLP125 (integrase)	TKELQKQITKIQNFRVYY	5	2,904	190	22,130.5	0.0148
7	Gag-OLP53 (p15)	KGQKRIKCFNCGKEGHL	3	918	192	22,028.5	0.0077
8	Env-OLP60 (gp120)	SGRINCVSNITGILLTR	3	391	192	314	0.0185

Table 8b. OLPs that showed a significant associated with high plasma HIV-RNA level (n = 195 of HIV-1 CRF01_AE infected patients)

No.	Proteins	Sequence	Responder (n)	Median VL (copies/ml)	Non responder (n)	Median VL (copies/ml)	*p* value
1	Env-OLP76 (gp41)	LRAIEAQQHLLQLTVWGI	41	36,643	154	20,450.5	0.0290
2	Vpu-OLP1	MTPLEISAIVGLIVALIL	20	37,801	175	20,434	0.0143
3	Env-OLP41 (gp120)	NCTRPSNNTRTSITI	6	178,294.5	189	21,025	0.0052

5.9 Potential novel CD8+ T cell epitopes

A total of 33 candidates that are likely to be novel epitopes were identified and summarized in the Table 9.

Table 9. Potential novel OLPs with or without low or high plasma HIV-RNA level association were ranked by the IFN-γ ELISpot responding rate in 195 HIV-1 CRF01_AE infected patients

No	Proteins	Sequence	Responder (n)	% responses	Average responses (SFU/10^6 PBMCs)	Associated with plasma HIV-RNA level	
						low	high
1	Gag-OLP38 (p24)	IVRMYSPVSILDIRQGPK	57	29.23	1,746		
2	Env-OLP75 (gp41)	IVQQQSNLLRAIEAQQHL	46	23.59	1,345		
3	Rev-OLP2	DEELLRAVRIIKILY	45	23.08	1,231		
4	Env-OLP76 (gp41)	LRAIEAQQHLLQLTVWGI	41	21.03	1,261		yes
5	Nef-OLP14	GLDGLIYSKKRQEILDLW	28	14.36	1,201		
6	Env-OLP30 (gp120)	IHYCTPAGYAILKCNDK	27	13.85	1,379		
7	Env-OLP7 (gp120)	VWRDADTTLFCASDAKAH	24	12.31	987		
8	Pol-OLP16 (protease)	KVRQYDQILIEICGKKAI	23	11.79	894	yes	
9	Vpu-OLP1	MTPLEISAIVGLIVALIL	20	10.26	1,079		yes
10	Gag-OLP12 (p17)	SLFNTVATLWCVHQRIEV	18	9.23	996	yes	
11	Env-OLP71 (gp41)	AMIFGFLGAAGSTMGAA	17	8.72	1,532		
12	Env-OLP113 (gp41)	WRAILHIPRRIRQGLERA	17	8.72	1,256		
13	Env-OLP51 (gp120)	GDLEITMHHFNCRGEFFY	16	8.21	1,561		
14	Env-OLP67 (gp120)	VVQIEPLGIAPTRAKRRV	16	8.21	839		
15	Env-OLP70 (gp41)	RAVGIGAMIFGFLGAA	16	8.21	982		
16	Vif-OLP13	NYSTQIDPDLADQLIHLQ	15	7.69	827		
17	Gag-OLP43 (p24)	EVKNWMTETLLVQNA	14	7.18	1,057	yes	
18	Env-OLP69 (gp41)	RVVEREKRAVGIGAMIF	14	7.18	860		
19	Vpu-OLP3	ILAIVVWTIVAIEFKKIL	14	7.18	659		
20	Rev-OLP7	RRRRWRARQRQIRAISER	12	6.15	754		
21	Env-OLP13 (gp120)	NVTENFNMWKNNMVEQMQ	11	5.64	1,770		
22	Env-OLP14 (gp120)	WKNNMVEQMQEDVISLW	11	5.64	1,094		
23	Rev-OLP1	MAGRSGSTDEELLRAVRI	6	3.08	1,270		
24	Vpr-OLP4	ELKNEAVRHFPRPWLHGL	6	3.08	663		
25	Gag-OLP52 (p15)	QRGNFKGQKRIKCF	5	2.56	534	yes	

No	Proteins	Sequence	Responder (n)	% responses	Average responses (SFU/10^6 PBMCs)	associated with plasma HIV-RNA level	
						low	high
26	Env-OLP66 (gp120)	ELYKYKVVQIEPLGIA	5	2.56	1,148		
27	Pol-OLP125 (IN)	TKELQKQITKIQNFRVYY	5	2.56	814	yes	
28	Pol-OLP33 (RT)	ELNKRTQDFWEVQLGIPH	4	2.05	1,328		
29	Pol-OLP31 (RT)	IKKKDSTKWRKLVDFREL	3	1.54	950		
30	Env-OLP23 (gp120)	NMTTELRDKKQKVHALFY	3	1.54	1,040		
31	Gag-OLP53 (p15)	KGQKRIKCFNCGKEGHL	3	1.54	337	yes	
32	Env-OLP60 (gp120)	SGRINCVSNITGILLTR	3	1.54	1,293	yes	
33	Tat-OLP9	SKDHQNPIPEQPLPIIR	1	0.51	440		

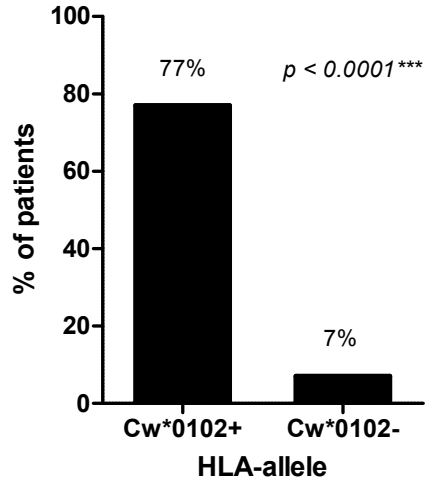
5.9.1 Identification and characterization of an immunodominant novel epitope in p24 region

As shown in Table 9, Gag-OLP38; $_{273}$IVRMYSPVSILDIRQGPK$_{290}$ was the most common novel OLP that recognized by up to a third of the subjects (the 7th most frequently targeted OLP) and with an average 1,746 SFU/10^6 PBMCs among the responders.

This OLP is located in p24 region which included a number of known epitopes restricted by various HLA alleles including -A*02, -B*52, and –Cw*18 (http://www.hiv.lanl.gov/content/immunology). However, none of these alleles were found in these responders. Interestingly, 39 of the 46 subjects (85%), who showed detectable IFN-γ CD8+ T cell responses against Gag-OLP38, expressed HLA-Cw*0102 ($p < 0.0001$).

In addition, of the 51 HLA-Cw*0102+ve subjects in this cohort, 39 (77%) had a detectable response against Gag-OLP38, ranging from 230 to 4,320 SFU/10^6 PBMCs (the mean of SFU/10^6 PBMCs in the responders was 1,650) (Figure 29). Thus it was the most frequently targeted peptide among the Cw*0102+ve subjects.

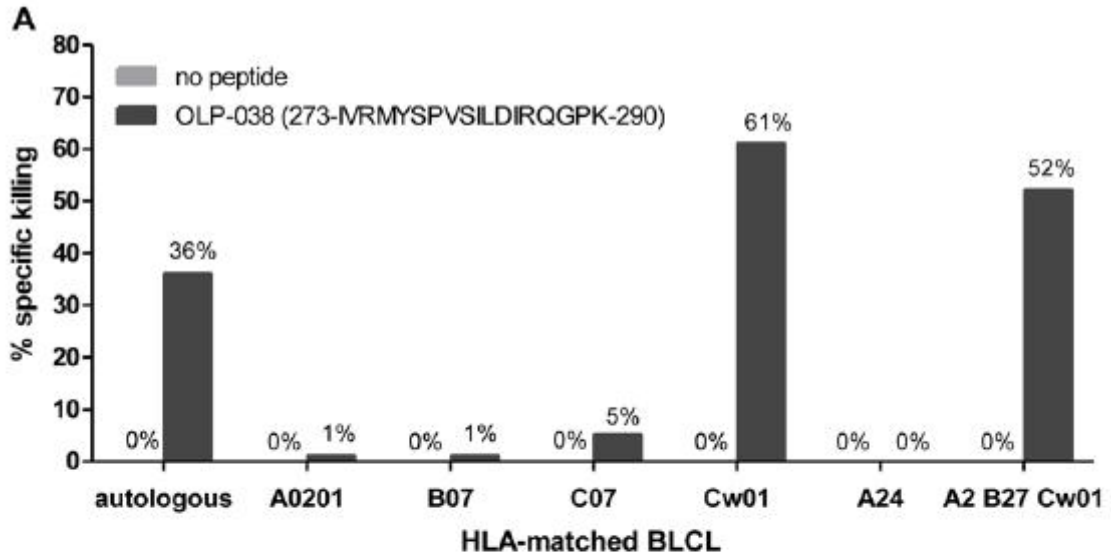
Figure 29. Predominant recognition of Gag-OLP038 in Cw*0102+ve subjects. The majority of Cw*0102+ve subjects (39/51; 77%) versus a minority of the Cw*0102-ve subjects (7/105; 7%) exhibited an ELISpot response against Gag-OLP38 ($p < 0.0001$).

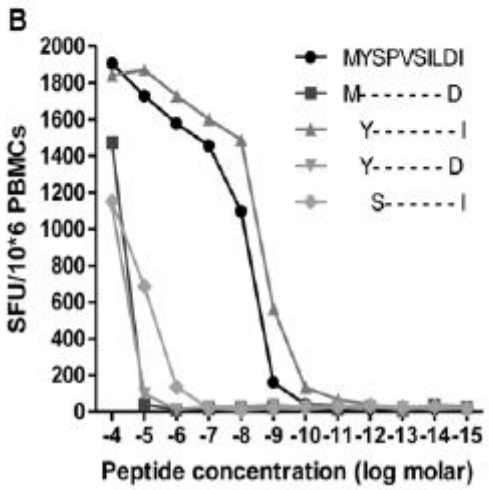


*5.9.2 Fine mapping of the novel Cw*0102-restricted CD8 epitope p24 $_{277}$YI9$_{285}$*

The HLA class I restriction results were shown in Figure 30a. In the ^{51}Cr-release assay, PBMC from the subject 0578 who carries HLA-A*0201, A*24; -B*7, -B*27; -Cw*01, Cw*07 had recognized Gag-OLP38. Only Cw*01 matched heterologous BLCLs containing experiments as antigen presenting cells revealed a high and comparable level of specific CTL killing as of the autologous BLCL results. This results had proven that Gag-OLP38 is presented and restricted to HLA-Cw*01 in this patient. Further, fine mapping of this epitope by using serial dilutions of truncated peptides in both IFN-γ ELISpot (Figure 30b) and ^{51}Cr-release assay were performed (Figure 30c). These assays determined the minimal optimal epitope. The YI9 or $_{277}$YSPVSILDI$_{285}$ is identified as a 9 amino acids optimal peptide. The depletion of CD4+ T cells and CD8+ T cells experiments had confirmed that this is a CD8 T cell epitope (Figure 31).

Figure 30. Identification of the novel Cw*0102-restricted CD8 epitope $_{277}$YI9$_{285}$ (a) autologous BLCL from subject 0578 (strongly responded to Gag-OLP38), and BLCL targets from 6 other individuals expressing one of HLA alleles found in subject 0578, were tested for their abilities to recognize peptide pulsed targets using a ^{51}Cr-release assay. (b) ELISpot and (c) ^{51}Cr-release assays were performed in identifying the 9 amino acids YI9 as the minimal optimal epitope.





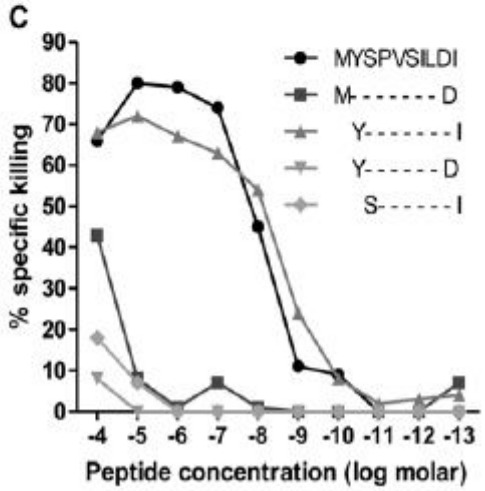
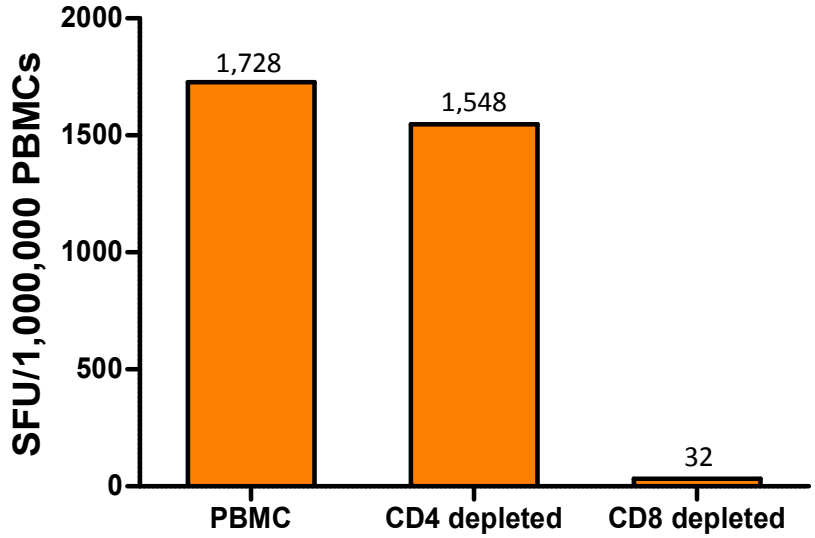


Figure 31. Confirmation of the novel Cw*0102-restricted $_{277}$YI9$_{285}$ epitope as a CD8 epitope by the CD4 or CD8 cell depletion and IFN-γ ELISpot experiments.



5.10 Novel Cw*0102-restricted CD8 epitope p24 $_{277}$YI9$_{285}$ and viral escape

To explore the patterns of CTL responses and its impact on viral escape, we analyzed their viral sequences of Gag $_{277}$YI9$_{285}$ region, their HIV-1 specific CD8+ T cell responses and their HLAs.

*5.10.1 Frequent viral escape from the Gag epitope in Cw*0102+ve with CRF01_AE infected subjects*

To determine whether YI9-restricted CD8+ T cell responses were capable of exerting immune selection pressure upon HIV-1, the sequence data of the Gag-region was generated from 156 subjects. As shown in Figure 32, irrespective of the expression of Cw*0102, 111/156 (71%) sequences from our cohort were almost conserved. Notably, in the absence of Cw*0102, the number of consensus sequences increased to 84/105 (80%).

As shown in Figure 32, a strong association between mutations in YI9 and the expression of Cw*0102 was observed. More specifically, in Cw*0102+ve subjects 24 of 51 (47%) versus only 21/105 (20%) in Cw*0102-ve subjects exhibited mutations in YI9 (p = 0.0007), with the S278X (p = 0.0059) and V280X (p = 0.0003) mutations predominating.

Figure 32. HLA-Cw*0102 associated footprints in the YI9 epitope. Gag sequences were generated from 156 subjects and sequence variation within the YI9 epitope compared between Cw*0102+ve and Cw*0102-ve subjects.

