การหลบหลีกภูมิคุ้มกันที่เกิดจากไซโตท็อกซิก ที ลิมฟ์โฟไซต์ในคนไข้เอชไอวี
ที่เป็น viraemic controller และ non-controller

นางสาวประทานพร แก้วปรีดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CTL-MEDIATED IMMUNE EVASION IN HIV VIRAEMIC CONTROLLERS AND NON-CONTROLLERS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Microbiology
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ประทานพร แก้วปรีดี : การหลบหลีกภูมิคุ้มกันที่เกิดจากไซโตท็อกซิก ที ลิมฟ์โฟไซต์ในคนไข้เอชไอวีที่เป็น viraemic controller และ non-controller (CTL-MEDIATED IMMUNE EVASION IN HIV VIRAEMIC CONTROLLERS AND NON-CONTROLLERS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ปกรัฐ หังสสูต, 125 หน้า.

วัตถุประสงค์: เพื่อเปรียบเทียบการตอบสนองของทีเซลล์และหากลไกที่เชื้อไวรัสเอชไอวีใช้ในการหลบหลีกจากภูมิคุ้มกัน

วิธีดำเนินการทดลอง: รวบรวมอาสาสมัครที่ติดเชื้อเอชไอวี ที่มีระดับซีดีสี่มากกว่า 450 cells/μL จากคลินิกนิรนาม สภากาชาดไทย โดยแบ่งอาสาสมัครเป็น 2 กลุ่ม ตามปริมาณไวรัสในกระแสเลือด (VL) เป็น viraemic controllers (VC) (VL<2,000 cp/mL) และ non-controllers (NC) (VL>2,000 cp/mL) จากนั้นเก็บเลือดเพื่อนำมาทดสอบการตอบสนองต่อโปรตีนของเชื้อเอชไอวีส่วน Gag p24 ด้วยวิธี IFNγ ELISpot assay และทำการหาลำดับสารพันธุกรรมเพื่อใช้ในการวิเคราะห์กลไกที่ใช้ในการหลบหลีกจากภูมิคุ้มกัน

ผลการทดลอง: VC มีจำนวน 23 คน และ NC มีจำนวน 41 คน โดยที่ปัจจัยต่างๆ ได้แก่ อายุ เพศ รสนิยมทางเพศ การมี protective alleles (HLA-B27, -B57, -B58) นั้นไม่แตกต่างกัน จากการทดสอบการตอบสนองต่อโปรตีน Gag p24 ด้วยวิธี IFNγ ELISpot assay โดยกระตุ้นด้วยเอพิโทปที่จำเพาะต่อชนิดของเอชแอลเอของอาสาสมัคร พบว่ามีอาสาสมัครจำนวนหนึ่งที่ไม่สามารถตอบสนองต่อเอพิโทปดังกล่าวได้ (Non-responder) กล่าวคือ อาสาสมัครที่มี HLA-B27 ที่ไม่ตอบสนองต่อ KK10 นั้น มีสาเหตุจากการมี HLA polymorphism เป็น HLA-B2706 แทนที่จะเป็น HLA-B2704 หรือ HLA-B2705 อาสาสมัครที่มี HLA-B57 เมื่อทดสอบด้วย TW10 นั้น พบว่าผู้ที่ไม่ตอบสนองมี mutation ที่ตำแหน่ง T242N และเมื่อทดสอบด้วย QW9 พบว่าผู้ที่ไม่ตอบสนอง มี mutation ที่ตำแหน่ง T3S ในขณะที่อาสาสมัครที่มี HLA-A11 เมื่อทดสอบด้วย AK11 นั้นไม่พบการกลายพันธุ์ในระดับโปรตีน แต่พบความแตกต่างอย่างมีนัยสำคัญในระดับนิวคลีโอไทด์ (codon usage) ที่ตำแหน่ง anchor residue ซึ่งตรงกับกรดอะมิโนไลซีน กล่าวคือ ผู้ที่มีการตอบสนองต่อ AK11 มักใช้ codon AAG ในขณะที่ผู้ที่ไม่ตอบสนองมักใช้ AAA ในการแปลรหัสเป็นกรดอะมิโนไลซีน โดยสรุปจากศึกษาในครั้งนี้สามารถนำความรู้ที่ได้เกี่ยวกับกระบวนที่ไวรัสใช้ในการหลบหลีกจากภูมิคุ้มกันไปประยุกต์ใช้ในการพัฒนาวัคซีนต่อไปในอนาคต

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5387787320 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: HIV / CTL / ESCAPE MUTATION

PRATHANPORN KAEWPREEDEE: CTL-MEDIATED IMMUNE EVASION IN HIV VIRAEMIC CONTROLLERS AND NON-CONTROLLERS. ADVISOR: ASST. PROF. POKRATH HANSASUTA, M.D., DPhil., 125 pp.

Objective: To compare T cell response and to identify the mechanism of escape in HIV-infected volunteers.

Methods: HIV-infected volunteers (CD4>450 cells/μL) were enrolled from the anonymous clinic. Volunteers were classified into two groups based on HIV plasma viral load; viraemic controllers (VC) (VL<2000 cp/mL) and non-controllers (NC) (VL>2000 cp/mL). EDTA-whole blood was collected and determined the response against Gag p24 by IFNγ ELISpot assay. Population sequencing within the Gag p24 region was performed to identify escape mutations.

Results: Demographic data [age, gender, sexual preference and the presence of protective alleles (HLA-B27, -B57 and –B58)] between VC (n=23) and NC (n=41) were no statistical significance. IFNγ-secreting cells response against Gag p24 as determining by ELISpot assay revealed that some volunteers could not respond to epitopes which are restricted by their HLA alleles (termed as non-responder). The HLA-B27-KK10 non-responders contain HLA polymorphism, HLA-B2706 which accommodates different amino acid with HLA-B2704 and HLA-B2705. The HLA-B57-TW10 and QW9, non-responders did not respond because escape mutation (T242N and T3S, respectively) was found within non-responders' sequences. Interestingly, HLA-A11-AK11 epitope at the amino acid level was not different among responders and non-responders. Codon usages were subsequently compared and it was found that at the C-terminal position (K; Lysine) preferred different codons for translating to lysine. AAG codon is more preferable in AK11-responders while AAA codon is preferred in AK11 non-responders. In conclusion, these findings may contribute to better knowledge on HIV pathogenesis and the way to improve vaccine efficacy in the future.

Field of Study: Medical Microbiology Student's Signature .....

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LIST OF ABBREVIATIONS

AICD	Activation induced cell death
AIDS	Acquired immune deficiency syndrome
ALP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
ART	Antiretroviral therapy
ATCC	American type culture collection
AZT	Azidothymidine/Zidovudine
B-LCL	B lymphoblastoid cell line
BSA	Bovine serum albumin
BV	Brilliant violet
CA	Capsid
cART	Combination antiretroviral therapy
CBC	Complete blood count
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
cp	Copies
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double-stranded
EDTA	Ethylenediaminetetraacetic acid

LIST OF ABBREVIATIONS (Continued)

EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	Glycoprotein
HEK	Human embryonic kidney
HD	Healthy donor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HTLV-III	Human T cell lymphotropic virus type III
IFN	Interferon
IL	Interleukin
IVDU	Intravenous drug user
kb	kilobase
kD	kilodalton
LAV	Lymphadenopathy-associated virus
LEDGF	Lens epithelium-derived growth factor
LTNP	Long-term non-progressor
LTR	Long terminal repeat
MA	Matrix
mAb	Monoclonal antibodies
MALT	Mucosal-associated lymphoid tissue
MIP	Macrophage inflammatory protein
mL	Milliliter

LIST OF ABBREVIATIONS (Continued)

mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
MTCT	Mother-to-child transmission
NC (Patient)	Non-controller
NC (Protein)	Nucleocapsid
NK	Natural killer
NNRTI	Non-nucleoside analog reverse transcriptase inhibitor
NR	Non-responder
NRTI	Nucleoside analog reverse transcriptase inhibitor
OI	Opportunistic infection
OLP	Overlapping peptide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-SSOP	Polymerase chain reaction-sequence specific oligonucleotide probes
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PRR	Pattern recognition receptor
PHA	Phytohemagglutinin
P-TEFb	Positive transcription elongation factor b
pVL	Plasma viral load
R	Responder
RNA	Ribonucleic acid
RPM	Round per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SFU	Spot forming unit

LIST OF ABBREVIATIONS (Continued)

SIV	Simian immunodeficiency virus
ss	Single-stranded
STD	Sexually-transmitted disease
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIM	Tripartite motif-containing protein
USFDA	United States food and drug administration
VC	Viraemic controller
VL	Viral load
WBC	White blood cell
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Human immunodeficiency virus (HIV) is known to be a cause of acquired immunodeficiency syndrome (AIDS). Three decades since HIV was first identified in 1981 (1), AIDS is still a major global health problem. Current data from World Health Organization (WHO) in 2012 shows that 35.3 million people worldwide are living with HIV (2). In Thailand, HIV prevalence from 2009 to 2013 was 0.5-0.6%. There are approximately 452,000 adults living with HIV (3). Populations classified as high risk groups are men who have sex with men (MSM), intravenous drug users (IVDU), commercial sex workers, blood recipients who received contaminated blood transfusions and children whose mothers are HIV seropositive have a chance of becoming infected during childbirth or breastfeeding (4). Although a number of HIV research have been performed, researchers do not known which immune cell is the key component to HIV protection.