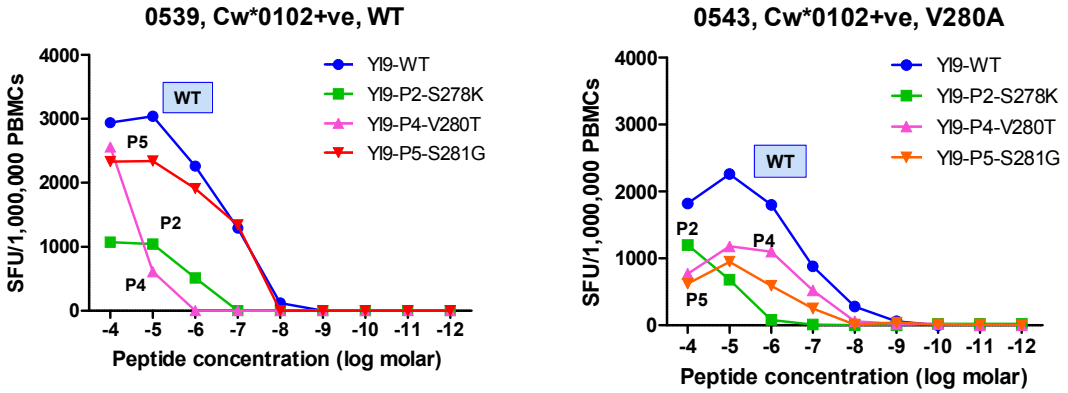
Cw*0102+ve (n = 51)												Cw*0102-ve (n = 105)											
Y	S	P	V	S	I	L	D	I		n	%	Y	S	P	V	S	I	L	D	I		n	%
.	.	.	.	.	.	.	.	.		27	52.9	.	.	.	.	.	.	.	.	.		84	80.0
.	.	.	T	.	.	.	.	.		9	17.6	.	.	.	.	G	.	.	.	.		15	14.3
.	.	.	.	G	.	.	.	.		6	11.8	.	.	.	T	.	.	.	.	.		3	2.9
.	K	.	.	.	.	.	.	.		4	7.8	.	.	.	I	.	.	.	.	.		1	1.0
.	.	.	I	.	.	.	.	.		1	2.0	.	C	.	.	.	.	.	.	.		1	1.0
.	R	.	.	.	.	.	.	.		1	2.0	.	R	.	.	.	.	.	.	.		1	1.0
.	.	.	A	.	.	.	.	.		1	2.0												
.	R	.	A	.	.	.	.	.		1	2.0												
.	K	.	.	G	.	.	.	.		1	2.0												

Position		Cw*0102+ve	Cw*0102-ve	*p*-value
	Total variation	47.06%	20.00%	**0.0007**
P2	**S278X**	13.73%	1.90%	**0.0059**
P4	**V280X**	23.59%	3.81%	**0.0003**
P5	**S281G**	13.73%	14.29%	1.0000

5.10.2 Immune escape to CD8+ T cell response of mutant peptides of YI9

To confirm the HLA-Cw*0102 associated with the mutation due to effect of viral escape, we conducted the ELISpot assay by using the serial dilutions of wild type (WT) and variant peptides of YI9. The variant peptides were consisted of P2 mutation S278K, P4 mutation V280T, and P5 mutation S281G. The patient with WT (0539) and the patient with V280A mutation (0543) were tested. The results showed the loss of recognition for both of the epitope with S278K and V280T mutation (Figure 33). Moreover, the V280T mutation resulted to a loss of CD8 T cell recognition greater than V280A.

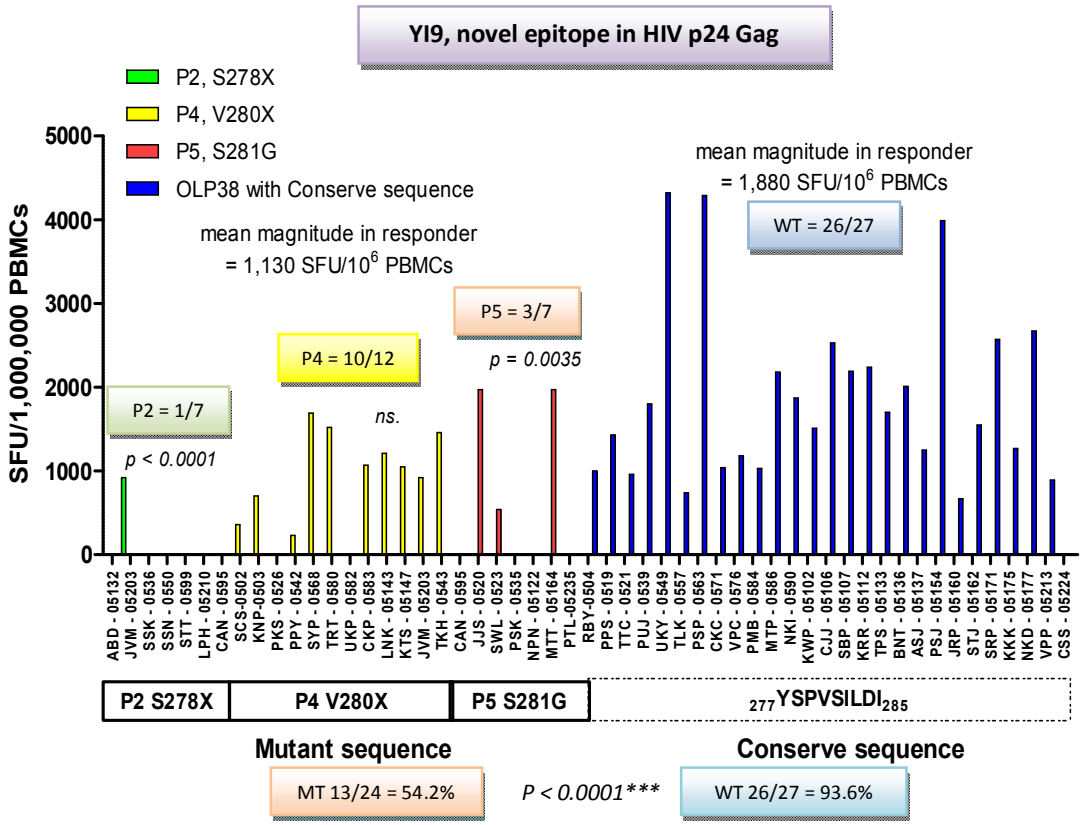
Figure 33. HLA-Cw*0102-associated mutations in YI9 impair CD8+ T cell recognition. P2 mutation S278K, P4 mutation V280T and P5 mutation S281G of HLA-Cw*0102-restricted YI9 CD8 epitope resulted to an immune escape. IFN-γ ELISpot assay were performed in these experiments.



*5.10.3 CD8+ T cell responses against wild type and mutants YI9 in Cw*0102+ve subjects*

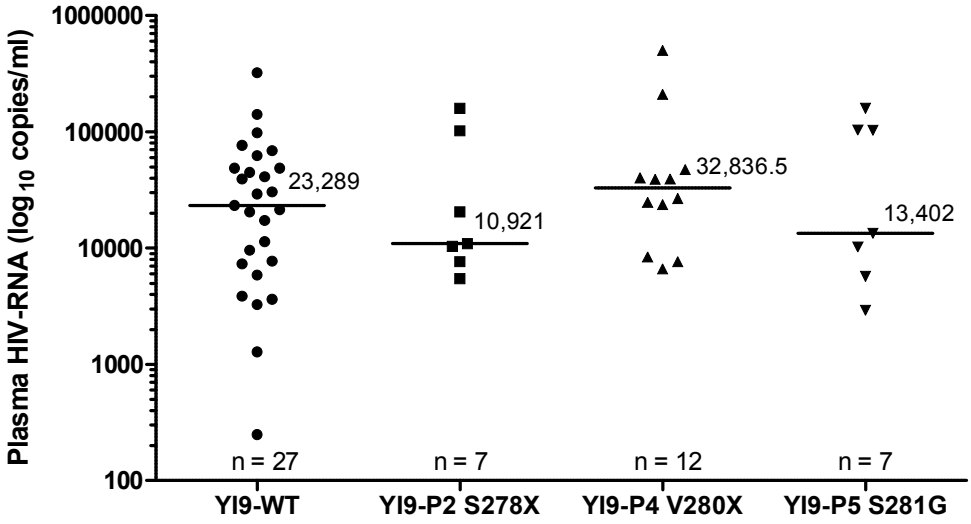
As shown in Figure 34, a comparison of the CD8+ T cell responses between patients with HLA-Cw*0102+ve with conserve and mutant at YI9 was analyzed. Most patients with HLA-Cw*0102+ve with a wild type sequence at YI9 (26 of 27 or 96.3%) showed CD8 T cell responses against YI9 containing Gag-OLP38 and with an average ELISpot response of 1,880 SFU/10^6 PBMCs among the responders (Figure 34). In contrast, among HLA-Cw*0102+ve patients who carried mutant sequence at YI9 only 13 of 24 (54.2%) had responses against this OLP and with a lower average magnitude of 1,130 SFU/10^6 PBMCs ($p < 0.0001$). More specifically only 1 of 7 (14%; $p < 0.0001$), and 3 of 7 (42%; $p = 0.0035$) in patients who carried S278X and S281X showed CD8 T cell ELISpot responses, respectively.

Figure 34. Magnitude of ELISpot response against YI9 containing Gag-OLP38 in the patients with HLA-Cw*0102 with conserve or mutant sequence at YI9 (N=51)



While mutations occurred at positions P2, P4 and P5, the most frequent mutation was a single amino acid substitution at position 4 i.e., V280X (23.5%; predominantly Threonine (T), 17.6%). Mutations at P4 reduced the response rate to 83.3% and diminished the magnitude of the ELISpot responses to an average of 1,021 SFU/10^6 PBMCs in responders (p = 0.0071). The amino acid mutation at P5, S281G, reduced the ELISpot response rate to 42.9%, with an average magnitude of the ELISpot response of 1,493 SFU/10^6 PBMCs in responders (p = 0.0150). More dramatically, mutations at P2, which occurred in six cases, appeared to completely impair ELISpot responses (p = 0.0002). However, there was no significantly different between each group in plasma HIV-RNA level (Figure 35).

Figure 35. The correlation between plasma HIV-RNA level in each group of WT and mutant YI9 responses in Cw*0102 (N = 51). The bar represents the median of plasma HIV-RNA level.



5.11 Prediction of HLA restricted OLPs that associated with low plasma HIV-RNA levels

To find the HLA restriction of OLPs associated with low plasma HIV-RNA level, we used the program from HIV database called as "Hopeful epitopes or Hepitopes". After put a set of HLA alleles and a set of patients who response with that peptide, the results exhibited possible sequential of the HLA restriction. The first top three hepitope predictions with $p < 0.05$ were compared with known reported HLA alleles from HIV database http://www.hiv.lanl.gov/content/immunology/maps/maps.html (Table 10). Among 8 OLPs, 6 OLPs were probable novel epitopes that have an association with low plasma HIV-RNA level and 2 OLPs (Gag-OLP48 and Pol-OLP125) were known reported epitopes association with low plasma HIV-RNA level.

Table 10. The first top three hepitope predictions with p value < 0.05 were compared with known reported HLA alleles from HIV database http://www.hiv.lanl.gov/content/immunology/maps/maps.html.

No	Proteins	Sequence	% responses	HLA restriction reported in HIV database	First top three Hepitope prediction (p value < 0.05)		
					1st HLA	2nd HLA	3rd HLA
1	Gag-OLP48 (p24)	ACQGVGGPSHKARVLAEA	14.36	A*11	A*1101	NS	NS
2	Pol-OLP16 (protease)	KVRQYDQILIEICGKKAI	11.79	B*13, Cw*0401	A*0203	B*3909	C*0702
3	Gag-OLP12 (p17)	SLFNTVATLWCVHQRIEV	9.23	B*58	B*5801	A*3303	C*0302
4	Gag-OLP43 (p24)	EVKNWMTETLLVQNA	7.18	B*53	B*5201	B*4006	B*4001
5	Pol-OLP125 (integrase)	TKELQKQITKIQNFRVYY	2.56	A*30, B*1503	B*1302	A*3001	B*1535
6	Gag-OLP52 (p15)	QRGNFKGQKRIKCF	2.56	NA	B*4801	B*1511	B*2705
7	Gag-OLP53 (p15)	KGQKRIKCFNCGKEGHL	1.54	A*11	B*1511	C*0702	B*4801
8	Env-OLP60 (gp120)	SGRINCVSNITGILLTR	1.54	B*56	C*0501	B*1535	B*4002

NS = no significant

5.12 Potential novel CD8+ T cell epitopes associated with low plasma HIV-RNA level in CRF01_AE infected subjects

Two OLPs; Gag-OLP48 and Pol-OLP16 were characterized in this cohort, because both have the highest responses rate that associated with low HIV-RNA level. Gag-OLP48 was a known reported epitope whereas Pol-OLP16 was a novel epitope. HLA restriction and fine mapping of the optimal epitope by IFN-γ ELISpot assay was performed.

5.13 Characterization of AK11 in Gag-OLP48 found associated with low plasma HIV-RNA levels

Gag-OLP48 was showed significantly associated with low HIV-RNA level (p = 0.0004) and was recognized by 34 (14.2%) subjects (the 28th frequently targeted OLP) with an average 1,191.5 SFU/10^6 PBMCs in responders. This OLPs is located in p24 region which includes epitopes restricted by various HLA alleles including -A*11, -B*07, and –B*35 (http://www.hiv.lanl.gov/content/immunology). This OLP sequence; $_{349}$ACQGVGGP**S**HKARVLAEA$_{366}$, has only one amino acid different from Gag clade B; ACQGVGGP**G**HKARVLAEA. Thus, the previously reported epitope AK11 of clade B is $_{349}$ACQGVGGP**G**HK$_{359}$ whereas clade CRF01_AE $_{349}$ACQGVGGP**S**HK$_{359}$. This epitope is restricted by HLA-A*11 based on the HIV database.

In this study, 85.7% (24/28) of patients responded with Gag-OLP48 expressed HLA-A*1101 ($p < 0.0001$). On the other hand, of the 95 HLA-A*1101+ve subjects in our cohort, 25.3% (24/95) had a detectable response against Gag-OLP48, ranging from 290 to 2,840 SFU/10^6 PBMCs (the mean of SFU/10^6 PBMCs in the responders was 1,168) (Figure 36) making it the most frequently targeted peptide in the HLA-A*1101+ve subjects. In contrast, there was only 4% recognition (4 subjects) in the HLA-A*1101-ve subjects with an average of 1,125 SFU/10^6 PBMCs. However, there were significantly different between both groups in plasma HIV-RNA level (p = 0.0009) as shown in Figure 37.

Figure 36. Predominant recognition of Gag-OLP48 in HLA-A*1101+ve subjects. ELISpot responses in patients with and without HLA-A*1101 were tested.

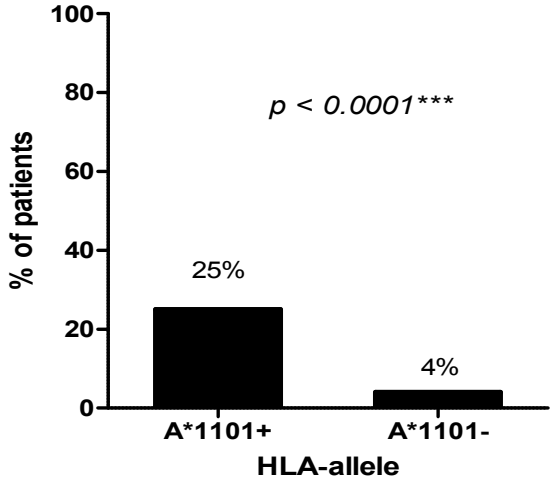
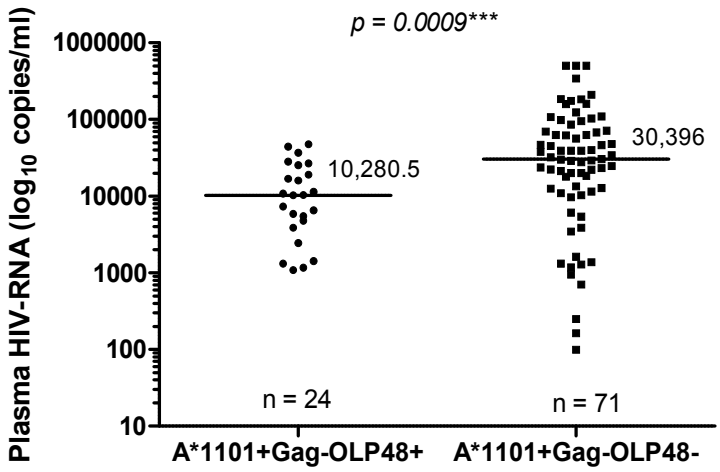


Figure 37. The correlation between plasma HIV-RNA level in both groups of Gag-OLP48 responses in A*1101 (N = 95). The bar represents the median of plasma HIV-RNA level.