HIV is a member of family *Retroviridae* and genus *Lentivirus*. The genetic material of HIV is composed of 2 identical negative sense single-stranded RNA strands. The unique characteristic of family *Retroviridae* is that they have a reverse transcriptase enzyme (RNA-dependent DNA polymerase) for reverse transcription process which converts single-stranded RNA to double-stranded DNA (5). HIV also has an ability to integrate its genome into the host genome using the integrase enzyme after reverse transcription and maintains itself for lifetime of the integrated cells. HIV has nine important genes which can be classified into three groups based on function. First, the structural genes which are *gag* (group-specific antigen; p17 matrix, p24 capsid, p7 nucleocapsid and p6), *env* (envelop) and *pol* (Polymerase; integrase, protease and reverse transcriptase). Second are the regulatory genes which are *Tat* (transcription *trans*-activator) and *rev* (regulator of expression of virion proteins). Lastly, the accessory genes which are *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein unique) and *nef* (negative factor) (5). One hundred million new virions are produced per day. The replication rate per year is approximately 150 to 300 replication cycles. Because of the lack of proof-reading ability, low fidelity and high replication

rate, HIV also has high mutation rate approximately 1 nucleotide per 10^4 to 10^5 nucleotides (6, 7). The important characteristic of HIV is an ability to become quasispecies which has many differences in viral population within one infected person (8).

HIV can infect both $CD4^+$ helper T cells and $CD4^+$ macrophages. It enters to host cell by using glycoprotein120 (gp120) binds to human CD4 molecule as a main receptor and also requires a co-receptor for entry. The two main co-receptors are CCR5 and CXCR4 which act as chemokine receptor for humans.

The natural history of HIV infection can be divided into three phases; acute infection, asymptomatic or chronic phase and AIDS phase. Acute infection is defined by the presence of the HIV p24 antigen but the absence of the antibody. Flu-like symptoms may or may not appear depending on each person. At this phase, $CD4^+$ T cells which are the HIV target are decreased due to the elimination of infected cells and the activation induced cell death (AICD). Plasma HIV viral load is greater than 10^6-10^7 copies/mL. Subsequently, plasma HIV viral load drops to a steady state which is called the viral set point. $CD4^+$ T cells rebound but not to the initial level. In the chronic phase there are usually no symptoms. Timing of the chronic phase can classify infected patients into three groups. The rapid progressor is a person who is infected with HIV for less than 3 years and progresses to the AIDS phase, the typical progressor usually develops AIDS within 5-7 years while the long-term non-progressor (LTNP) takes more than 10 years. The last phase is the AIDS phase in which patients can develop opportunistic infections and antiretroviral therapy has to be initiated.

HIV can trigger both innate immunity such as natural killer cells, dendritic cells and macrophages and adaptive immunity such as B cells which produce neutralizing antibodies and T cells. The strong evidence in macaque models has shown that $CD8^+$ T cells (CTL) play an important role in controlling HIV viral load. Macaques were infected with simian immunodeficiency virus (SIV) which develops similar immunopathogenesis as HIV in humans. When plasma SIV load was stable, $CD8^+$ T cells were depleted by monoclonal antibody against CD8 molecules. After CD8 molecules depletion, the results show that macaques could no longer control viral replication. Both plasma SIV load and proviral DNA increased. When $CD8^+$ T cells

were presented, the plasma SIV load and proviral DNA were simultaneously dropped (9).

$CD8^+$ T cells recognize HIV antigens via HLA-peptide complex on the surface of antigen presenting cells (APCs). First HIV antigens are processed by proteasomes into small peptides in cytosol. Then, TAP protein transports small peptides into the endoplasmic reticulum for amino-terminal trimming (6). The optimal peptide length between 8 to 12 amino acids, or epitope, binds to HLA class I molecule using anchor residues of the epitope which locate at position 2 (P2) and C-terminal embedded into B-pocket and F-pocket of HLA class I molecule respectively. $CD8^+$ T cells use T cell receptor (TCR) to recognize HLA-peptide complex contacting at TCR residues of the epitope. After antigen recognition, T cell signaling initiates to respond against HIV. $CD8^+$ T cell modulates HIV replication by proliferation, cytotoxicity using perforin and granzyme, cytokines and chemokine secretion such as macrophage inflammatory protein 1-beta (MIP-1β), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2). A several reports have shown that $CD8^+$ T cells with polyfunctionality are associated with low HIV plasma viral load (10, 11). Some HLA class I molecules are believed to have a protective effect and in controlling HIV. The studies in Caucasian populations have shown that infected patients who carry HLA-B57, HLA-B57 and HLA-B58 were associated with good clinical outcome (6, 12). These patients tended to have higher $CD4^+$ T cell count, low plasma HIV load and slower progression to the AIDS phase when compared to patients who do not carry the protective alleles (13). The exact mechanism in which HLA class I molecules contribute to protection still unclear. Several studies have shown that these protective alleles present HIV antigens which have high fitness cost. For example, HLA-B27-restricted KK10 (KRWIILGLNK) epitope is high functional constraint because it is important for capsid protein dimerization (14, 15).

$CD8^+$ T cells have a strong selective pressure which drives HIV to escape from immune responses. HIV is forced to select an escape variant to become a major population instead of the wild type for survival. Some escape variants effect the HIV replicative capacity and also the host immune response (15). Evidence has shown that when an escape variant was transmitted from a patient with selective pressure such as HLA protective alleles to one without pressure, the major population was reverted to

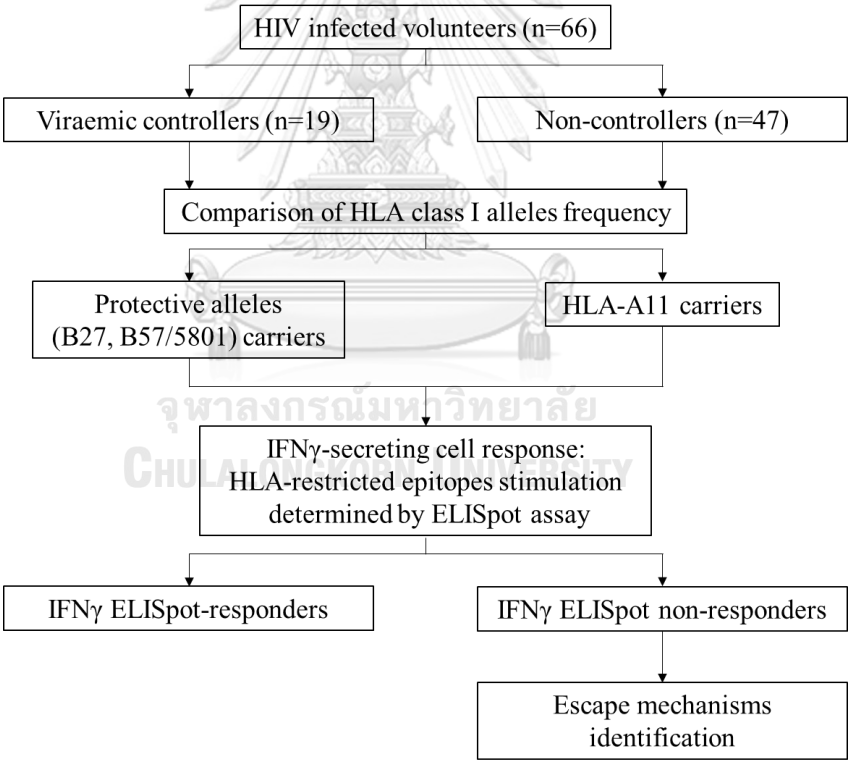
the wild type (16). There are 2 types of HIV escape mechanism: mutational and non-mutational mechanisms. Mutational escape is the change in sequences in intraepitopic region which may affect binding to HLA class I molecules and/or T cell receptors and in the flanking region which may affect antigen processing and presentation. Non-mutational escapes are the down-regulation of HLA class I molecules especially HLA-A and HLA-B by Nef protein, destruction of $CD4^+$ T cells and ability to become latent (6, 17). Translational efficiency is one of the possible mechanisms which may affect T cell response. A report has shown that even though the amino acid sequences in the Gag-RY10 epitope were similar, T cell responses were different (18). They proposed that these may be a difference in the densities of the HLA-peptide complex on the surface of target cells.

Due to the fact that the human codon has 61 different codons for translation into 20 amino acids; one amino acid may have more than one codon available for translation. Codon bias is a phenomenon which organisms prefer to use synonymous codons in different frequencies (19). Factors that contribute to use different have been reported such as nucleotide composition, percentage of cytosine (C) and guanine (G) in the genome, the availability of tRNA pool and mutation selection (20-25). Studies have shown that human and HIV prefer to use different codons for translating synonymous amino acid (19, 26-28). For example, Lysine (K) can be translated by two codons; AAA and AAG. Humans prefer AAG codon while HIV prefers AAA codon. Codon optimization from non-preferable codon to preferable codon can enhance the level of protein expression (29, 30). However, there is no direct evidence that shows different codon usage may contribute to different specific epitope presentation and also affect CTL response. This study aims to determine the role of codon usage on epitope expression and the effect of escape mutation on CTL response.

CHAPTER II
OBJECTIVES

1. To analyze longitudinal CTL response in HIV controllers and non-controllers
2. To compare CTL response in HLA-matched HIV-infected individuals
3. To analyze sequences of CTL targets
4. To identify mechanism of escape in HIV-infected individuals

Work flow



CHAPTER III
LITERATURE REVIEWS

Human Immunodeficiency Virus

Human Immunodeficiency Virus or HIV causes acquired immunodeficiency syndrome (AIDS). The HIV term derives from ‘human’ as a host, ‘immunodeficiency’ as a following condition after infection and this organism is a ‘virus’. The first few cases report of HIV were presented during 1981. In the past, HIV has been called LAV or HTLV-III. Since 1986, HIV has been officially nomenclated by the International Committee on the Taxonomy of Viruses (31).

HIV-1 is originated from apes. It has been thought that zoonotic infection was initiated in bush meat hunters. Phylogenetic analysis has been shown that there are 4 groups of HIV; M, N, O and P. Group M, N and O were transmitted from chimpanzees when group P was transmitted from gorillas (32). While group M causes global HIV pandemic, group N, O and P are restricted only in Africa. Group M can be further classified into different subtypes which are subtype A, B, C, D, F, G, H, J, and K. B subtype is predominant in the Americas, Western Europe and Australia while C subtype is mainly found in Africa and accounted for 50% in global as shown in Figure 1. Moreover, there are recombinant forms of subtypes called circulating-recombinant forms (CRFs). In Thailand and Southeast Asia, a recombinant between A and E subtypes named CRF_01AE is the most dominant (33).

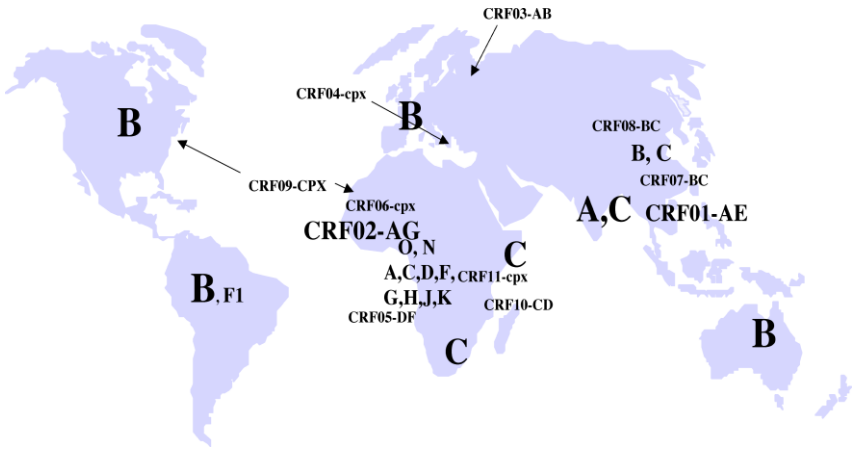


Figure 1: HIV subtypes distribution (34).

As World Health Organization report on global AIDS epidemic, there are 36.7 million people who are living with HIV. Most of them are adults (34.9 million). In 2015, HIV incidence rate was 2.1 million and mortality rate was 1.1 million (35). In Thailand, there are approximately 440,000 people living with HIV with 1.1% incidence rate (36).

HIV genome

HIV is a lentivirus which belongs to family *Retroviridae*. Viruses in this family have a common feature which are able to alternate their ssRNA genome into dsDNA using RNA-dependent DNA polymerase or reverse transcriptase (RT) enzyme. HIV genome contains two negative sense single-stranded RNA. Genome size is approximately 9.7kb (HXB2 reference strain, GenBank Accession Number K03455). Long terminal repeats or LTRs are responsible for transcription initiation and polyadenylation (37).

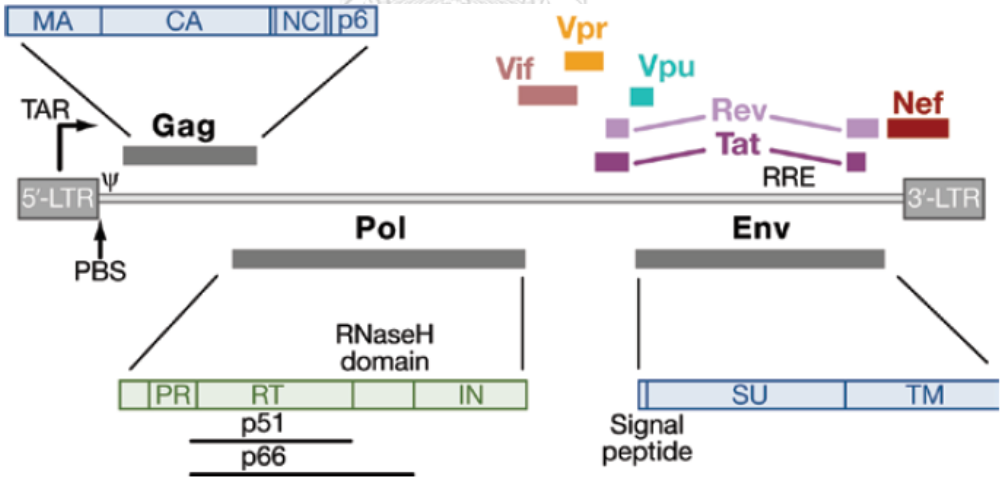


Figure 2: HIV genome (38).

HIV proteins

Nine encoded-proteins of HIV are classified into 3 groups based on their functions. Structural proteins, Gag, Pol and Env, are essential for viral structure. Regulatory proteins consist of Tat and Rev. Accessory proteins are Vif, Vpr, Vpu and Nef.

Structural proteins

Gag

A 55-kD protein so called $Pr55^{Gag}$ as Gag precursor protein. $Pr55^{Gag}$ is further cleaved into 4 proteins; p17 matrix (MA), p24 capsid (CA), p7 nucleoprotein (NC) and p6 C-terminal protein. Matrix is myristoylated protein which locates in N-terminal of $Pr55^{Gag}$ and attaches to inner membrane of viral lipid bilayer. It is responsible for stabilizing viral particle and importing viral genome into nucleus (39). Capsid is a core structure of virus and interacts with host cyclophillin A to construct a viral particle as a conical core. This process can inhibit by cyclosporine A (40). Nucleoprotein can recognize HIV packaging signal to promote viral assembly. NC also involves in reverse transcription (41). Polyprotein p6 is responsible for Vpr incorporation (42) and virion budding. $Pr55^{Gag}$ also produces small peptides p1 and p2 but the exact function is still unknown (43).