*5.13.1 CD8+ T cell immune selective pressure on the Gag AK11 epitope in A*1101+ve subjects*

To determine whether AK11-restricted CD8+ T cell responses were capable of exerting immune selection pressure upon HIV-1, sequence data of the Gag-region was generated from all subjects. As shown in Figure 38, irrespective of the expression of A*1101, 186/189 (98%) sequences from this cohort was conserved.

Remarkably, in HLA-A*1101+ve or HLA-A*1101-ve subjects, the number of consensus sequences (97.9% vs. 98.9%) and mutation in AK11 (2.1% vs. 1.1%) were observed without any different. Particularly, patients with Gag-OLP48 responses with and without HLA-A*1101 showed the similar conserve sequence. In addition, there were not different in an average ELISpot responses in both A*1101+ve or A*1101-ve subjects (1,168 vs. 1,125 SFU/10^6 PBMCs) in responder. All of subject with expressed mutation AK11 was a single amino acid substitution at position 9, mutation S357G. No significant difference in plasma HIV-RNA level could be found between the different groups.

Figure 38. HLA-A*1101 association in amino acid sequences of the AK11 epitope. Gag sequences were generated from 189 subjects and sequence variation within the AK11 epitope compared between A*1101+ve and A*1101-ve subjects. Rare and not significant mutations in AK11 were observed.

A*1101+ve (n = 95)				A*1101-ve (n = 94)			
A C Q G V G G P S H K	n	%		A C Q G V G G P S H K	n	%	
. . . . .	93	97.9		. . . . .	93	98.9	
. . . . . G . .	2	2.1		. . . . . G . .	1	1.1	

Position		A*1101+ve	A*1101-ve	*p*-value
	Total variation	2.00%	1.06%	1.000
P9	S357G	2.00%	1.06%	1.000

*5.13.2 CD8+ T cell responses against wild type and mutants AK11 in A*1101+ve subjects*

As shown in Figure 39a, a comparison of the CD8+ T cell responses between patients with HLA-A*1101+ve with conserve and mutant at AK11 was analyzed. The patients with HLA-A*1101+ve with a wild type sequence at AK11 (24 of 93 or 25.8%) showed CD8 T cell responses against AK11 containing Gag-OLP48 and with an average ELISpot response of 1,168 SFU/10^6 PBMCs among the responders (Figure 39). In contrast, only two HLA-A*1101+ve patients who carried mutant sequence at AK11 showed no responses against this OLP. Moreover, these patients (S357G mutation) showed lower plasma HIV-RNA levels than the patients who have WT sequence (Figure 40).

Figure 39. Magnitude of ELISpot response against AK11 containing Gag-OLP48 in the patients with HLA-A*1101 with conserve or mutant sequence at AK11 (N=95)

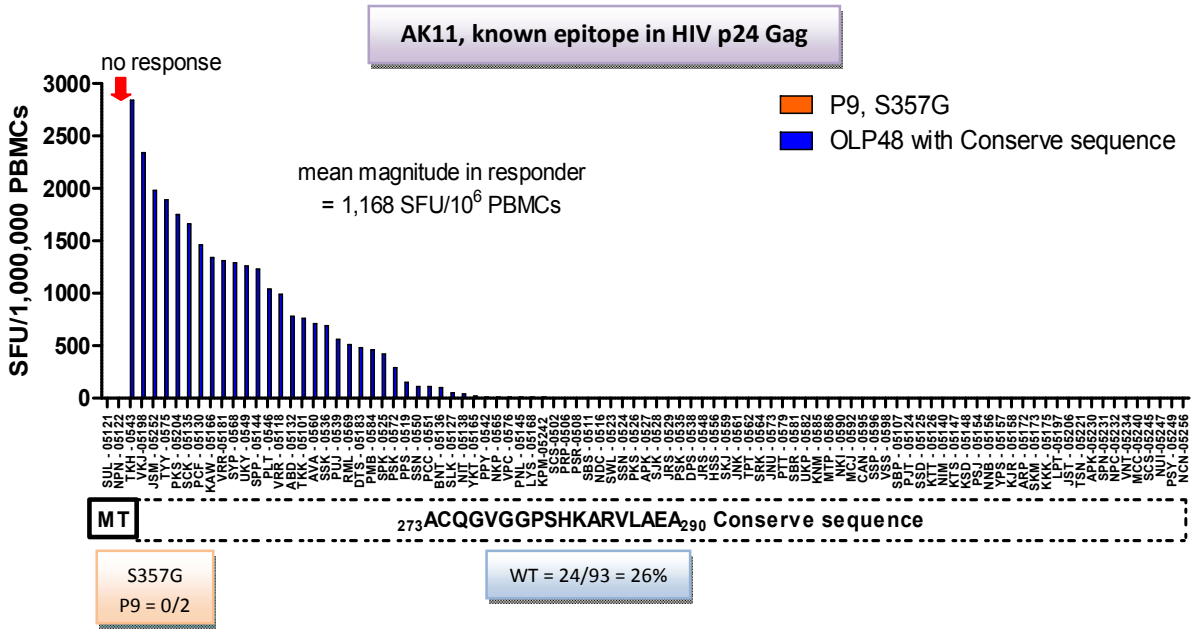
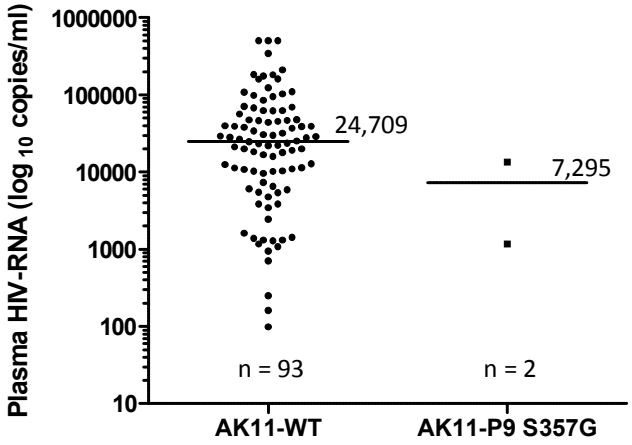


Figure 40. The correlation between plasma HIV-RNA level in both groups of WT and S357G mutation of Gag-OLP48 responses in A*1101 (N = 95). The bar represents the median of plasma HIV-RNA level.

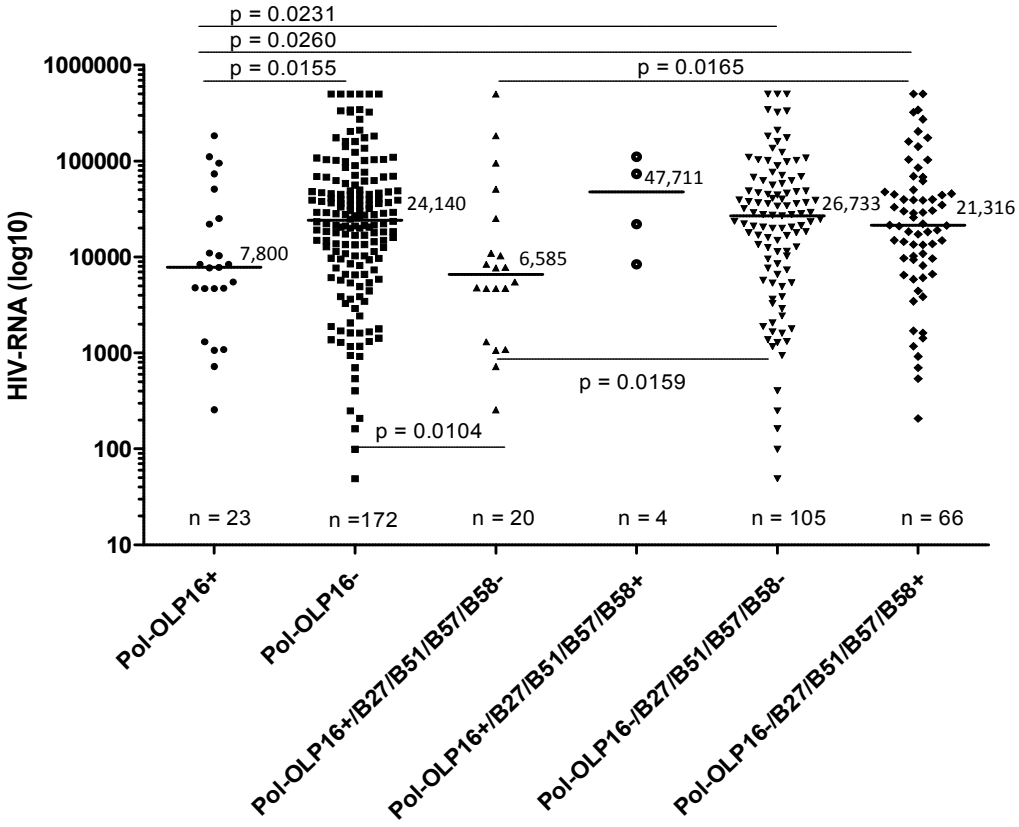


5.14 Characterization of Pol-OLP16: a novel epitope associated with low plasma HIV-RNA level

Pol-OLP16 was identified significantly associated with low HIV-RNA level (p = 0.0155) and was recognized by 11.8% of the subjects (the 40th frequently targeted OLP) with an average 894 SFU/10^6 PBMCs among the responders.

To investigate whether the Pol-OLP16 responses that associated with low plasma HIV-RNA level was independent from all currently known protective alleles, we further analyzed by excluding HLA-B*27, -B*51, -B*57 and -B*58 alleles and have shown the same significant result. However, of interest, patients with Pol-OLP16 responses but with no those protective alleles showed lower in median plasma HIV-RNA level than those without Pol-OLP16 together with or without protective alleles (p = 0.0165 and 0.0159, respectively) (Figure 41). This finding confirms that Pol-OLP16 responses were associated with low plasma HIV-RNA level.

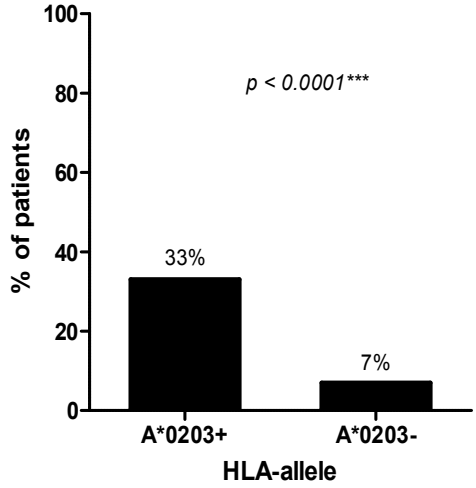
Figure 41. The patients with Pol-OLP16 responses with and without protective alleles were compared regarding the association with plasma HIV-RNA level (log_{10} copies/ml) among 195 HIV-1 infected individuals.



This OLP is located in Pol-protease region which based on the available database, it contains a number of epitopes restricted by various HLA alleles including -B*13, and -Cw*0401 (http://www.hiv.lanl.gov/content/immunology). Surprisingly, only few responders carry those allele i.e., 2 of 23 (8%) have HLA-B*1301, 2 of 23 (8%) have HLA-B*1302, and one of 23 (4%) have HLA-Cw*0401. Interestingly, 12 of 23 (52%) of the responders expressed HLA-A*0203 ($p < 0.0001$). Thus, this is a novel HLA-restricted candidate epitope associated with viral control that warranted for further characterization.

When analyzed the frequency of response against this Pol-OLP16 among 12 who expressed and 11 who did not expressed HLA-A*0203+ve, 33% versus only 7% had a detectable ELISpot response against Pol-OLP16. The response was ranging from 210 to 3,050 with the mean of 924.4 SFU/10^6 PBMCs among the A*0203 responders (Figure 42).

Figure 42. Predominant recognition of Pol-OLP16 in HLA-A*0203+ve subjects. HLA-A*0203+ve subjects (12/36; 33%) exhibited a CD8+ T cell response against Pol-OLP16, versus a minority of HLA-A*0203-ve subjects (11/159; 7 percent) ($p < 0.0001$).

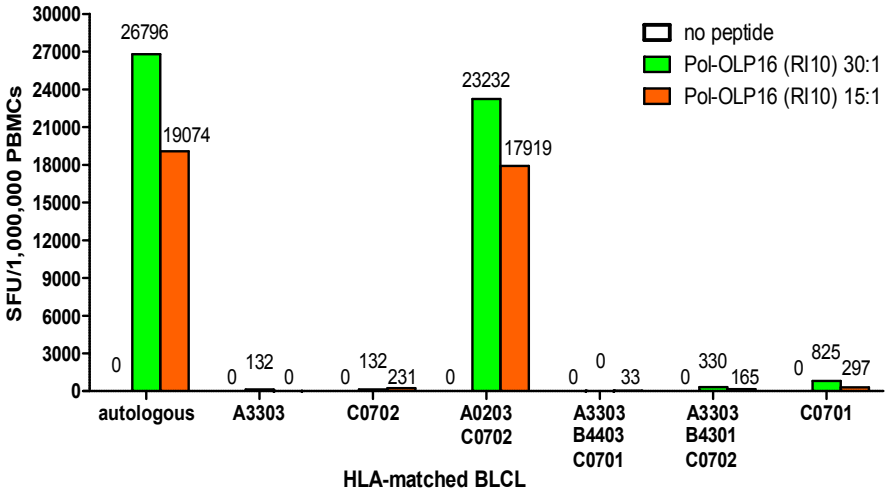


*5.14.1 Fine mapping of the novel A*0203-restricted CD8 epitope pol-protease $_{113}$RI10$_{122}$*

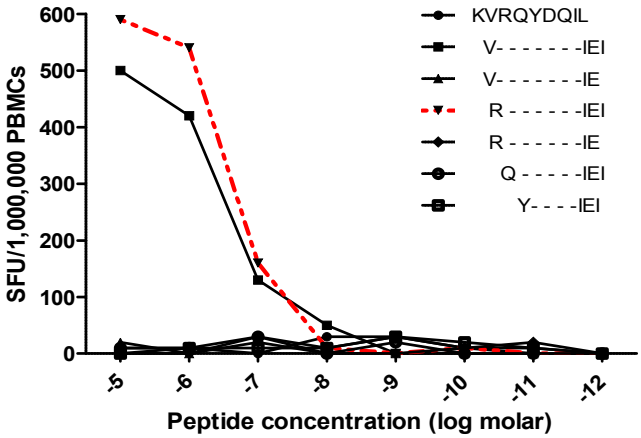
In ELISpot assay, PBMC from the subject 0522 who carries HLA-A*0203, -A*3303; -B*3801, -B*4403; -Cw*0701, -Cw*0702 had recognized Pol-OLP16 only when presented by BLCL from subjects expressing HLA-A*0203 (Figure 43a). This results had proven that Pol-OLP16 is presented and restricted to HLA-A*0203 in this patient. After that, fine mapping of this epitope by using serial dilutions of truncated peptide in IFN-γ ELISpot assay (Figure 43b) were performed. This assays established the minimal optimal epitope. The RI10 or $_{113}$RQYDQILIEI$_{122}$ is identified as a 10 amino acids optimal peptide.

Figure 43. Identification of the novel HLA-A*0203-restricted CD8 epitope $_{113}$RI10$_{122}$ (a) autologous BLCL from subject 0522 (strongly responded to Pol-OLP16) and BLCL targets from 6 other individuals expressing one of HLA alleles found in subject 0522, were tested for their ability to recognize peptide pulsed targets using a ELISpot assay. (b) ELISpot assay were performed in identifying the 10 amino acids RI10 as the minimal optimal epitope.

(43a)



(43b)



*5.14.2 Immune selective pressure on the novel Pol-epitope RI10 viral sequence in A*0203+ve subjects*

To determine whether RI10-restricted CD8+ T cell responses were capable of exerting immune selection pressure upon HIV-1, sequencing data of the Pol-region was generated from 64 subjects. As shown in Figure 44, irrespective of the expression of A*0203, 37/64 (58%) sequences from this cohort were conserved. Notably, in the absence of A*0203, the number of consensus sequences increased to 33/54 (61%).

As shown in Figure 44, a strong association between mutations in RI10 and the expression of HLA-A*0203 was observed. In addition, in HLA-A*0203+ve subjects 6 of 10 (60%) versus 21 of 54 (38.9%) in A*0203-ve subjects exhibited mutations in RI10 showed insignificantly (p = 0.2295).