Pol

Pol protein is cleaved by protease from Gag-Pol polyprotein. There are 3 enzymes encoded by Pol; protease (PR), reverse transcriptase (RT) and integrase (IN). Protease is essential for Gag-Pol Polyproteins processing and maturation (44). Reverse transcriptase or RT is a characteristic of *Retroviridae* family. RT converts viral RNA into viral DNA within 6 hours after entry into host cells (45). RNase H domain of RT degrades RNA template to generate complementary DNA. According to RT has no ability to proof-read, mutation is prone to occur easily (46, 47). RT is one the most important drug targets for antiretroviral therapy. Integrase is responsible for viral genome integration. Three major roles are trimming viral DNA by an exonuclease, cleaving host DNA at integration site by an endonuclease and lastly ligating viral DNA with host genome by a ligase with covalent bonds (48).

Env

A 160-kD glycosylated protein also known as glycoprotein 160 (gp160). Glycoprotein 160 is synthesized in endoplasmic reticulum and transported to Golgi bodies for glycosylation. This glycosylation is important for viral infectivity. The gp160 is then cleaved by protease into surface glycoprotein (SU, gp120) as trimeric molecules and transmembrane glycoprotein (TM, gp41). Glycoprotein 120 exposes on the surface and binds to CD4 receptor and CCR5, CXCR4 as co-receptors on the host cell to facilitate virus attachment and entry (49). Virus later uses gp41, transmembrane domain, for fusion with host cell membrane and entry.

Regulatory proteins

Tat

Tat is a transcriptional *trans*-activator protein found in nuclei of an infected cell. Tat binds directly to transactivation responsive region (TAR) at N-terminal of HIV RNA. This protein is function to promote transcription elongation to generate complete transcript by binding to its cofactor, P-TEFb, which is a transcriptional elongation factor (50, 51). Without Tat protein, transcript can be as short as less than 100 nucleotides.

Rev

A 13-kD sequence specific RNA binding protein encoded by 2 exons (52). Rev binds to a specific part of RNA elongated stem-loop structure called Rev-responsive element (RRE). Rev is responsible for late phase transcription and exporting viral RNA from nucleus to cytoplasm. Virion cannot be produced in the absence of Rev because of degrading unspliced RNA in nucleus and lack of late phase transcription (51).

Accessory proteins

Functions of accessory proteins are not clearly determined. Basically, the role of these proteins is to facilitate viral infectivity and to ensure that virus can survive in host cells (53).

Vif

A 23-kD virion infectivity factor (Vif) protein which is responsible for viral replication with particular cells; lymphocyte in periphery and macrophage. Vif can suppress APOBEC3G and APOBEC3F which function to inhibit infection.

Vpr

Viral protein R or Vpr is a protein that can incorporated into virions. This occasion is triggered by an interaction with $Pr55^{Gag}$ precursor. Vpr facilitates virus to infect non-dividing cell. It can enhance infectivity after viral entry by G2/M cell cycle arrest.

Vpu

Viral protein U or Vpu is a 16-kD phosphoprotein locates in internal membrane. Vpu is translated by the same mRNA as Env protein but in a lower level of expression. Vpu has been found to down-regulate CD4 molecule and promote viral release from host cell (54).

Nef

A 27-kD myristoylated protein functions as a negative factor. Nef is an early gene and is the first protein that can be translated after infection (52). Nef can down-regulate CD4 as well as Vpu by increasing endocytosis and lysosomal degradation of CD4 (55). Not only CD4 molecules, but Nef can down-regulate HLA class I molecules (56). This protein also activates T cell, enhance viral infectivity and apoptosis blockage.

Replication cycle

Cell tropisms for HIV refer to cells that express CD4 molecules which are $CD4^+$ T cell, monocyte, macrophage and dendritic cell. Not only CD4 which function as main receptor, but co-receptors such as CCR5 and CXCR4 are also required for infection.

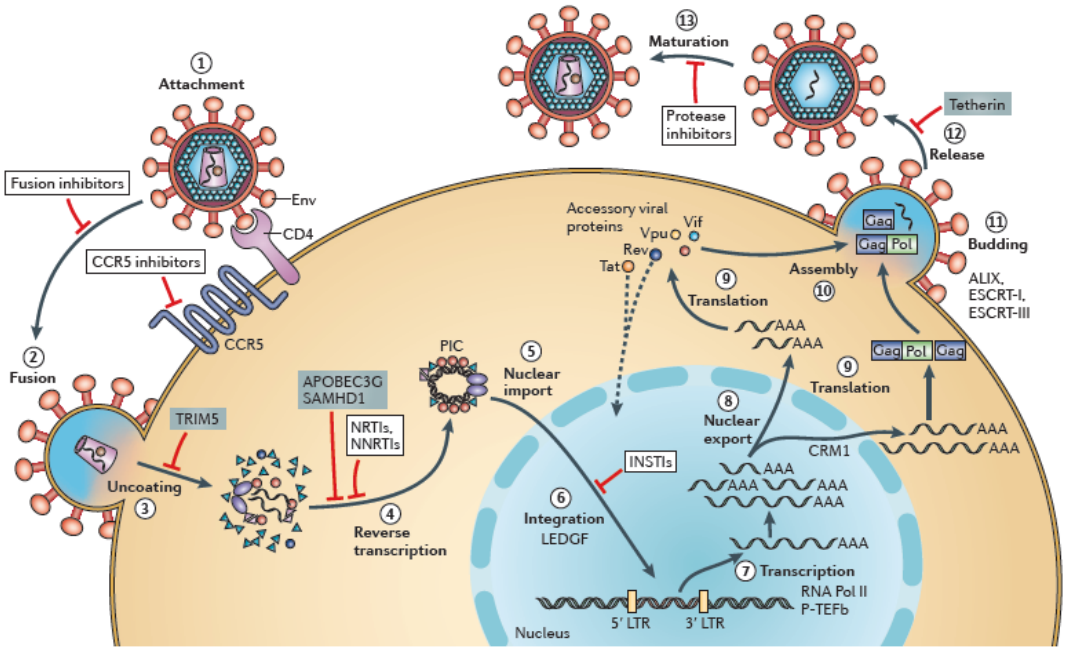


Figure 3: HIV replication cycle (57)

Firstly, glycoprotein 120 attaches to CD4 receptor (58) and follows by binding to co-receptor on the cell surface. This interaction resulting in viral fusion with host cell membrane using glycoprotein 41. Conformational change of gp41 is required for fusion. Capsid protein partially uncoats to release viral genetic material into host cell. RNA genome is then reverse transcribed by reverse transcriptase in cytoplasm into pre-integration complex (PIC). PIC is imported inside the nucleus and integrates with host genome by integrase enzyme with help from host chromatin-binding protein, LEDGF. Transcription is processed by host RNA polymerase II and P-TEFb in different sizes. Messenger RNA is exported out of nucleus to serve as template for viral protein translation. Proteins are assemble in viral particle. New virions bud and release from host cell. Lastly, in order to generate infectious virions, maturation which mediates by protease enzyme is followed (57). New virions can be produced approximately 10^9 per day (59).

HIV Transmission

Transmission occurs through mucosal tissue and injured tissue by directly contacting with HIV seropositive people. HIV can stay in regional lymph nodes for 2 days and travel into bloodstream after 5 days (60). Higher viral load was associated with increasing risk of transmission (61). Transmission rate was increased 2.4-fold for every 1 $\log_{10}$ viral load increase (62).

The most common route of HIV transmission is sexual transmission via vaginal or rectal mucosa. HIV can transmit through blood in particular circumstances such as mother-to-child transmission (MTCT) during laboring, needle or syringe sharing among intravenous drug user (IVDU) and blood transfusion. HIV can also found in certain body fluids which are pre-seminal fluids, seminal fluids, vaginal fluids, rectal fluids and breast milk. Some sexually transmitted diseases (STDs) such as HSV-2 infection and bacterial vaginosis have been reported that they were associated with increased risk of transmission (63). Other factors are also related with transmission rate; pregnancy, receptive anal intercourse, long-term contraception by injection, many sexual partners and concurrent sexual partnerships (64). On the other hand, male circumcision is associated with decreased risk of transmission (65).

High risk groups are men who have sex with men (MSM), commercial sex workers and intravenous drug users.

Table 1: HIV transmission rate in different type of exposure (66) .

Type of exposure	Estimated median (range) risk of HIV transmission per exposure
Receptive anal intercourse	1.11% (0.042%–3.0%)
Insertive anal intercourse	0.06% (0.06%–0.065%)
Receptive vaginal intercourse	0.1% (0.004%–0.32%)
Insertive vaginal intercourse	0.082% (0.011%–0.38%)
Receptive oral sex (fellatio)	0.02% (0%–0.04%)
Insertive oral sex (receiving fellatio)	0%
Blood transfusion (one unit)	(90%–100%)
Needlestick injury	0.3% (95% CI: 0.2%–0.5%)
Sharing injecting equipment	0.67%
Mucous membrane exposure	0.63% (95% CI: 0.018%–3.47%)

HIV Pathogenesis

After infection establishes at local site, HIV-infected cell travels to draining lymph nodes for further infection in other cells. Virus is bound to DC-SIGN on the surface of dendritic cell. DC-SIGN molecule has been suggested to be a Trojan horse for mediating HIV infection (67, 68). This mechanism facilitates viral replication and spreads throughout the body especially in GALT and MALT where majority of $CD4^+$ T cells reside (69) and a recent study shown that it may be blocked by TRIM5α (70).

The natural history of HIV infection can be described into 3 phases; acute infection phase, chronic infection phase and AIDS phase as shown in Figure 4.

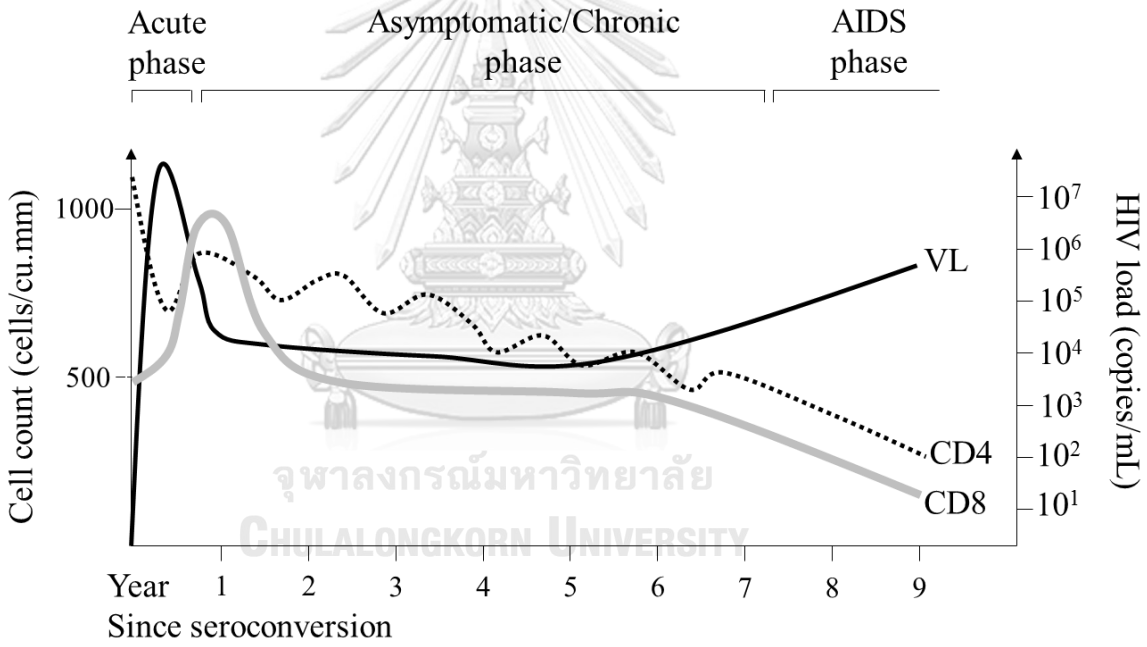


Figure 4: HIV natural history adapted from Goulder and Watkins (6) represented CD4 count (dotted line), CD8 count (grey line) and HIV viral load (black line).

Acute phase

Viral RNA cannot be detected during approximately 10 days after HIV transmission termed as eclipse phase. Even though, there is no clinical symptoms in eclipse phase, virus still propagates in $CD4^+$ T cell in mucosa and lymph nodes.

Two weeks after infection flu-like symptoms may present in some people. Clinical symptoms that may be observed are fever, rhinorrhea, fatigue, sore throat, myalgia, headache, arthralgia, swollen glands and diarrhea (71).

Virus can be detected in this phase. Peak viremia is as high as 10^7 copies/mL during acute infection. Currently, Fiebig staging is commonly used to identify phase of acute infection based on viral appearance (72, 73). Fiebig stage I can only detect viral RNA in plasma. Fiebig stage II can detect p24 antigen by ELISA assay. HIV specific antibody detected by ELISA is presented in Fiebig stage III. Inconclusive western blot for HIV specific antibody and p31 non-reactive are presented in Fiebig stage IV. In Fiebig stage V, Western blot for HIV specific antibody positive and p31 reactive are presented as shown in Figure 5.

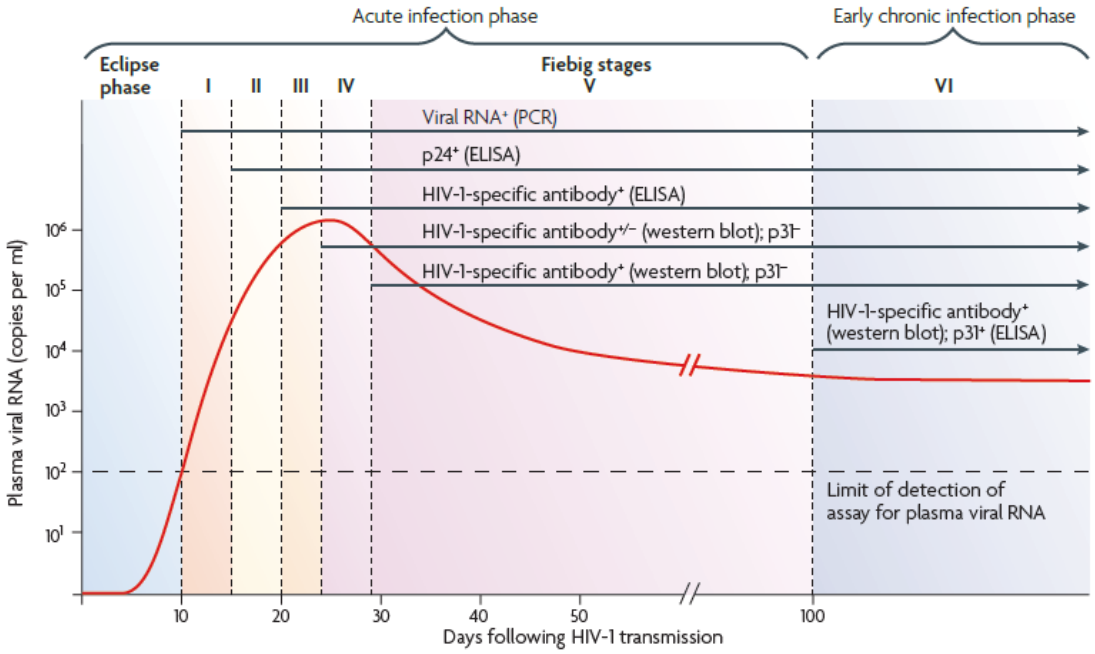


Figure 5: Fiebig staging in HIV acute infection (72).

Asymptomatic/Chronic phase

This phase also called clinical latency due to the absence of clinical symptoms. HIV viral load is dropped to nearly stable called viral set point. Even though infected people may not present any symptoms, virus still replicates and $CD4^+$ T cell is declined 50-100 cells per year. Median time from seroconversion to AIDS is normally 5-10 years but it may differ in each ethnic. Median time to clinical AIDS is 7.2 years in Thai population (74) and 9.4 years in African population (75).

AIDS phase

AIDS phase is defined by level of CD4 count less than 200 cells/μL. The immune system is extremely damaged, being immunocompromised and has a greater risk to get life-threatening opportunistic infections (OIs). The most common disease causes by OIs is tuberculosis. Others OIs diseases are pneumococcal pneumonia, toxoplasmic encephalitis, candidiasis, oral thrush and cryptococcal meningitis (76, 77) OIs are also correlated with level of CD4 count as shown in Table 2 (78, 79).

Table 2: AIDS-related opportunistic infections.