Figure 44. HLA-A*0203 associated with the footprints in the RI10 epitope. Pol sequences were generated from 64 subjects and sequence variation within the RI10 epitope compared between A*0203+ve and A*0203-ve subjects. Mutations in RI10 were no significantly elevated between A*0203+ve and A*0203-ve subjects (p = 0.2995), and L119X is the mutations predominant in RI10 (4/10, 40%).

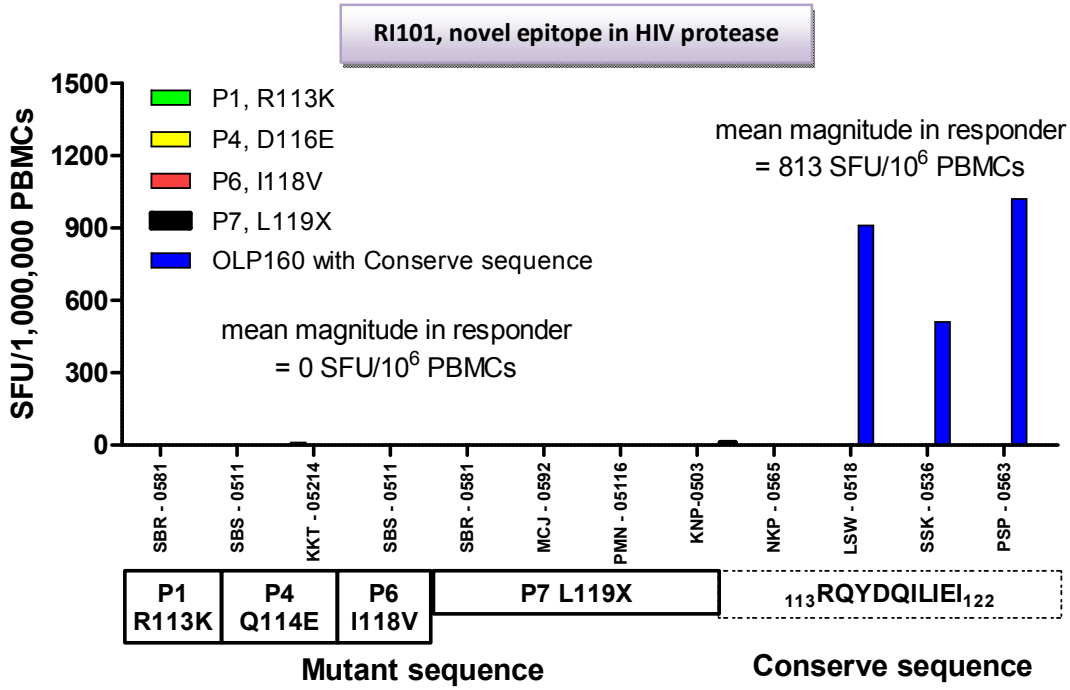
A*0203+ve (n= 10)			A*0203-ve (n = 54)		
R Q Y D Q I L I E I	n	%	R Q Y D Q I L I E I	n	%
. . . . .	4	40.0	. . . . .	33	61.1
. . . . . S . . .	2	20.0	K	5	9.3
K . . . . . S . . .	1	10.0	. . . . . P . . .	5	9.3
. . . . . P . . .	1	10.0	. . . E	2	3.7
. . . E	1	10.0	. . . . . S . . .	2	3.7
. . . E . V	1	10.0	K . . . . V P . . .	1	1.9
			. E	1	1.9
			. . . . . V	1	1.9
			. . . . . V H . . .	1	1.9
			. . . . . V S . . .	1	1.9
			. . . . . Q . . .	1	1.9
			. . . . . V . D .	1	1.9

Position		A*0203+ve	A*0203-ve	*p*- value
	Total variation	60.00%	38.90%	0.2995
P1	R113K	10.00%	11.10%	1.0000
P2	Q114E		1.90%	1.0000
P4	D116E	20.00%	3.70%	0.1119
P6	I118V	10.00%	7.40%	1.0000
P7	L119X	40.00%	22.20%	0.2517
P9	E121D		1.90%	1.0000

*5.14.3 CD8+ T cell responses against RI10 in A*0203+ve subjects*

The patients with HLA-A*0203+ve with a wild-type sequence at RI10 showed 3 of 4 (75%) responses against Pol-OLP16, with an average ELISpot response of 813 SFU/10^6 PBMCs in responders (Figure 45). In contrast, none of patients with HLA-A*0203+ve showed expressing mutations at RI10. (No significant, p = 0.0870). This is an interesting observation and it indicates that the although mutations in RI10 affected on immune escape but may also affect viral fitness to therefore did not affect overall viral control outcome.

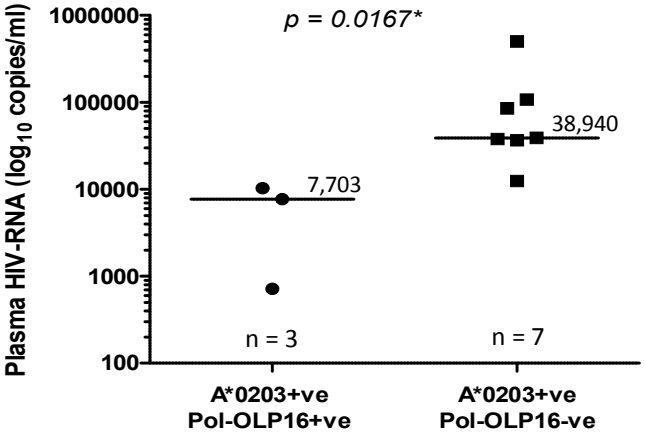
Figure 45. Magnitude of ELISpot response against RI10 in the patients with HLA-A*0203 with conserve and any mutant sequence at RI10 (N=10)



As shown in Figure 45, while 3 of 4 HLA-A*0203+ve subjects who carried RI10 wild-type virus showed the ELISpot responses to the Pol-OLP16, there was no responses among all of 6 who carried RI10 mutants.

Mutations occurred at all positions except P3, P5, P8 and P10. The most common mutation was a single amino acid substitution at position 7 which is L119X (40%; predominantly Proline, 44%). Interestingly, HLA-A*0203+ve/Pol-OLP16+ve subjects had lower median plasma HIV-RNA level than those who had HLA-A*0203+ve but not respond to Pol-OLP16 significantly (p = 0.0167) (Figure 46).

Figure 46. Comparison of the plasma HIV-RNA levels (log_{10} copies/ml) between HLA-A*0203 patients who responded and not responded to Pol-OLP16 (p = 0.0167). (Total number = 10 and the bar represent median values)



5.15 Characterization of Elite controllers in this cohort

5.15.1 *Clinical and demographic data*

In this study, we found four HIV-1 infected, 2 female and 2 male who had undetectable plasma HIV-RNA level (< 50 copies/ml). All were infected with HIV-1 clade CRF01_AE. Age ranged from 28 to 66 years old with the mean of 45 years old. Median duration after first HIV-1 infection diagnosis was 9.5 years (range 5 to 14 years). All were heterosexuals. Median CD4+ T cell counts 776 cells/cu.mm. (range 448 to 1,292 cells/cu.mm.) (Table 11).

Table 11. Demographic and Clinical data of 4 elite controllers (had undetectable plasma HIV-RNA level and high CD4 T cell count)

Elite controller	Total
Number of patients	4
Mean age (year, range)	45 (28-66)
Median sero-diagnosis (year, range)	9.5 (5-14)
Median CD4+ T cells	776
(cells/cu.mm., range)	(448-1,292)
HIV-1 clade	CRF01_AE
Risk of transmission (n, %)	
- Heterosexual intercourse	4 (100%)

5.15.2 HLA typing and CD8+ T cell responses data among elite controllers

Among these 4 elite controllers, patient 0558 and 0577 had been infected for 14 and 13 years, respectively. They have protective alleles including HLA-B*1301, -B*2704 and B*5801. Both of them had high breadth of p24 Gag CD8+ T cell responses (2 and 3 OLPs, respectively). Patient 05186 had been infected for 6 years. She has a protective alleles, i.e., HLA-B*1301 and had a breadth of Gag CD8+ T cell responses (6 OLPs of Pol and 2 OLPs of Env). Patient 0545 who had been infected for 5 years have also HLA-B*1301 as his protective alleles. He had no Gag CD8+ T cell responses. But had high breadth of Env CD8+ T cell responses (2 OLPs) and a breadth of Pol CD8+ T cell responses. Interestingly, both patients 0545 and 0558 also had heterozygous and homozygous of HLA-A*0203, respectively (table12).

Table 12. HLA and CD8+ T cell response profiles of elite controllers (N = 4)

No.	Patient ID	CD4	HLA typing						duration time	Breadth of responses		
			HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	of infection	Gag	Pol	Env
1	NRU - 0545	673	**0203**	2402	**1301**	3802	0406	0702	5	0	1	2
2	RSW - 0558	448	**0203**	**0203**	**1301**	4601	0102	0406	14	2	0	1
3	PJJ - 0577	878	**1101**	3303	**2704**	**5801**	0302	1202	13	3	0	0
4	VMK-05186	1292	**1101**	2410	**1301**	3802	ND	ND	6	1	6	2

ND= not done

CHAPTER VI

DISCUSSIONS

HIV-specific CTLs have been shown associated with protection, viral control and the delay in disease progression. Understanding the CTL responses in HIV-infected Thai individuals may provide essential information for specific HIV vaccine design and development.

6.1 Clinical data and demographic data in comparison to other cohorts

Based on the literature review as summarized in Table 13, there are 3 clade B, 1 clade C and 1 clade CRF01_AE HIV-1 infected study populations. In our study, 85% of the subjects were infected with clade CRF01_AE. The sample size was varied from 25 to 153 patients, whereas we have the largest sample size as of 240. Female was included of 57% in this study, compared with 18-78% from those reports. All studies including ours had median CD4+ T count of >300 cells/mm^3 (range 336-461), however we have the lowest median CD4+ T count i.e., 336. The median plasma HIV-RNA level of this study was 4.32 log_{10} copies/ml which is similar to most reports except for the one report by Mothe. B., et al. 2012 that had median plasma HIV-RNA level = 5.0 log_{10} copies/ml. Thus, overall our study has a larger sample size but with a bit lower median CD4+ T count than the others.

Table 13. Comparison of gender, clade, CD4 and plasma HIV-RNA level in among ethnic groups

			Gender			Median		
	N	Ethnic	Male	Female	HIV-1 Clade	CD4 (cell/mm^3)	HIV-RNA (log10)	References
1	45	African	37 (82%)	8 (18%)	B	417	4.53	2006, Zuniga R et al.
2	153	Chinese	83 (54%)	70 (46%)	B	378	4.34	2010, Meng Feng Y et al.
3	50	Caucasian	30 (60%)	20 (40%)	B	370	5.00	2012, Mothe B et al.
4	25	Indian	11 (44%)	14 (56%)	C	360	ND	2005, Kaushik S et al.
5	137	Thais	30 (22%)	107 (78%)	CRF01_AE	461	4.22	2011, Mori M et al.
6	240	Thais	103 (43%)	137 (57%)	CRF01_AE	336	4.32	This study

ND = not done

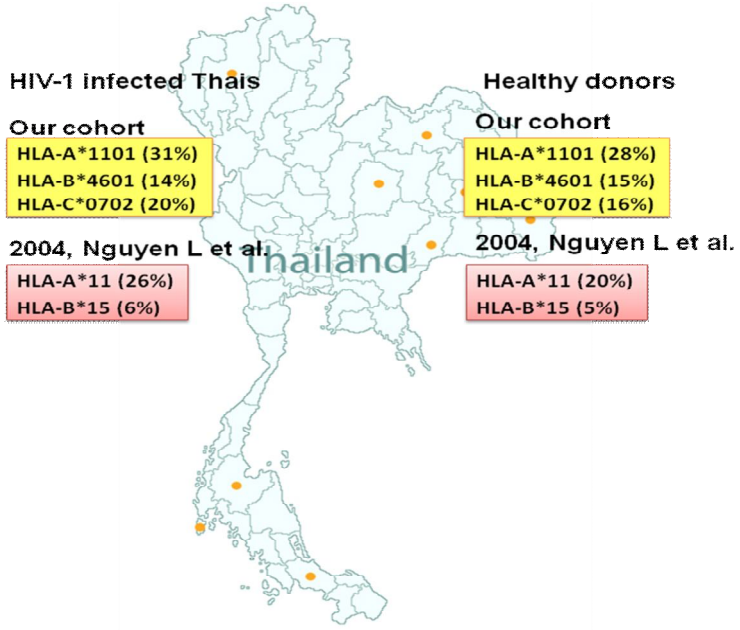
CD4+ T cells are the target cells of HIV. After HIV infection, the increase of plasma HIV-RNA level and the decrease of CD4+ T cell were observed in acute and chronic infection phase. However, when the immune system flights back the plasma HIV-RNA levels and CD4+ T cell shows inversion correlation. In our study, we

found the inverse correlation between CD4+ T cells and plasma HIV-RNA level (p <0.0001) as shown in Figure 7, which was consistent with current knowledge[98, 104].

6.2 Common HLA class I alleles and the frequency of known protective alleles

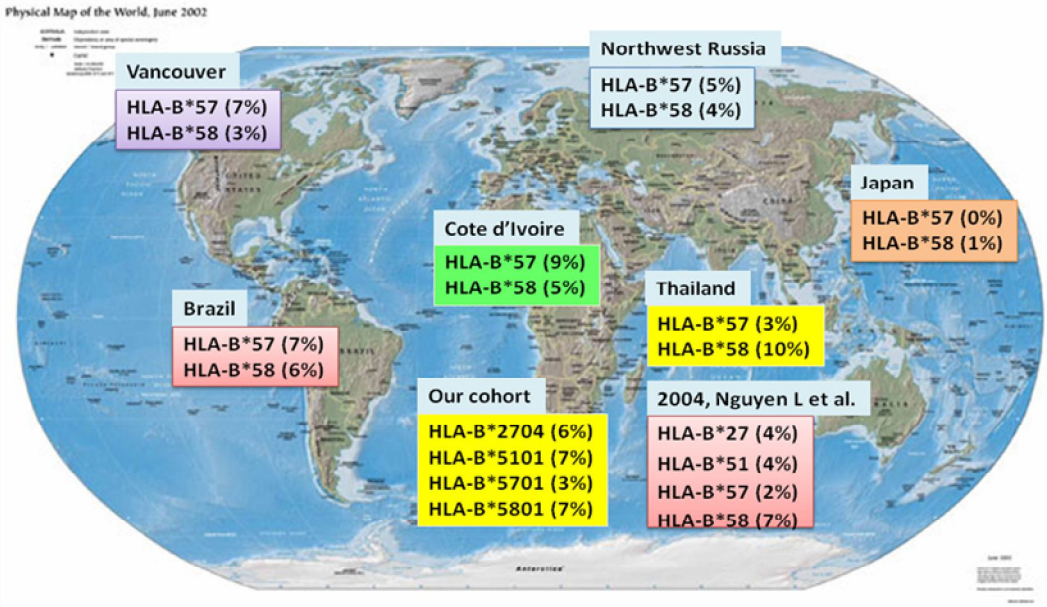
HLA allele frequencies vary greatly among different ethnic groups. The common HLA class I molecules around the world shows in Figure 6. In this study, the most common HLA class I molecule among HIV-1 infected individuals and uninfected Thais were HLA-A*1101 (31% vs. 28%), B*4601 (14% vs. 15%) and -C*0702 (20% vs. 16%) as shown in figure 10. The HLA class I allele's frequencies between HIV-1 infected patients and healthy volunteers were insignificantly different. Comparison data with other Thais studies[33] were also shown in Figure 47.

Figure 47. Comparison between common HLA class I alleles among HIV seropositive and seronegative Thais in this cohort and other reported cohorts



In other ethic predominantly infected with clade B, there are a number of known protective alleles associated with control of HIV-1 infection[24-27] such as HLA-B*27, -B*51, -B*57 and -B*5801. In this study, we found 6% HLA-B*2704, 7% HLA-B*5101, 3% HLA-B*5701 and 7% HLA-B*5801. Comparison with other previous studies[23,33] (Figure 48), Japanese had the lowest prevalence of both B*5701 and B*5801 i.e., 0 and 1%, respectively. Our results are similar to the previous Thai cohort –reported data.