CD4 count	Opportunistic infections/diseases
> 500 cells/μL	Minor risk for fungal infection Kaposi's Sarcoma
200 – 500 cells/μL	Candidiasis/oral thrush Pulmonary tuberculosis VZV dermatitis
100 – 200 cells/μL	Histoplasmosis/Coccidioidomycosis Miliary/extrapulmonary tuberculosis Non-Hodgkin's lymphoma Pneumococcal pneumonia (PCP) Progressive multifocal leukoencephalopathy (PML)
50 – 100 cells/μL	Aspergillosis Cryptococcosis Cryptosporidiosis Cytomegalovirus: retinitis, encephalitis, gastroenteritis, pneumonitis Toxoplasmosis
< 50 cells/μL	CNS lymphoma *Mycobacterium avium* complex (MAC)

To prevent those opportunistic conditions, antiretroviral therapy (ART) has been applied to treat HIV-infected patients. The first USFDA approved drug was zidovudine or AZT in 1987. Mechanism of action of antiretroviral drugs is to block replication processes such as reverse transcription (RT inhibitor; nucleoside analog reverse transcriptase inhibitors (NRTIs) and non-nucleoside analog reverse transcriptase inhibitors (NNRTIs)), integration (Integrase inhibitor) and Gag maturation (Protease inhibitor) or even block viral entry such as fusion inhibitor (80) and CCR5 antagonists (81). Monotherapy was discarded because the efficacy was only last for 2-3 years (82). Combination antiretroviral therapy (cART) or highly active antiretroviral therapy (HAART) has been used for more effective to suppress viral replication (83).

Control of HIV infection

1. **Host factors**

Genetic background

Human leukocyte antigen (HLA) has been proposed to play an essential on HIV control. HLA interacts with both NK cell and T cell. GWAS data shown that SNPs that have significant difference between controllers and progressors locate in chromosome 6 on HLA region (84). HLA-B27, -B57 and -B58 alleles in Caucasians have been correlated to slow progression so called protective alleles (12, 13, 85). On the other hand, HLA-B35, -B8, -B45 and -B53 are associated with rapid progression (86, 87)

Another genetic factor that has been shown contribution on HIV control is CCR5 chemokine receptor molecule which acts as co-receptor of HIV (88). A 32bp deletion named CCR5Δ32 has reduced 31% risk of HIV infection (89) and associated with delay disease progression (90). Berlin patient who is the only patient until now that absolutely cured from HIV infection since he has been received bone marrow transplantation from CCR5 Δ32/Δ32 donor (91).

Immunity

Innate immune cells and adaptive immune cells has been reported that they play an important role on HIV control as shown in Figure 6.

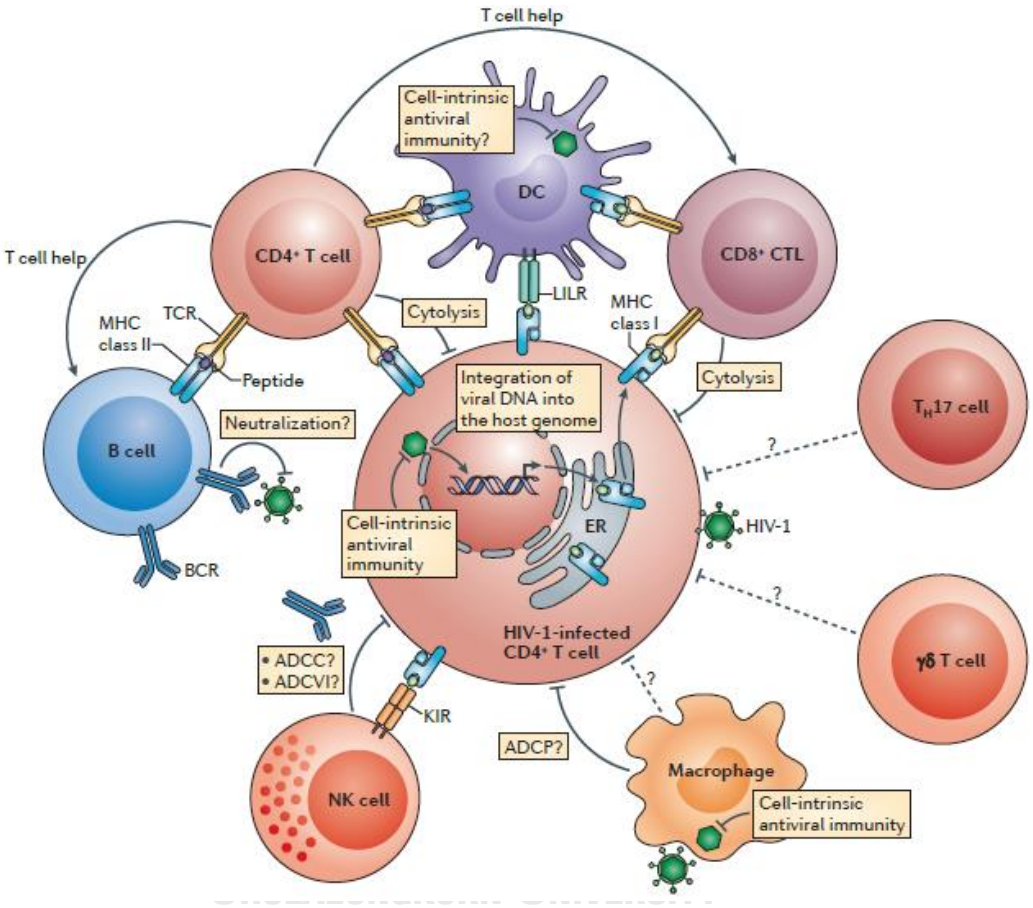


Figure 6: Schematic of HIV immunity (92).

Innate immunity

Innate immune cells consists of phagocyte, monocyte and macrophage which function to prime for antigen elimination, natural killer (NK) cell and γδ T cell which function to direct killing infected cells and dendritic cell which functions as professional antigen presenting cells (APCs) to prime and initiate adaptive immune response.

Pattern recognition receptors or PRRs particularly Toll-like receptors (TLRs) have been reported to recognize HIV. TLR3, 7, and 8 recognize RNA pathogen-associated molecular patterns (PAMPs) while TLR9 recognizes DNA PAMPs (93). Signal triggering through TLR7/8 can induce DC activation. After that, type I IFNs and TNFα are largely produced by DC to inhibit viral replication (94). As similar to TLR7/8, TLR9 has also suggested to play a role on DC and NK cell activation by binding to gp120 of HIV envelop (95).

NK cell recognize foreign antigen via a number of stimulating and inhibitory receptors named killer immunoglobulin-like receptor (KIR) on its surface. By sensing down-regulation or absence of HLA class I (96), NK cell can release cytotoxic substance to kill target cell. Some KIRs have been associated with control of HIV such as KIR3DL1 (97-99) and KIR3DS1 (100). NK cell also has an impact on HIV by placing footprints on its genome (101). Another innate cell that plays role on cytotoxicity is γδ T cell. Gamma/delta T cell which express Vγ2α2 TCR has been found to be increased and maintained in HIV controllers rather than non-controllers (102).

Dendritic cell is function to crosstalk between innate and adaptive immune response. Myeloid dendritic cells from HIV controllers had higher ability to induce type I interferons and ability to process and present antigen (103). Moreover, studies showed that DCs from elite controller effectively stimulated $CD8^+$ T cell response (104). While plasmacytoid dendritic cell has less evidence on HIV control.

Adaptive immunity

Adaptive immunity is classified into humoral immune response and cell-mediated immune response. Key component on humoral immune response is B cell. Even though some broadly neutralizing antibodies (bNAbs) have been suggested to block HIV entry (105), they have been generated in a limited number (106). Viral escape from neutralizing antibodies has been observed (107, 108).

$CD4^+$ T cell or T helper cell is essential for maintaining immune response against chronic HIV infection. HIV-specific $CD4^+$ T cells with potential to proliferate and respond to p24 has shown negative correlation with viral load (109). $CD4^+$ T cells from elite controller have high avidity and polyfunctionality (110). Moreover, $CD4^+$ T cells also help to generate functional memory $CD8^+$ T cells (111).

As showed in the natural history (Figure 4), $CD8^+$ T cell or cytotoxic T lymphocyte (CTL) may provide an effect on HIV control. Evidence in macaques have shown that SIV viral load and proviral DNA were rapidly increased after CD8 depletion in vivo (112, 113) and decreased when $CD8^+$ cells returning back (9). Studies in ex vivo and in vitro also present an ability of $CD8^+$ T cells to suppress viral replication as seen in animal model (114-116). HIV Gag-specific $CD8^+$ T cell response is associated with low plasma viral load (117-120). Polyfunctionality such as MIP-1β, IFNγ, TNFα, CD107a as degranulation marker and IL-2 (11, 121) and effective proliferative capacity (122) have been remarked in HIV controllers.