Figure 48. Prevalence of known reported protective HLA class I alleles among different ethnic cohorts.



6.3 HIV-1 clade distribution

Among 240 HIV-1 infected individuals, we found predominant HIV-1 clade CRF01_AE (85%) based on 231 sequences of Gag, Nef and full-length sequencing. The 60 full-length sequences had also confirmed clade CRF01_AE as the majority subtype. Comparison with other countries in Asia, we found that other countries such as Indonesia[106], Vietnam[107] and Singapore[108] were HIV-1 clade CRF01_AE as well.

6.4 HLA class I alleles associated with low or high plasma HIV-RNA level

We determined the correlation between HLA class I allele of HIV-1 infected volunteers with plasma HIV-RNA level. We found that HLA-*B1302 in HIV-1 infected patients was associated with low plasma HIV-RNA level significantly (p = 0.0044) as shown in Figure 11. This finding was confirmed even after other known protective alleles were excluded (p = 0.0014) as shown in Figure 12. Our finding supports the role of HLA-B*13 associated with low plasma HIV-RNA level even though without a protective alleles previous reported in subtype C[94,109].

In addition, HLA-A2410 in HIV-1 infected patients was associated with low plasma HIV-RNA level (p = 0.0348) in Figure 11. Although, this cohort had only 3% or 7 individuals who have HLA-A2410+ve, after the adjustment by excluding other

known protective alleles, the association was even significantly stronger compared with those who did not carry this allele (p = 0.0085) as shown in Figure 13. Based on the literature review, such association of HLA-A*2410 allele with low plasma HIV-RNA level has not yet been reported.

In this study, we found the association of HLA-C*0304 with high plasma HIV-RNA level (p = 0.0033) as shown in Figure 11. The possible reason for this finding may be due to HLA-C allele can enhance viral infectivity as had been shown *in vitro* it fused more rapidly with HIV-1 gp120/gp41 from CHO cells expressing HLA-C positive cells and produced larger syncytia than HLA-C negative cells[112-113]. Recently, a study identified a single nucleotide polymorphism (SNP) at 35 kb upstream of the HLA-C locus that associated with disease outcome[110]. The -35C was associated with low plasma HIV-RNA level and slow disease progression whereas -35T was associated with high plasma HIV-RNA level and rapid disease progression. Whether -35T SNP may play a role in our finding regarding, HLA-Cw*0304 is not known and warranted for further investigation.

6.5 Frequency of HIV-1 specific CD8+ T cell responses

To determine the frequency, breadth and magnitude of HIV-1 specific CD8+ T cell responses and the association with their clinical outcomes, we analyzed all HIV-1 CRF01_AE proteins (413 OLPs) CD8+ T cell responses in 240 HIV-infected Thais. We found that the most common top 4 responses of HIV-1 target are Pol (91%), Gag (87%), Env (83%) and Nef (78%). Notably, the gag CD8+ T cell response rate is similar to another Thais study[80] (82%) as shown in Table 14. In a French, clade B study, with a comparable median CD4+ T cell count, the CD8 T cell response rates to Gag, Pol, Env and Nef, were all lower than this cohort and other studies[14,20]. The markedly differences among studies may be due to the use of recombinant vaccinia (rVV) and ^{51}Cr release assays in most more remote studies that has a lower sensitivity than ELISpot assay.

The Ethiopians studies of which was a clade C HIV-1 infected population, also showed a low Gag CD8+ T cell responses (58%)[114]. There were 2 Indian, clade C studies have been reported with a contradictory results i.e., one had 73% versus the other had 100% HIV-1 Gag specific responses[97-98]. The reasons for these variations in the same clade responses may be due to the number and peptide designs. The Ethiopians studies used the 49 OLPs from clade C synthetic Gag peptides based on one isolate 96ZM651.8. The peptides length was 20 amino acids overlapping 10 amino acids. In contrast, both Indian studies used the 121 OLPs from consensus HIV-

1 clade C Gag and the peptides length was 15±1 amino acids overlapping the next peptide by 11 amino acids.

The response rates to Pol, Env and Nef were also varied between the studies as shown in Table 14. These vast differences observed between cohorts are likely due to a selection bias, small sample size, regional variation of the patients and/or different immune status.

Table 14. Percentage of HIV-1 target proteins in among different ethnic cohorts.

				median		Frequency percentage				
	Ethnics	**Clade**	**N**	**CD4 (cells/mm^3)**	**VL (log10)**	**Gag**	**Pol**	**Env**	**Nef**	**References**
1	French	B	148	329	4.38	57%	57%	36%	30%	Chouquet, C., et al. 2002
2	African	B	45	417	4.53	80%	63%	37%	74%	Zuniga, R., et al. 2006
3	Indian	C	25	360	ND	100%	NA	NA	NA	Kaushik, S., et al. 2005
4	Ethiopians	C	48	387	3.65	58%	NA	NA	NA	Tsegaye, A., et al. 2007
5	Indian	C	30	319	35263	73%	NA	NA	90%	Mendiratta, S., et al. 2009
6	Thais	CRF01_AE	137	461	4.22	82%	NA	NA	NA	Mori, M., et al. 2011
7	Thais	CRF01_AE	240	336	4.32	87%	91%	83%	78%	This study

ND = not done, NA = not analyze

6.6 HIV-1 proteins specific CD8+ T cell response and its potential low or high plasma HIV-RNA level

Then, we compared the frequency of CD8+ T cells with plasma HIV-RNA level, and found that the frequency of Gag responses were associated with low plasma HIV-RNA level ($p = 0.0054$ and $p < 0.0001$) whereas Env responses were associated with high plasma HIV-RNA level ($p = 0.0013$ and $p = 0.0069$). In 2007, Kiepiela P *et al*. demonstrated Gag-specific CD8+ T cell responses was associated with low plasma HIV-RNA level ($p = 0.0006$) while Env and Nef-specific CD8+ T cell responses were associated with high plasma HIV-RNA level ($p = 0.0005$ and 0.0302, respectively)[112]. Moreover, Mori M *et al*. presented that Gag-specific CTL response was associated with low plasma HIV-RNA level ($p = 0.0018$)[80]. Our results supported both previous studies that HIV-1 Gag-specific CD8+ T cell response was associated with low plasma HIV-RNA level whereas Env-specific CD8+ T cell response was associated with high plasma HIV-RNA level (Table 15).

The mechanisms of why CD8 responding to Gag but not the other HIV targets is beneficial on the viral control are not been elucidated. There are some possible

explanation including the Env is the most diversified region that results to frequently and subsequently immune escape, those escape mutations within the Env do not affect the viral fitness or may even be more enhancing viral replication, In contrast, Gag is more conserved and evidences have supported that some immune escape mutants may compromise the viral fitness and thus maintaining the viral control effect[111].

Table 15. Correlation between frequency of responses and plasma HIV-RNA level of HIV-1 target proteins in other cohorts and this cohort

				median		Frequency of responses vs. plasma VL correlation			
	Ethnics	**Clade**	**N**	**CD4 (cells/mm^3)**	**VL (log10)**	**Gag**	**Env**	**Nef**	**References**
1	South African	C	578	NA	35329	low VL, p=0.0006	high VL, p=0.0005	high VL, p=0.0302	Kiepiela P et al. (2007)
2	Thais	CRF01_AE	137	461	4.22	low VL, p=0.0018	NA	NA	Mori M et al. (2011)
3	Thais	CRF01_AE	240	336	4.32	low VL, p=0.0054	high VL, p=0.0013	NS	This study

NA = not analyze, NS = no significant

6.7 Breadth responses of HIV-1 specific CD8+ T cell responses

Most studies reported breadth responses of p24-Gag HIV-1 specific CTL responses were associated with disease outcome[20, 80, 112]. In this study, we found the breadth responses against Gag-HIV-1 specific CD8+ T cell responses which were associated with low plasma HIV-RNA level ($p < 0.0001$) while the breadth responses against Env-HIV-1 specific CD8+ T cell responses which were associated with high plasma HIV-RNA level ($p = 0.0069$). This finding was similar to the study in clade B[20], clade CRF01_AE[80] and clade C[112] (Table 16). These finding may be supported previous studies that breadth responses of p24-Gag HIV-1 specific CTL responses were associated with slow disease outcome.

Table 16. Correlation between breadth and plasma HIV-RNA level of HIV-1 target proteins among different ethnic cohorts.

				median		Breadth vs. VL association			
	Ethnics	**Clade**	**N**	**CD4 (cells/mm^3)**	**VL (log10)**	**Gag**	**Env**	**Nef**	**References**
1	African	B	45	417	4.53	low VL, p=0.0105	NA	NA	Zuniga R et al. (2006)
2	South African	C	578	NA	35329	low VL, p<0.0001	high VL, p<0.0001	NA	Kiepiela P et al. (2007)
3	Thais	CRF01_AE	137	461	4.22	low VL, p=0.0015	NA	NA	Mori M et al. (2011)
4	Thais	CRF01_AE	240	336	4.32	low VL, p<0.0001	high VL, p=0.0069	NS	This study

NA = not analyze, NS = no significant

6.8 Magnitude responses of HIV-1 specific CD8+ T cell responses

Many researchers found that magnitude response of Gag-responses was associated with low plasma HIV-RNA level and favorable clinical outcome[80]. Several studies found the inverse correlation between magnitude responses of HIV-1 Gag-specific CD8+ T cell responses and plasma HIV-RNA level [20, 98] (Table 17). Our study were also found similarly inverse correlation between magnitude responses of HIV-1 Gag-specific CD8+ T cell responses and plasma HIV-RNA level (p = 0.0011). But, we found the direct correlation between magnitude responses of HIV-1 Env and Nef-specific CD8+ T cell responses and plasma HIV-RNA level (p = 0.0032 and 0.0180, respectively). These finding confirm that Gag-specific CD8+ T cell responses were associated with low plasma HIV-RNA level.

Table 17. Correlation between magnitude and plasma HIV-RNA level of HIV-1 target proteins among different ethnic cohorts

				Median		Magnitude vs. VL association			
	Ethnics	Clade	N	CD4 (cells/mm^3)	VL (log10)	Gag	Env	Nef	References
1	African	B	45	417	4.53	low VL, p=0.022	NA	NA	Zuniga R et al. (2006)
2	Ethiopians	C	48	387	3.65	NS	NA	NA	Tsegaye A et al. (2007)
3	Indian	C	30	319	35263	low VL, p=0.001	NA	NS	Mendiratta S et al. (2009)
4	Thais	CRF01_AE	137	461	4.22	low VL, p=0.0084	NA	NA	Mori M et al. (2011)
5	Thais	CRF01_AE	240	336	4.32	low VL, p=0.0011	high VL, p=0.0032	high VL, p=0.0180	This study

VL = plasma HIV-RNA level, NA = not analyze, NS = no significant

Frequency, breadth and magnitude of CD8+ T cell responses are important factors to be further characterized. Gag protein was targeted by 87% because amino acid sequences were very conserve in this area. Nef protein was the earliest responses in HIV-1 infection. We found Nef targeted was 78% and the immunodominant regions were located in central conserved domains[98]. Pol protein, which the epitope-rich regions were located in RT was the most targeted 91% in our cohort. Env protein was targeted 83% that spread all regions. In addition, the regulatory protein targeted at N-terminal and central region except Vif and Vpr protein targeted all regions as Env protein.

6.9 Novel CD8+ T cell epitope with viral escape of HIV-1 CRF01_AE infected Thai individuals

Although, Gag-specific CTL responses are associated with low plasma HIV-RNA level, we found one OLP from p24 Gag associated with viral escape. This OLP was Gag-OLP38; $_{273}$IVRMYSPVSILDIRQGPK$_{290}$ that restricted by HLA*Cw0102. It contained epitope $_{277}$YSPVSILDI$_{285}$ or YI9 which was only the 7th most frequently targeted epitope in this cohort. However, more than 80% of responders against Gag-OLP38 were expressed Cw*0102 ($p < 0.0001$).

This YI9 epitope is located in a highly conserve region p24 Gag. The mutation in YI9 were found 47.06% of all Cw*0102+ve subjects. The most occurring mutation was a V280T mutation at position 4 (17.6% of all Cw*0102+ve subjects), which was a significantly higher than in Cw*0102-ve subjects ($p = 0.0003$). The position 4 represents a region for binding of the TCR to peptide in T cell. The frequency of Cw*0102 in the Thai population, and the frequent of viral escape patterns were observed in this epitope, suggested that they were likely reverse of this escape mutation back to wildtype upon transmission to a new host. These data may suggest the ability of this mutation to impair viral replication as observed for other escape mutations in the region of p24[115-116].

Position 2 represents an anchor position for binding of the peptide to the HLA class I groove, and thus a potentially more promising pathway to viral escape phenomenon. Here, the mutations at position 2 were present in 13.73% of all Cw*0102+ve subjects and rarely in Cw*0102-ve subjects ($p = 0.0059$). Moreover, mutation at P2 appeared completely to impair the ELISpot response.

The YI9 epitope was observed 77% in this cohort suggests that it was the most frequent immunodominant in CRF01_AE infections. In comparison to extensive immunodominance data available for clade B infection[117], only a handful of CD8 epitopes are as commonly targeted as YI9 in acute or chronic infection[118]. Notably, this peptide maps are close to the region of Gag containing immunodominant CD8 epitopes restricted by HLA-B*27 ($_{263}$KRWIILGLNK$_{272}$) and HLA-B*57 ($_{240}$TSTLQEQIGW$_{249}$). Both of these HLA alleles are associated with a beneficial outcome in clade B infections[117, 119-121]. However, in our cohort there is no significant difference in plasma HIV-RNA levels between Cw*0102+ve and Cw*0102-ve patients (mean 53,096 vs. 66,948.54 copies/ml, $p = 0.6708$), nor in CD4 T cell counts (mean 316.5 vs. 314 cells/mm^3, $p = 0.8709$). This may be the result of the high rate of viral escape within this epitope[122].

Further analysis of the HLA data showed linkage disequilibrium (LD) between Cw*0102 with B*4601 ($p = 9.8399*10^{-23}$), another common HLA epitope in the Thai population (allelic frequency 17.2%, NCBI). While we saw significantly more mutations in YI9 in Cw*0102+ve subjects than in Cw*0102-ve subjects ($p = 0.0007$), there was no significant difference in the occurrence of YI9 mutations in B*4601+ve (24.8%) versus B*4601-ve (39.5%) patients ($p = 0.0778$).

Thus, this argues against B4601 as the restricting HLA type, as does the strong data showing restriction of this response in Cw*0102 positive cells (Figure 30A).

HIV is known to down-regulate HLA-A and HLA-B alleles on the cell surface[123], leaving HLA-C intact in order to protect cells from attack by NK cells[124]. As such, HLA-A or HLA-B restricted CD8+ T cells may be partially compromised in their ability to recognize and destroy HIV-infected cells as compared to HLA-C restricted responses. However, few HLA-C restricted epitopes have been identified to date (http://www.hiv.lanl.gov/content/immunology/tables/ctl_summary.html). Therefore, focus on the identification and characterization of HLA-C restricted responses may be warranted to aid in the development of an effective HIV vaccine.

There are many studies reported that the HLA class I allele associated with viral escape and rapid progression such as HLA-B*3502, -B*3503, -B*5802 and -B*1503. Here, we summarized the previous studies including our cohort as shown Table 18.

Table 18. Viral escape associated with HLA class I molecules

	Sample		HLA class I molecule	Epitope implicated	References
	N	Population			
1	498 850	Caucasian Caucasian	HLA-B*35 HLA-B*3502 HLA-B*3503	ND	Carrington M et al. (1999) Honeyborne I et al. (2007)
2	128	Thailand	HLA-B*15 (Clade CRF01_AE)	ND	Gao X et al. (2001)
3	423 202	Zambian, clade C Rwandan, clade A	HLA-B*5802		Ferrer A et al. (2005)
4	248 169	Clade B, Boston area and on the island of Barbados Clade C, Durban, South Africa	HLA-B*1503 (clade C)		Frahm N et al. (2006)
5	195	Clade CRF01_AE, Thailand	HLA-Cw*0102	YSPVSILDI (Gag 277-285)	This study

ND = not done

6.10 A known reported epitope associated with low plasma HIV-RNA level of HIV-1 CRF01_AE infected Thai individuals

In this study, we found Gag-OLP48 which is a known reported epitope and was associated with low plasma HIV-RNA level (p = 0.0004). This Gag-OLP48 (p24); ACQGVGGPSHK or AK11 restricted by HLA-A*1101. It was the 28th frequently targeted epitope. Indeed, 85.7% of responders against Gag-OLP48 were expressed HLA-A*1101 ($p < 0.0001$).

The AK11 is located in a highly conserve region of p24 Gag. The conserve sequence showed over 95% in both HLA-A*1101+ve and HLA-A*1101-ve subjects. The S357G mutation at position 9 in the patients with HLA-A*1101 showed 2% compared with 1.1% in the patients without HLA-A*1101. No mutation found difference in both patients with and without HLA-A*1101 (p = 1.000).

This peptide are located close to the region of Gag containing immunodominant CD8 epitopes restricted by HLA-B*51($_{325}$NANPDCKTIL$_{334}$),

HLA-B*57 ($_{308}$QASQEVKNWM$_{317}$) and HLA-B*58 ($_{308}$QASQEVKNW$_{316}$). These epitopesare associated with slow disease progression in clade B infections[117, 119-121]. Moreover, there is no significant difference in plasma HIV-RNA level between HLA-A*1101+ve and HLA-A*1101-ve patients (mean 56,221 vs. 63,953 copies/ml, p = 0.8212), nor in CD4+ T cell counts (mean 311 vs. 312 cells/cu.mm, p = 0.8722). Moreover, further analysis of HLA data showed no LD in HLA-A*1101.

6.11 Novel CD8+ T cell epitope with low plasma HIV-RNA level of HIV-1 CRF01_AE infected Thai individuals

Next, we determined a novel CTL epitope that was associated with low plasma HIV-RNA levels and its impact. We found one OLP; Pol-OLP16 that was associated with low plasma HIV-RNA level (p = 0.0155) and strongly significant confirmed by excluding protective alleles (p = 0.0104). This Pol-OLP16; $_{111}$KVRQYDQILIEICG KKAI$_{128}$ was restricted to HLA-A*0203. It was contained novel epitope $_{113}$RQYDQILIEI$_{122}$ or RI10 that was the 40th frequently targeted epitope in our cohort. Fifty-two percentage of responder against Pol-OLP16 were also expressed HLA-A*0203 ($p < 0.0001$).

This RI10 epitope is located in a highly conserve region of protease Pol. The mutation in RI10 were found 60% of all A*0203+ve subjects. The D116E mutation at position 4 (20%) of HLA-A*0203+ve subjects were higher than in A*0203-ve subjects (3.7%). The position 4 represents a T cell region for binding of the TCR to peptide. The patients with HLA-A*0203 allele with RI10 mutation showed completely impair CD8+ T cell responses. Moreover, we observed the frequent escape mutation in RI10 epitope and more frequency of HLA-A*0203 in this cohort (allelic frequency 11%), we may suggest the likely conversion of this epitope mutation back to WT as found in YI9 novel escape epitope. However, this information need more sample size to confirm.

Mutation at P2 is an efficiency impact to occur the viral escape. In this study, we found more conserve at this position (glutamine) in both patients with and without HLA-A*0203. This finding may suggest the processing and binding of RI10 on HLA-A*0203 allele and presenting on the cell surface without ELISpot responses. This finding may occur from viral escape at P4 which are TCR binding region.

Previous studies showed that HLA-B*13 linked with successful immune control[125-126]. This epitope (RI10) had been reported that restricted by HLA-B*1302[93]. We analyzed the mutation in RI10 of HLA-B*1302+ve versus HLA-B*1302-ve subjects and found insignificantly difference in both groups (p = 0.5684).

Notably, this peptide maps close to the region of Pol containing immunodominant CD8 epitopes restricted by HLA-B*51 ($_{63}$QRPLVTIKIG$_{72}$) and HLA-B*57, -B*58 and -B*63 ($_{126}$KAIGTVLV$_{133}$). Both of HLA-B*51 and -B*57 are associated with a beneficial outcome in clade B infections[117, 119-121]. In addition, HLA-B*63 is associated with control HIV replication and shares the epitope binding anchor motif with HLA-B*57 and -B*58[28].

Besides, there was no significantly different in plasma HIV-RNA levels between HLA-A*0203+ve and HLA-A*0203-ve subjects (median 18,286 vs. 22,690 copies/ml, p = 0.7647), and in CD4+ T cell counts (mean 313 vs. 323 cells/mm^3, p = 0.6858). Interestingly, the patients with HLA-A*0203+ve who recognized RI10 had a significant lower plasma HIV-RNA level than those who did not (median 7,703 vs. 38,940 copies/ml, p = 0.0167), but not in CD4+ T cell counts (mean 418 vs. 201 cells/mm3, p = 0.0667). This data may support the low plasma HIV-RNA level from viral fitness of RI10.

Further analysis of the HLA data showed LD between A*0203 with B*3802 (p = 4.102*10^{-7}), another common HLA epitope in the Thai population (allelic frequency 6%). However, the mutation in RI10 in B*3802+ve and B*3802-ve subjects were no significant difference (p = 0.4427).

There are many studies reported the HLA class I allele associated with HIV delay progression such as HLA-B*5701, -B*5702, -B*5703, -B*5801, -B*63, -B*1503 and -B*1302. In this study, we found HLA*A0203 with RI10 responses associated with low plasma HIV-RNA level. We summarized all of the studies including our cohort in Table 19.

Table 19. Viral control associated with HLA class I molecules

	Sample		HLA class I molecule	Epitope implicated	References
	N	Populations			
1	13 200	clade B, LTNP progressor	HLA-B*5701, HLA-B*5702 HLA-B*5703	ISPRTLNAW (Gag 147–155) KAFSPEVIPMF (Gag 162–172) TSTLQEQIAW (Gag 240–249) QASQEVKNW (Gag 308-316)	Migueles SA et al. (2000)
2	423 202	Zambian, clade C Rwandan, clade A	HLA-B*5703 HLA-B*5801		Ferrer A et al. (2005)
3	21 7	clade B, United States, Barbados, and South Africa clade C, United States, Barbados, and South Africa	HLA-B*63	RSLYNTVATLY (Gag76-86) FSPEVIPMF (Gag 164–172) YTPGPGIRY (Nef 127-135) LTFGWCFKL (Nef 137-145) KAIGTVLV (Pol 70-77)	Kiepiela P et al. (2004)
4	248 169	Clade B, Boston area and on the island of Barbados Clade C, Durban, South Africa	HLA-B*1503 (clade B)	TKIQNFRVYY (Pol) KQNPDIVIY (Pol 328-336) VKVVEEKAF (Gag) KQEFGIPY (Pol) TQIGCTLNF (Pol) TKGLGISY (Tat)	Crrington M et al. (1999)
5	578	Clade C, Durban, South Africa	HLA-B*1302	VQNLQGQMV (Gag 135-143) GQMREPRGSDI (Gag 226-236) RQANFLGKI (Gag 429-437) RQDILDLWV (Nef 106-114) RQYDQILIEI (Pol 113-122) GQGQWTYQI (Pol 488- 496)	Gong X et al. (2006)
6	195	Clade CRF01_AE, Thailand	HLA-A*0203	RQYDQILIEI (Pol 113-122)	This study

6.12 HIV-1 CRF01_AE infected patients identified as Elite controllers

In this study, we found that four HIV-1 infected patients had undetectable of plasma HIV-RNA level. The duration time of HIV-1 infection after diagnostic ranged from 5 to 14 years with the median of 9.5 years. The CD4+ T cell counts ranged from 448 to 1,292 cells/cu.mm. with the median of 776 cells/cu.mm. The other studies showed LTNP with the duration time of HIV-1 infection after diagnostic more than 5 years with the CD4+ T cell counts higher than 500 cells/cu.mm[61-62].

Furthermore, all subjects showed the protective alleles such as HLA-B*1301, -B*2704, B*5801 and A*0203 that found in this study. The patient 0558, 0577 and 05186 showed breadth of Gag-CD8+ T cell responses whereas the patient 0545 showed breadth of Env-CD8+ T cell responses.

These patients were screened for HIV-1 specific CD8+ T cell responses with a set of 413 OLPs IFN-γ ELISpot assay. The patient 0545 was performed HLA typing (HLA-A*0203, -A*2402, -B*1301, -B*3802, -C*0406, -C*0702). He had HLA-B*1301 allele as protective allele. However, he did not respond to other OLPs that associated with HLA-B*1301. Notably, he recognized with Pol-OLP16 or RI10 which were restricted by HLA-A*0203. This finding may support our study that the HLA-A*0203 allele was associated with low HIV-RNA level.

The second patient 0558 has HLA typing; HLA-A*0203, -A*0203, -B*1301, -B*4601, -C*0406, -C*0102. He responded with Gag-OLP20 and -OLP21 which recognized with HLA-A*02 epitope or RV9 and Env-OLP51 which recognized with three OLPs such as HLA-A*02 or PS7, HLA-Cw*0401 or SY11 and HLA-Cw*0407 or SY11 epitopes. The reported HLA-A*02 epitope may present as HLA-A*0203 allele in this patient. We need to characterize more information of this epitope. Also, she has no respond with HLA-B*1302 epitope. This data may support our study that the HLA-A*0203 allele can control viral replication.

The third patient 0577 has HLA typing; HLA-A*1101, -A*3303, -B*2704, -B*5801, -C*0302, -C*1202. He has 2 alleles of protective alleles which are HLA-B*2704 and HLA-B*5801. He responded with Gag-OLP33 and -OLP34 which recognized with HLA-A*02 epitope or SM10. He also responded HLA-B*58 epitope or SW9 and Gag-OLP44 which recognized with HLA-A*11 epitope or VK9. He did not respond to HLA-B*2704 epitope. This finding suggests that the HLA-B*5801 alleles is the protective allele and supports our study that the HLA-A*1101 is associated with low HIV-RNA level.

The last patient 05186 has HLA typing; HLA-A*1101, -A*2410, -B*1301, -B*3802. She responded with 11 OLPs. The Gag-OLP27 recognized with HLA-A*11 or HK9 epitope, the Pol-OLP67 recognized with HLA-A*11 or QK11 epitope and HLA-B*13 or GI9 epitope. This finding suggests that the HLA-B*1301 alleles as protective allele and supports our study that the HLA-A*1101 was associated with low HIV-RNA level.

Nonetheless, there are some limitations of this study to take into the consideration. Firstly, it was a cross-sectional study which cannot predict the longer term outcomes. Secondly, we used on univariate factor to assess the association with plasma HIV-RNA levels. Thirdly, our patients were in chronic infection phase may not represent the phenomenon during very early phase of infection. And lastly, the specific CD8+ T cell response was based only on IFN-γ ELISpot assay.

Recently, Thai phase III (RV144 trial) results did not see a correlation of T cell responses and protection. In contrast, they found higher level of IgG Ab to variable region 1 and 2 (V1V2) of HIV-1 Env protein than placebo.

Last but not least, there were several new knowledges from this study. In HLA allele aspect, we found new HLA-A*2410 allele which was associated with low plasma HIV-RNA level. In epitope aspect, we found novel YI9 CD8+ T cell epitope was associated with viral escape. In contrast, we found novel RI10 and known AK11 CD8 epitopes were associated with low plasma HIV-RNA level.

In conclusion, our data indicate the potential influences of other epitopes recognized by the same HLA alleles. This data may suggest the impact of both HLA class I allele and targeted proteins of immune responses.

In Summary: HLA-B*1302 and HLA-A*2410 were associated with low plasma HIV-RNA level. Pol, Gag, Env and Nef are four most common HIV targets of CD8 T cells, however, only HIV-1 Gag was found associated with low plasma HIV-RNA level. Three CD8 epitopes: YI9, RI10 and AK11 restricted by HLA-Cw*0102, -A*0203 and -A*1101, respectively; were characterized. RI10 and AK11, but not YI9, were associated with lower plasma HIV-RNA level that possibly to HIV viral control. Further characterization of the rest of possible novel epitopes is warranted.

CHAPTER VII

CONCLUSION

From these Thai patients predominantly infected with subtype CRF01_AE (85%) and had modest immunocompromise status (as shown by the median CD4+ T cell count of 336 cells/mm^3 and median plasma HIV-RNA level of 20,994 copies/ml), we identified 2 HLA class 1 alleles: HLA-B*1302 and HLA-A*2410 alleles (after exclude HLA-B*27, -B*51, -B*57, and -B*58 known as protective alleles) were associated with low plasma HIV-RNA level (p = 0.0014 and p = 0.0085, respectively). But, HLA-C*0304 allele was found associated with high plasma HIV-RNA level (p = 0.0033). In term of the frequency of responses, there are 4 most common target recognized by CD8 IFN-γ ELISpot including Pol (91%), Gag (87%), Env (83%) and Nef (78%). Moreover, the frequency, breadth and magnitude of Gag responses are associated with low plasma HIV-RNA level ($p < 0.01$).

Two novel epitopes were identified and characterize i.e., Gag epitope $_{277}$YSPVSILDI$_{285}$ or YI9 restricted by HLA-Cw*0102 and Pol epitope $_{113}$RQYDQILIEI$_{122}$ or RI10 restricted by HLA-A*0203. The Gag YI9 was found associated with foot printed mutations among patients who had and not had Cw*0102, and the mutations in the P2 (S278X) and P4 (V280X) were resulted to the CD8 immune escape.

The Pol RI10 epitope was associated with low plasma HIV-RNA level. Of note, immune escape mutations of RI10 were remained associated with low plasma HIV-RNA level. This may be due to such mutation has led to the reduction of viral fitness. Additional new finding is that the known reported Gag epitope $_{349}$ACQGVGGPSHK$_{359}$ or AK11 that was restricted by HLA-A*1101 was associated with low plasma HIV-RNA level. However, there are several more novel epitopes that further characterization is warrant.

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APPENDICES

APPENDIX A

REAGENTS, MATERIALS AND INSTRUMENTS

A. Reagents

Alkaline Phosphatase (AP) conjugate Substrate Kit (Bio Rad Labs., Hercules, CA)
Anti-human IFN-γ mAb 1-D1K (Mabtech, Nacka, Sweden)
Biotinylated anti-human IFN-γ mAb 7-B6-1 (Mabtech, Nacka, Sweden)
Chromium-51 (Amersham, UK)
Clorox
Cyclosporin A (Sigma, UK)
DMSO (Sigma, UK)
Fetal Bovine Serum (Bio Whittaker, Maryland, U.S.A)
IL-2 (Genzyme, U.S.A)
IL-7 (Genzyme, U.S.A)
L-Glutamine (Sigma, UK)
PBS (Sigma, UK)
Penicillin G (General Drugs House, Thailand)
Peptide (Obtained from Boston, U.S.A)
PHA (Sigma, UK)
RPMI medium1640 (GIBCO, U.S.A)
Streptavidin-alkaline phosphatase (Mabtech, Nacka, Sweden)
Streptomycin (General Drugs House, Thailand)
Triton-X 100 (Sigma, UK)
Trypan blue (Sigma, UK)
Tween-20 (Sigma, UK)

B. Materials

24-well flat plate (Costar, U.S.A)
96-well U plate (Costar, U.S.A)
ACD tube (Becton-Dickinson, U.S.A)
Automatic pipette (Gilson, France)
Conical tube 50, 15 ml (Falcon, U.S.A)
Counting chamber
Cryotube (Sarstedt, Germany)
Disposable serological pipette 25, 10, 5, 2, 1 ml (Costar, U.S.A)

EDTA tube (Becton-Dickinson, U.S.A)
ELISpot plate (Millipore, U.S.A)
Flask 25,75 cm^3 (Nunc, Denmark)
Glove
Lead shield
Multichannel Autopipettor
Microcentrifuge (Eppendrof, U.S.A)
Microcentrifuge tube
Pipette boy
Pipette tip

C. Instruments

Biological Safety Cabinet Class II
Centrifuge
CO_2 incubator (Forma Scientific, U.S.A)
ELISpot Automatic reader (Carl Zeiss, Germany)
ELISpot washer
Freezer – 70^oC
Gamma counter
Geiger counter (Ludlum, U.S.A)
Liquid nitrogen tank
Mixer-Vortex-Genic (Scienfic industries, U.S.A)
Refrigerator
Water bath (Shel-lab, U.S.A)