2. Viral factor

Viral fitness

Viral fitness is a term to define viral replicative capacity in a particular condition (123). In environment with pressure such as antiretroviral therapy and immune cells especially CTL, mutation may occur and reduce viral fitness in addition to survive (124). Reversion of escape mutation or compensatory mutation could be happened after absence of the pressure (125).

Mechanisms of mutational escape

Basically, there are two types of mutational escape which are non-synonymous and synonymous mutation. Non-synonymous mutation is a mutation that changing amino acid. As shown in Figure 7, non-synonymous mutation can be presented either in flanking regions which may interfere proteasome processing (126) and amino-terminal trimming (127) or an intraepitope region which may affect HLA binding (14) or TCR binding (125, 128).

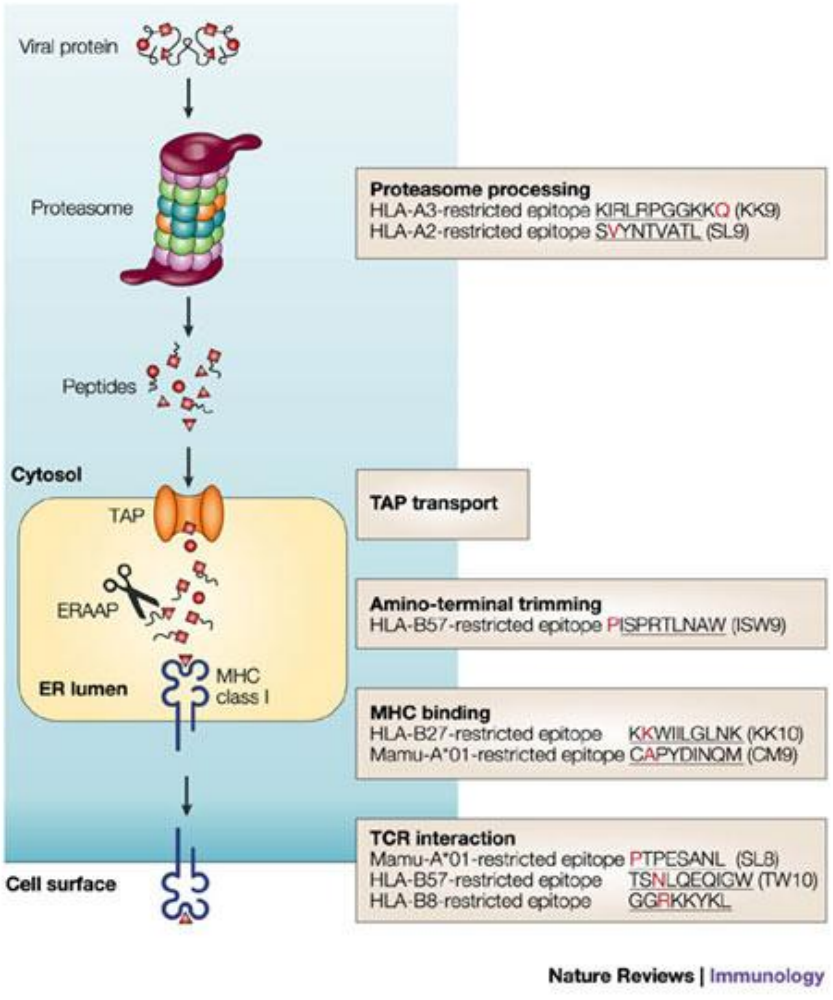


Figure 7: Possible mechanisms to escape from CTL (6).

On the contrary, synonymous mutation does not change amino acid residue but may affect splicing, stability or folding of RNA (129).

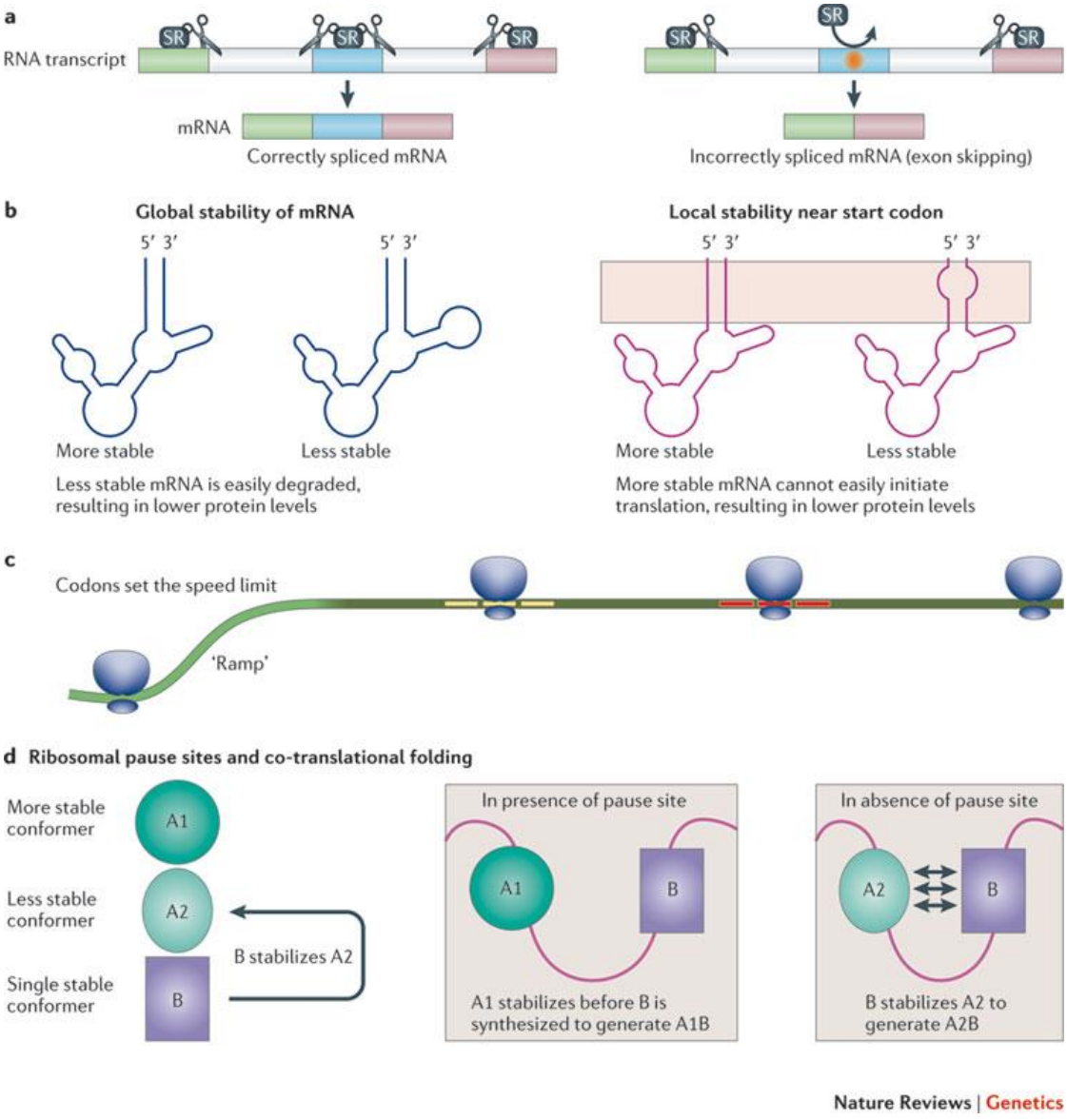


Figure 8: Effects of synonymous mutation (129).

3. Environmental factors

The environmental factors that may affect HIV infection are economics, education and culture. Male circumcision is preferable tradition among Muslim to remove foreskin since early childhood (130). The efficacy of male circumcision is approximately 30-50% protection against HIV infection in intervention group (131-133). Not only HIV infection but male circumcision can protect HSV-2, HPV infection and syphilis similarly (134).

CHAPTER IV
MATERIALS AND METHODS

Study subjects

HIV-infected volunteers (n= 66) who had CD4 count greater than 450 cells/µL were recruited from the anonymous clinic, Thai Red Cross AIDS Research Center. Patients were longitudinal follow up every 3-6 months. Healthy donors (n=52) were also included as control group. This study was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB: 610/57). Informed consents were legally signed by all volunteers. All of HIV-infected volunteers were naïve for antiretroviral therapy (ART) and absence of opportunistic infections.