APPENDIX B

REAGENTS AND PREPARATIONS

1. Reagent for PBMCs thawing
   - 1.1 RPMI medium 1640 (ready to used)
   - 1.2 Penicillin 10,000 Units/ml
     - 1.2.1 Stock penicillin 100,000 Units/ml
       Penicillin G 1,000,000 Units per ampoule was reconstituted with sterile DW 10 ml and mixed
     - 1.2.2 Working penicillin 10,000 Units/ml

Stock penicillin 100,000 Units/ml	1	ml
RPMI1640	9	ml

   - 1.3 Streptomycin 10,000 μg/ml
     - 1.3.1 Stock Streptomycin 100,000 μg/ml
       Streptomycin 1 gm was reconstituted with sterile DW 10 ml and mixed
     - 1.3.2 Working Streptomycin 10,000 μg/ml

Stock Streptomycin 100,000 μg/ml	1	ml
RPMI1640	9	ml

   - 1.4 R10

RPMI1640 + 100 units/ml Streptomycin + 100 units/ml Penicillin	90	ml
Fetal Bovine Serum (FBS)	10	ml

   RPMI 1640 + 10% FBS and kept cold to 2-8^{o}C prior to use

2. Reagent for ELISpot washing buffer
   - 2.1 Phosphate Buffer Saline 1X (PBS) pH 7.4
     One pouch dissolved in 1 litre of deionized water

3. Reagent for culture
   - 3.1 R10

RPMI1640 + 100 units/ml Streptomycin + 100 units/ml Penicillin	90	ml
Fetal Bovine Serum (FBS)	10	ml

   - 3.2 R20

RPMI1640 + 100 units/ml Streptomycin + 100 units/ml Penicillin	80	ml
Fetal Bovine Serum (FBS)	20	ml

4. Cyclosporin A 1 μg/ml
   - 4.1 Stock CSA 1 mg/ml
     - CSA 50 mg /ml — 100 μl
     - Normal Saline Sterile — 4900 μl
   - 4.2 Stock CSA 10^{-4} g/ml
     - Stock CSA 1 mg/ml — 100 μl
     - R20 — 900 μl
   - 4.3 CSA 1 μg/ml
     - Stock CSA 10^{-4} g/ml — 100 μl
     - R20 — 900 μl

5. Peptide preparation
   - 5.1 Stock peptide 1 mg/ml
     - Peptide — 1 mg
     - 1% DMSO in PBS — 1 ml
   - 5.2 Peptide 200 μg/ml
     - Stock peptide 1 mg/ml — 200 μl
     - Sterile PBS — 800 μl

APPENDIX C

SEQUENCE OF GAG A PEPTIDE

1. Abbreviation for amino acids

A	alanine	L	leucine
R	arginine	K	lysine
N	asparagine	M	methionine
D	aspartic acid	F	phenylalanine
C	cysteine	P	proline
Q	qlutamine	S	serine
E	glutamic acid	T	threonine
G	glycine	W	trytophan
H	histidine	Y	tyrosine
I	isoleucine	V	valine

2. HIV-1 clade CRF01_AE genome (413 OLPs, Obtained from Boston, U.S.A)

OLP	Sequence	Protein OLP
001	MGARASVLSGGKLDAWEK	p17 GAG-AE-1
002	SGGKLDAWEKIRLRPGGK	GAG-AE-2
003	EKIRLRPGGKKKYRMKHL	GAG-AE-3
004	GKKKYRMKHLVWASREL	GAG-AE-4
005	KHLVWASRELERFAL	GAG-AE-5
006	ASRELERFALNPGLL	GAG-AE-6
007	ERFALNPGLLETAEGCQ	GAG-AE-7
008	GLLETAEGCQQIIEQL	GAG-AE-8
009	EGCQQIIEQLQSTLKTGS	GAG-AE-9
010	QLQSTLKTGSEELKSLF	GAG-AE-10
011	TGSEELKSLFNTVATLW	GAG-AE-11
012	SLFNTVATLWCVHQRIEV	GAG-AE-12
013	LWCVHQRIEVKDTKEAL	GAG-AE-13
014	IEVKDTKEALDKIEEVQNK	GAG-AE-14
015	LDKIEEVQNKSQQKTQQA	GAG-AE-15
016	NKSQQKTQQAAAGTGSSSKV	GAG-AE-16
017	AAGTGSSSKVSQNYPIV	GAG-AE-17
018	SKVSQNYPIVQNAQGQMV	p24 GAG-AE-18
019	IVQNAQGQMVHQPLSPR	GAG-AE-19

OLP	Sequence	Protein OLP
020	QMVHQPLSPRTLNAWVKV	GAG-AE-20
021	PRTLNAWVKVVEEKGF	GAG-AE-21
022	WVKVVEEKGFNPEVIPMF	GAG-AE-22
023	GFNPEVIPMFSALSEGA	GAG-AE-23
024	PMFSALSEGATPQDLNMM	GAG-AE-24
025	GATPQDLNMMLNIVGGH	GAG-AE-25
026	NMMLNIVGGHQAAMQMLK	GAG-AE-26
027	GHQAAMQMLKETINEEAA	GAG-AE-27
028	LKETINEEAAEWDRVHPV	GAG-AE-28
029	AAEWDRVHPVHAGPIP	GAG-AE-29
030	VHPVHAGPIPPGQMREPR	GAG-AE-30
031	IPPGQMREPRGSDIA	GAG-AE-31
032	MREPRGSDIAGTTSTL	GAG-AE-32
033	SDIAGTTSTLQEQIGWM	GAG-AE-33
034	STLQEQIGWMTNNPPIPV	GAG-AE-34
035	WMTNNPPIPVGDIYKRWI	GAG-AE-35
036	PVGDIYKRWIILGLNKIV	GAG-AE-36
037	WIILGLNKIVRMYSPVSI	GAG-AE-37
038	IVRMYSPVSILDIRQGPK	GAG-AE-38
039	SILDIRQGPKEPFRDYV	GAG-AE-39
040	GPKEPFRDYVDRFYKTLR	GAG-AE-40
041	YVDRFYKTLRAEQATQEV	GAG-AE-41
042	LRAEQATQEVKNWMTETL	GAG-AE-42
043	EVKNWMTETLLVQNA	GAG-AE-43
044	MTETLLVQNANPDCKSIL	GAG-AE-44
045	NANPDCKSILKALGTGA	GAG-AE-45
046	SILKALGTGATLEEMMTA	GAG-AE-46
047	GATLEEMMTACQGVGGPSH	GAG-AE-47
048	ACQGVGGPSHKARVLAEA	GAG-AE-48
049	SHKARVLAEAMSQAQHA	p15 GAG-AE-49
050	EAMSQAQHANIMMQR	GAG-AE-50
051	QHANIMMQRGNFKGQKR	GAG-AE-51
052	QRGNFKGQKRIKCF	GAG-AE-52
053	KGQKRIKCFNCGKEGHL	GAG-AE-53
054	CFNCGKEGHLARNCRAPR	GAG-AE-54
055	HLARNCRAPRKKGCWK	GAG-AE-55
056	RAPRKKGCWKCGKEGHQM	GAG-AE-56
057	WKCGKEGHQMKDCTERQA	GAG-AE-57
058	QMKDCTERQANFLGKIW	GAG-AE-58

OLP	Sequence	Protein OLP
059	RQANFLGKIWPSNKGR	GAG-AE-59
060	GKIWPSNKGRPGNFPQSR	GAG-AE-60
061	GRPGNFPQSRPEPTA	GAG-AE-61
062	FPQSRPEPTAPPAENWGM	GAG-AE-62
063	TAPPAENWGMGEEITSLLK	GAG-AE-63
064	GEEITSLLKQEQKDKEH	GAG-AE-64
065	LKQEQKDKEHPPPLVSLK	GAG-AE-65
066	KEHPPPLVSLKSLFGNDPL	GAG-AE-66
067	LKSLFGNDPLSQ	GAG-AE-67
068	MGGKWSKSSIVGWPQVR	NEF-AE-1
069	SSIVGWPQVRERIKQT	NEF-AE-2
070	PQVRERIKQTPPAAEGV	NEF-AE-3
071	KQTPPAAEGVGAVSQDL	NEF-AE-4
072	EGVGAVSQDLDKHGAV	NEF-AE-5
073	SQDLDKHGAVTSSNM	NEF-AE-6
074	GAVTSSNMNNADCVWL	NEF-AE-7
075	MNNADCVWLRAQEEEEEV	NEF-AE-8
076	WLRAQEEEEEVGFPVRPQV	NEF-AE-9
077	EVGFPVRPQVPLRPMTYK	NEF-AE-10
078	QVPLRPMTYKGAFDLSFF	NEF-AE-11
079	YKGAFDLSFFLKEKGGL	NEF-AE-12
080	SFFLKEKGGLDGLIYSKK	NEF-AE-13
081	GLDGLIYSKKRQEILDLW	NEF-AE-14
082	KKRQEILDLWVYNTQGFF	NEF-AE-15
083	LWVYNTQGFFPDWQNY	NEF-AE-16
084	QGFFPDWQNYTPGPEIRY	NEF-AE-17
085	QNYTPGPEIRYPLCFGWCF	NEF-AE-18
086	IRYPLCFGWCFKLVPV	NEF-AE-19
087	FGWCFKLVPVDPREVEED	NEF-AE-20
088	PVDPREVEEDNKGENNCL	NEF-AE-21
089	EDNKGENNCLLHPMSQH	NEF-AE-22
090	NCLLHPMSQHGIEDEER	NEF-AE-23
091	SQHGIEDEEREVLMWKF	NEF-AE-24
092	EEREVLMWKFDSALARKH	NEF-AE-25
093	KFDSALARKHIARELH	NEF-AE-26
094	ARKHIARELHPEYYKDC	NEF-AE-27
095	MAGRSGSTDEELLRAVRI	REV-AE-1
096	DEELLRAVRIIKILY	REV-AE-2
097	RAVRIIKILYQSNPY	REV-AE-3

OLP	Sequence	Protein OLP
098	IKILYQSNPYPSSEGTR	REV-AE-4
099	NPYPSSEGTRQTRKNRRR	REV-AE-5
100	TRQTRKNRRRRWRARQR	REV-AE-6
101	RRRRWRARQRQIRAISER	REV-AE-7
102	QRQIRAISERILSTCLGR	REV-AE-8
103	ERILSTCLGRSTEPVPL	REV-AE-9
104	LGRSTEPVPLQLPPLERL	REV-AE-10
105	PLQLPPLERLHLDCSEDC	REV-AE-11
106	RLHLDCSEDCGTSGTQQSQGTETGV	REV-AE-12
107	DCGTSGTQQSQGTETGVGRPQISG	REV-AE-13
108	QQSQGTETGVGRPQISGESSVIL	REV-AE-14
109	QISGESSVILGPGTKN	REV-AE-15
111	MELVDPNLEPWNHPGSQPT	TAT-AE-1
112	PWNHPGSQPTTACSKCY	TAT-AE-2
113	QPTTACSKCYCKKCCWH	TAT-AE-3
114	KCYCKKCCWHCQLCFLKK	TAT-AE-4
115	WHCQLCFLKKGLGISYGR	TAT-AE-5
116	KKGLGISYGRKKRKHRRG	TAT-AE-6
117	GRKKRKHRRGTPQSSKDH	TAT-AE-7
118	RGTPQSSKDHQNPIPE	TAT-AE-8
119	SKDHQNPIPEQPLPIIR	TAT-AE-9
120	IPEQPLPIIRGNPTDPK	TAT-AE-10
121	IIRGNPTDPKESKKEV	TAT-AE-11
122	TDPKESKKEVASKAETDPC	TAT-AE-12
123	VASKAETDPCD	TAT-AE-13
124	MTPLEISAIVGLIVALIL	VPU-AE-1
125	IVGLIVALILAIVVWTIV	VPU-AE-2
126	ILAIVVWTIVAIEFKKIL	VPU-AE-3
127	IVAIEFKKILRQRKIDRL	VPU-AE-4
128	ILRQRKIDRLVKRIRERA	VPU-AE-5
129	RLVKRIRERAEDSGNESE	VPU-AE-6
130	RAEDSGNESEGDTDELAK	VPU-AE-7
131	SEGDTDELAKLVEMGDFD	VPU-AE-8
132	AKLVEMGDFDPWVGDNL	VPU-AE-9
145	FFRENLAFQQGKAGEF	Gag/Pol POL-AE-1
146	AFQQGKAGEFSSEQTRA	POL-AE-2
147	GEFSSEQTRANSPTSRKL	POL-AE-3
148	RANSPTSRKLGDGGR	POL-AE-4
149	TSRKLGDGGRDNLLTEA	POL-AE-5

OLP	Sequence	Protein OLP
150	GRDNLLTEAGAERQGTSSSF	POL-AE-6
151	GAERQGTSSSFSFPQITLW	POL-AE-7
152	SSFSFPQITLWQRPLVTVK	protease POL-AE-8
153	LWQRPLVTVKIGGQLKEA	POL-AE-9
154	VKIGGQLKEALLDTGA	POL-AE-10
155	LKEALLDTGADDTVLEDI	POL-AE-11
156	GADDTVLEDINLPGKWK	POL-AE-12
157	EDINLPGKWKPKMIGGI	POL-AE-13
158	KWKPKMIGGIGGFIKVR	POL-AE-14
159	GGIGGFIKVRQYDQILI	POL-AE-15
160	KVRQYDQILIEICGKKAI	POL-AE-16
161	LIEICGKKAIGTVLV	POL-AE-17
162	GKKAIGTVLVGPTPVNII	POL-AE-18
163	LVGPTPVNIIGRNMLTQI	POL-AE-19
164	IIGRNMLTQIGCTLNFPI	POL-AE-20
165	QIGCTLNFPISPIDTVPV	POL-AE-21
166	PISPIDTVPVTLKPGM	RT POL-AE-22
167	TVPVTLKPGMDGPKVKQW	POL-AE-23
168	GMDGPKVKQWPLTEEKIK	POL-AE-24
169	QWPLTEEKIKALTEI	POL-AE-25
170	EEKIKALTEICKEMEE	POL-AE-26
171	LTEICKEMEEEGKISKI	POL-AE-27
172	MEEEGKISKIGPENPY	POL-AE-28
173	ISKIGPENPYNTPVFAIK	POL-AE-29
174	PYNTPVFAIKKKDSTKWR	POL-AE-30
175	IKKKDSTKWRKLVDFREL	POL-AE-31
176	WRKLVDFRELNKRTQDFW	POL-AE-32
177	ELNKRTQDFWEVQLGIPH	POL-AE-33
178	FWEVQLGIPHPAGLKKKK	POL-AE-34
179	PHPAGLKKKKSVTVLDV	POL-AE-35
180	KKKSVTVLDVGDAYFSV	POL-AE-36
181	LDVGDAYFSVPLDESFRK	POL-AE-37
182	SVPLDESFRKYTAFTI	POL-AE-38
183	SFRKYTAFTIPSINNETPGI	POL-AE-39
184	PSINNETPGIRYQYNVL	POL-AE-40
185	PGIRYQYNVLPQGWK	POL-AE-41
186	QYNVLPQGWKGSPAIF	POL-AE-42
187	QGWKGSPAIFQSSMTKIL	POL-AE-43
188	IFQSSMTKILEPFRI	POL-AE-44

OLP	Sequence	Protein OLP
189	MTKILEPFRIKNPEMVIY	POL-AE-45
190	RIKNPEMVIYQYMDDLYV	POL-AE-46
191	IYQYMDDLYVGSDLEI	POL-AE-47
192	DLYVGSDLEIGQHRTKI	POL-AE-48
193	LEIGQHRTKIEELRAHLL	POL-AE-49
194	KIEELRAHLLSWGFTTPDK	POL-AE-50
195	LSWGFTTPDKKHQKEPPF	POL-AE-51
196	DKKHQKEPPFLWMGYELH	POL-AE-52
197	PFLWMGYELHPDRWTV	POL-AE-53
198	YELHPDRWTVQPIELPEK	POL-AE-54
199	TVQPIELPEKDSWTVNDI	POL-AE-55
200	EKDSWTVNDIQKLVGKL	POL-AE-56
201	NDIQKLVGKLNWASQIYA	POL-AE-57
202	KLNWASQIYAGIKVKQL	POL-AE-58
203	IYAGIKVKQLCKLLRGAK	POL-AE-59
204	QLCKLLRGAKALTDIVPL	POL-AE-60
205	AKALTDIVPLTEEAELEL	POL-AE-61
206	PLTEEAELELAENREILK	POL-AE-62
207	ELAENREILKTPVHGVYY	POL-AE-63
208	LKTPVHGVYYDPSKDLVA	POL-AE-64
209	YYDPSKDLVAEVQKQGQDQW	POL-AE-65
210	EVQKQGQDQWTYQIY	POL-AE-66
211	GQDQWTYQIYQEPFKNLK	POL-AE-67
212	IYQEPFKNLKTGKYARKR	POL-AE-68
213	LKTGKYARKRSAHTNDVR	POL-AE-69
214	KRSAHTNDVRQLTEVVQK	POL-AE-70
215	VRQLTEVVQKIATESIVI	POL-AE-71
216	QKIATESIVIWGKTPKFR	POL-AE-72
217	VIWGKTPKFRLPIQRETW	POL-AE-73
218	FRLPIQRETWETWWMEYW	POL-AE-74
219	TWETWWMEYWQATWIPEW	POL-AE-75
220	YWQATWIPEWEFVNTPPL	POL-AE-76
221	EWEFVNTPPLVKLWYQL	POL-AE-77
222	PPLVKLWYQLEKDPIVGA	POL-AE-78
223	QLEKDPIVGAETFYVDGA	POL-AE-79
224	GAETFYVDGAASRETKL	POL-AE-80
225	DGAASRETKLGKAGYV	POL-AE-81
226	ETKLGKAGYVTDRGRQKV	POL-AE-82
227	YVTDRGRQKVVSLTETTNQK	POL-AE-83

OLP	Sequence	Protein OLP
228	VSLTETTNQKTELHAIHL	POL-AE-84
229	QKTELHAIHLALQDSGS	POL-AE-85
230	IHLALQDSGSEVNIV	POL-AE-86
231	QDSGSEVNIVTDSQYAL	POL-AE-87
232	NIVTDSQYALGIIQA	POL-AE-88
233	SQYALGIIQAQPDRSESEV	POL-AE-89
234	AQPDRSESEVVNQIIEEL	POL-AE-90
235	EVVNQIIEELIKKEKVYL	POL-AE-91
236	ELIKKEKVYLSWVPAHK	POL-AE-92
237	VYLSWVPAHKGIGGNEQV	POL-AE-93
238	HKGIGGNEQVDKLVSSGI	POL-AE-94
239	QVDKLVSSGIRKVLFL	POL-AE-95
240	SSGIRKVLFLDGIDKA	POL-AE-96
241	VLFLDGIDKAQEEHERYH	Integrase POL-AE-97
242	KAQEEHERYHSNWRTMA	POL-AE-98
243	RYHSNWRTMASDFNLPPI	POL-AE-99
244	MASDFNLPPIVAKEIVA	POL-AE-100
245	PPIVAKEIVANCDKCQLK	POL-AE-101
246	VANCDKCQLKGEAMHGQV	POL-AE-102
247	LKGEAMHGQVDCSPGIW	POL-AE-103
248	GQVDCSPGIWQLDCTHL	POL-AE-104
249	GIWQLDCTHLEGKVILVA	POL-AE-105
250	HLEGKVILVAVHVASGYI	POL-AE-106
251	VAVHVASGYIEAEVIPA	POL-AE-107
252	GYIEAEVIPAETGQETAY	POL-AE-108
253	PAETGQETAYFLLKLAGR	POL-AE-109
254	AYFLLKLAGRWPVKVIH	POL-AE-110
255	AGRWPVKVIHTDNGSNF	POL-AE-111
256	VIHTDNGSNFTSAAVKAA	POL-AE-112
257	NFTSAAVKAACWWANVR	POL-AE-113
258	KAACWWANVRQEFGIPY	POL-AE-114
259	NVRQEFGIPYNPQSQGVV	POL-AE-115
260	PYNPQSQGVVESMNKELK	POL-AE-116
261	VVESMNKELKKIIGQVR	POL-AE-117
262	ELKKIIGQVREQAEHLK	POL-AE-118
263	QVREQAEHLKTAVQMAVF	POL-AE-119
264	LKTAVQMAVFIHNFKRK	POL-AE-120
265	AVFIHNFKRKGGIGGYSA	POL-AE-121
266	RKGGIGGYSAGERIIDII	POL-AE-122

OLP	Sequence	Protein OLP
267	SAGERIIDIIATDIQTK	POL-AE-123
268	DIIATDIQTKELQKQITK	POL-AE-124
269	TKELQKQITKIQNFRVYY	POL-AE-125
270	TKIQNFRVYYRDSRDPIW	POL-AE-126
271	YYRDSRDPIWKGPAKLLW	POL-AE-127
272	IWKGPAKLLWKGEGAVVI	POL-AE-128
273	LWKGEGAVVIQDNSDIKV	POL-AE-129
274	VIQDNSDIKVVPRRKAKI	POL-AE-130
275	KVVPRRKAKIIRDYGKQM	POL-AE-131
276	KIIRDYGKQMAGDDCVA	POL-AE-132
277	KQMAGDDCVAGRQDED	POL-AE-133
278	MEQAPEDQGPQREPYNEW	VPR-AE-1
279	GPQREPYNEWTLELLEEL	VPR-AE-2
280	EWTLELLEELKNEAVRHF	VPR-AE-3
281	ELKNEAVRHFPRPWLHGL	VPR-AE-4
282	HFPRPWLHGLGQYIYNNY	VPR-AE-5
283	GLGQYIYNNYGDTWEGV	VPR-AE-6
284	NNYGDTWEGVEAIIRIL	VPR-AE-7
285	EGVEAIIRILQQLLFVHF	VPR-AE-8
286	ILQQLLFVHFRIGCQHSR	VPR-AE-9
287	HFRIGCQHSRIGIIPGRR	VPR-AE-10
288	SRIGIIPGRRGRNGAGRS	VPR-AE-11
289	MRVKETQMNWPNLWKW	ENV-AE-1
290	QMNWPNLWKWGTLILGLV	Signal ENV-AE-2
291	KWGTLILGLVIICSAS	ENV-AE-3
292	LGLVIICSASDNLWVTVY	gp120 ENV-AE-4
293	ASDNLWVTVYYGVPVWR	ENV-AE-5
294	TVYYGVPVWRDADTTLF	ENV-AE-6
295	VWRDADTTLFCASDAKAH	ENV-AE-7
296	LFCASDAKAHETEVHNVW	ENV-AE-8
297	AHETEVHNVWATHACV	ENV-AE-9
298	HNVWATHACVPTDPNPQEI	ENV-AE-10
299	VPTDPNPQEIHLENV	ENV-AE-11
300	NPQEIHLENVTENFNMWK	ENV-AE-12
301	NVTENFNMWKNNMVEQMQ	ENV-AE-13
302	WKNNMVEQMQEDVISLW	ENV-AE-14
303	QMQEDVISLWDQSLKPCV	ENV-AE-15
304	LWDQSLKPCVKLTPLCV	ENV-AE-16
305	PCVKLTPLCVTLNCTNA	ENV-AE-17

OLP	Sequence	Protein OLP
306	LCVTLNCTNANLTNVNNT	ENV-AE-18
307	NANLTNVNNTTVSNIIGN	ENV-AE-19
308	VNNTTVSNIIGNITDEVR	ENV-AE-20
309	IIGNITDEVRNCSFNM	ENV-AE-21
310	DEVRNCSFNMTTELRDKK	ENV-AE-22
311	NMTTELRDKKQKVHALFY	ENV-AE-23
312	KKQKVHALFYKLDIVQI	ENV-AE-24
313	LFYKLDIVQINNNSSEYR	ENV-AE-25
314	QINNNSSEYRLINCNTSV	ENV-AE-26
315	YRLINCNTSVIKQACPKI	ENV-AE-27
316	SVIKQACPKISFDPIPIH	ENV-AE-28
317	KISFDPIPIHYCTPAGYA	ENV-AE-29
318	IHYCTPAGYAILKCNDK	ENV-AE-30
319	GYAILKCNDKNFNGTGPC	ENV-AE-31
320	DKNFNGTGPCKNVSSV	ENV-AE-32
321	TGPCKNVSSVQCTHGIK	ENV-AE-33
322	SSVQCTHGIKPVVSTQLL	ENV-AE-34
323	IKPVVSTQLLLNGSLA	ENV-AE-35
324	TQLLLNGSLAEEEIIIR	ENV-AE-36
325	SLAEEEIIIRSENLTNNA	ENV-AE-37
326	IRSENLTNNAKTIIVHL	ENV-AE-38
327	NNAKTIIVHLNKSVEI	ENV-AE-39
328	IVHLNKSVEINCTRPSNNTR	ENV-AE-40
329	NCTRPSNNTRTSITI	ENV-AE-41
330	SNNTRTSITIGPGQVFY	ENV-AE-42
331	ITIGPGQVFYRTGDII	ENV-AE-43
332	QVFYRTGDIIGDIRKAY	ENV-AE-44
333	DIIGDIRKAYCEINGTKW	ENV-AE-45
334	AYCEINGTKWNETLKQV	ENV-AE-46
335	TKWNETLKQVTEKLKEHF	ENV-AE-47
336	QVTEKLKEHFNNKTIIF	ENV-AE-48
337	EHFNNKTIIFQPPSGGDLEI	ENV-AE-49
338	QPPSGGDLEITMHHF	ENV-AE-50
339	GDLEITMHHFNCRGEFFY	ENV-AE-51
340	*HFNCRGEFFYCNTTKLF*	*ENV*-AE-52
341	FFYCNTTKLFNSTCIGNE	ENV-AE-53
342	LFNSTCIGNEMEGCNGTI	ENV-AE-54
343	MEGCNGTIILPCKIK	ENV-AE-55
344	GTIILPCKIKQIINMW	ENV-AE-56

OLP	Sequence	Protein OLP
345	CKIKQIINMWQGVGQAMY	ENV-AE-57
346	MWQGVGQAMYAPPISGRI	ENV-AE-58
347	MYAPPISGRINCVSNI	ENV-AE-59
348	SGRINCVSNITGILLTR	ENV-AE-60
349	SNITGILLTRDGGNNNTT	ENV-AE-61
350	TRDGGNNNTTNETFR	ENV-AE-62
351	NNNTTNETFRPGGGNIK	ENV-AE-63
352	TFRPGGGNIKDNWRSELY	ENV-AE-64
353	IKDNWRSELYKYKVVQI	ENV-AE-65
354	ELYKYKVVQIEPLGIA	ENV-AE-66
355	VVQIEPLGIAPTRAKRRV	ENV-AE-67
356	IAPTRAKRRVVEREKRAV	ENV-AE-68
357	RVVEREKRAVGIGAMIF	gp41 ENV-AE-69
358	RAVGIGAMIFGFLGAA	ENV-AE-70
359	AMIFGFLGAAGSTMGAA	ENV-AE-71
360	GAAGSTMGAASITLTVQA	ENV-AE-72
361	AASITLTVQARQLLSGIV	ENV-AE-73
362	QARQLLSGIVQQQSNLLR	ENV-AE-74
363	IVQQQSNLLRAIEAQQHL	ENV-AE-75
364	LRAIEAQQHLLQLTVWGI	ENV-AE-76
365	HLLQLTVWGIKQLQARVL	ENV-AE-77
366	GIKQLQARVLAVERYLK	ENV-AE-78
367	RVLAVERYLKDQKFLGLW	ENV-AE-79
368	LKDQKFLGLWGCSGKII	ENV-AE-80
369	GLWGCSGKIICTTAVPW	ENV-AE-81
370	KIICTTAVPWNSTWSNK	ENV-AE-82
371	VPWNSTWSNKSYEEIW	ENV-AE-83
372	WSNKSYEEIWNNMTWIEW	ENV-AE-84
373	IWNNMTWIEWEREISNY	ENV-AE-85
374	IEWEREISNYTNQIYEIL	ENV-AE-86
375	NYTNQIYEILTESQNQQDR	ENV-AE-87
376	LTESQNQQDRNEKDLLEL	ENV-AE-88
377	DRNEKDLLELDKWASLW	ENV-AE-89
378	LELDKWASLWNWFDITNW	ENV-AE-90
379	LWNWFDITNWLWYIKIFI	ENV-AE-91
380	NWLWYIKIFIMIVGGLI	ENV-AE-92
381	IFIMIVGGLIGLRIIFAV	ENV-AE-93
382	LIGLRIIFAVLSIVNRVR	ENV-AE-94
383	AVLSIVNRVRQGYSPLSF	ENV-AE-95

OLP	Sequence	Protein OLP
384	VRQGYSPLSFQTPTHHQR	ENV-AE-96
385	SFQTPTHHQREPDRPERI	ENV-AE-97
386	QREPDRPERIEEGGGEQ	ENV-AE-98
387	ERIEEGGGEQGRDRSVRL	ENV-AE-99
388	EQGRDRSVRLVSGFLALA	ENV-AE-100
389	RLVSGFLALAWDDLRSL	ENV-AE-101
390	ALAWDDLRSLCLFSYHRL	ENV-AE-102
391	SLCLFSYHRLRDFILIA	ENV-AE-103
392	HRLRDFILIAARTVELL	ENV-AE-104
393	LIAARTVELLGHSSLKGLRRGWEGL	ENV-AE-105
394	LLGHSSLKGLRRGWEGLKYLGNLL	ENV-AE-106
395	EGLKYLGNLLLYWGQELK	ENV-AE-107
396	LLLYWGQELKISAISLL	ENV-AE-108
397	ELKISAISLLDATAIAVA	ENV-AE-109
398	LLDATAIAVAGWTDRVI	ENV-AE-110
399	AVAGWTDRVIEVAQRAWR	ENV-AE-111
400	VIEVAQRAWRAILHIPRR	ENV-AE-112
401	WRAILHIPRRIRQGLERA	ENV-AE-113
402	RRIRQGLERALL	ENV-AE-114
403	MENRWQVMIVWQVDRMRI	VIF-AE-1
404	IVWQVDRMRIRTWNSLVK	VIF-AE-2
405	RIRTWNSLVKHHMYISKK	VIF-AE-3
406	VKHHMYISKKAKKWFYRH	VIF-AE-4
407	KKAKKWFYRHHYESQHPK	VIF-AE-5
408	RHHYESQHPKVSSEVHI	VIF-AE-6
409	HPKVSSEVHIPLGEARLV	VIF-AE-7
410	HIPLGEARLVIRTYWGLQ	VIF-AE-8
411	LVIRTYWGLQTGEKDWQL	VIF-AE-9
412	LQTGEKDWQLGHGVSIEW	VIF-AE-10
413	QLGHGVSIEWRQRNY	VIF-AE-11
414	VSIEWRQRNYSTQIDPDL	VIF-AE-12
415	NYSTQIDPDLADQLIHLQ	VIF-AE-13
416	DLADQLIHLQYFDCF	VIF-AE-14
417	LIHLQYFDCFSDSAIRKA	VIF-AE-15
418	CFSDSAIRKAILGQVV	VIF-AE-16
419	IRKAILGQVVRRRCEYPS	VIF-AE-17
420	VVRRRCEYPSGHNKVGSL	VIF-AE-18
421	PSGHNKVGSLQYLALKAL	VIF-AE-19
422	SLQYLALKALTTPKRIR	VIF-AE-20

OLP	Sequence	Protein OLP
423	KALTTPKRIRPPLPSVKK	VIF-AE-21
424	IRPPLPSVKKLTEDRWNK	VIF-AE-22
425	KKLTEDRWNKPQKIRGHR	VIF-AE-23
426	NKPQKIRGHRENPTMNGH	VIF-AE-24

BIOGRAPHY

Miss Supranee Buranapraditkun was born on May 17, 1972 in Bangkok, Thailand. She graduated with the Bachelor degree of Science in Medical Technology from the Faculty of Medicine, Chulalongkorn University in 1995 and Master in Interdepartment of Microbiology from the Faculty of Graduate School, Chulalongkorn University in 2001. Since 1995 to present, she has been working at the Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok. She entered the degree of Doctor of Philosophy Program in Interdepartment of Microbiology from the Faculty of Graduate School, Chulalongkorn Chulalongkorn since 2007. She has obtained the scholarship from the Royal Golden Jubilee Ph.D. Program Thailand Research Fund.