Sample Collection and PBMC isolation

Thirty milliliters of EDTA whole blood was collected from HIV-infected volunteers. EDTA whole blood was centrifuged at 3,000 rpm for 10 minutes and EDTA plasma was collected and stored at -80°C in cryotubes (Sarstedt, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated by a density gradient centrifugation method using Ficoll-Hypaque® at a density gradient of 1.077 g/L (IsoPrep, Robbins Scientific Corporation, USA). The remaining EDTA whole blood was diluted with RPMI-1640 medium (Gibco, USA) at ratio 1:1, then gently overlaid on Ficoll-Hypaque® in 50 mL tubes. Blood-containing tubes were centrifuged at 1,500 rpm for 30 minutes break-off. Buffy coat was collected using a serological pipette (Eppendorf, Germany) into new sterile tubes and washed twice with 1X PBS (Sigma-Aldrich, Germany) at 2,000 rpm for 10 minutes and 1,500 rpm for 5 minutes, respectively. Cell viability was determined by trypan blue staining (Sigma-Aldrich, Germany), the mixture of cells and dye was loaded into a hemocytometer and examined number of viable cells under a light microscope.

Number of cells (cells/mL) = (No. viable cells/4) × 10,000 × dilution factor

Complete blood count, CD4 count and plasma HIV load determination

Three milliliters of EDTA whole blood was collected for complete blood count (CBC) and CD4 count. CBC was performed on Sysmex xs-1000i™ automated hematology analyzer. EDTA whole blood was stained with BD Tritest™ CD4/CD8/CD3 (BD Biosciences, USA) which contains monoclonal antibody against PerCP-labeled-CD3 (clone SK7), FITC-labeled CD4 (clone SK3) and PE-labeled CD8 (clone SK1) and was analyzed by BD FACSCalibur™ flow cytometer. One point two milliliters of EDTA plasma was taken for HIV plasma viral load (VL) by Real-Time PCR assay using COBAS® AmpliPrep/COBAS® Taqman® HIV-1 test (Roche, USA). CBC, CD4 count and HIV viral load detection were performed by ISO 15189 certified-medical laboratories at King Chulalongkorn Memorial Hospital.

HLA typing

Genomic DNA was extracted from 10^6 PBMCs using Gentra Puregene Blood Kit (Qiagen, Germany). Extracted DNA was quantified by NanoDrop™ Spectrophotometer (Thermo Scientific, USA). High resolution HLA typing for HLA class I locus A, B and C was performed by using PCR-SSOP at Proimmune (United Kingdom) and BGI clinical laboratories (China).

Culture medium, cryopreservation and thawing

PBMCs were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), US origin (Gibco, USA), called R10 medium, in the presence of 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Gibco, USA).

For cryopreservation, 10^7 PBMCs were frozen per vial in 90% heat-inactivated FBS supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and kept overnight at -80°C in a Nalgene® Mr. Frosty (Sigma-Aldrich, Germany). On the following day, vials were transferred into a liquid nitrogen tank (-196°C) for long term preservation.

For cell thawing, PBMCs were rapidly thawed at 37°C for 2 minutes in a water bath incubator. PBMCs were subsequently washed twice with R10 medium in the

presence of 100 µg/mL deoxyribonuclease I (Sigma-Aldrich, Germany) at 1,500 rpm for 5 minutes. Cell viability should be determined prior to use in experiments.

Interferon gamma ELISpot assay for T cell response

Multiscreen hydrophobic polyvinylidene fluoride (PVDF) 96-well plates (Merck Millipore, USA) were coated with 100 µL of 5 µg/mL of anti-human IFN-γ mAb 1-D1K (Mabtech, Sweden) diluted with 1X PBS and were incubated at 37°C for 3 hours. The coated plates were washed 6 times with 200 µL of 1X PBS per well and were blocked by R10 medium for 1 hour at room temperature. Then, 2.5×10^5 PBMCs were added into each well and were stimulated at 37°C, 5% CO_2 for 15 hours with 10 µg/mL of HIV p24 Gag overlapping peptides (20 mers overlapped by 10 mers) as listed in Table 3 (11, 116) and HLA-A11 restricted epitopes (p24 Gag and Nef) (Mimotopes, Australia). HLA-B27 restricted epitopes (p24 Gag, Pol, Rev and Nef) in Table 4 and HLA-B57/5801 restricted epitopes (p17 Gag, p24 Gag, Pol and Nef) in Table 5 were provided by Prof. Philip Goulder, University of Oxford, United Kingdom (135). All peptides were tested in duplicates. Phytohemagglutinin (PHA; Sigma-Aldrich, Germany) was used as a positive control while PBMCs in R10 medium was used as a negative control. Recombinant CMV pp65 (Miltenyi Biotec, Germany) was also used as an irrelevant peptide control. After 15 hours stimulation, culture supernatant was collected and stored at -80°C. The ELISpot plates were washed 6 times with 200 µL per well of 1X PBS in the presence of 0.05% Tween20 (Sigma-Aldrich, Germany) followed by 1 time with 200 µL per well of 1X PBS. Fifty microliters of biotinylated anti-human IFN-γ mAb 7-B6-1 (Mabtech, Sweden) was added at a concentration of 1 µg/mL. Plates were incubated for 3 hours at room temperature and were washed as similar as a previous step. Fifty microliters of streptavidin-conjugated alkaline phosphatase (ALP) (Mabtech, Sweden) was added at a concentration of 1 µg/mL and incubated for 1 hour at room temperature and plates were washed as similar as a previous step. Lastly, NBT/BCIP or ALP substrate (Bio-Rad, USA) was added to each well. Plates were kept away from light and incubated for 5 minutes at room temperature. After that, plates were immediately washed with tap water for 10 times and left to air dry.

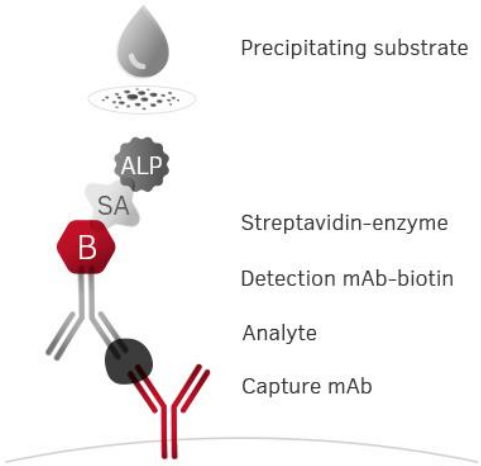


Figure 9: Enzyme-linked immunospot (ELISpot) assay from Mabtech

ELISpot results were determined after plates completely dry by an automated ELISpot reader (Carl Zeiss, Germany) and were calculated into spot forming unit per one million PBMCs (SFU/10^6 PBMCs). Spots which were greater than 80 SFU/10^6 PBMCs and higher than a negative control at least 4 times were considered as a positive result.

Magnitude (SFU/10^6 PBMC) = (no. of spot in test well – no. of spot in negative) × 4

Table 3: Gag p24 overlapping peptides

Peptide name	Sequence
OLP1	PIVQNAQGQMIHQSLSPRTL
OLP2	IHQSLSPRTLNAWVKVVEEK
OLP3	NAWVKVVEEKGFSPEVIPMF
OLP4	GFSPEVIPMFSALSEGAVPQ
OLP5	SALSEGAVPQDLNMMLNIVG
OLP6	DLNMMLNIVGGHQAAMQMLK
OLP7	GHQAAMQMLKETINEEAAEW
OLP8	ETINEEAAEWDRLHPVHAGP
OLP9	DRLHPVHAGPIPPGQMREPR
OLP10	IPPGQMREPRGSDIAGTTST
OLP11	GSDIAGTTSTLQEQIGWMTS
OLP12	LQEQIGWMTSNPPIPVGDIY
OLP13	NPPIPVGDIYKRWIILGLNK
OLP14	KRWIILGLNKIVRMYSPVSI
OLP15	IVRMYSPVSILDIRQGPKEP
OLP16	LDIRQGPKEPFRDYVDRFYK
OLP17	FRDYVDRFYKTLRAEQATQE
OLP18	TLRAEQATQEVKNWMTETLL
OLP19	VKNWMTETLLIQNANPDCKS
OLP20	IQNANPDCKSILKALGTGAT
OLP21	ILKALGTGATLEEMMTACQG
OLP22	LEEMMTACQGVGGPSHKARV
OLP23	VGGPSHKARVLAEAMSHAQQ

Table 4: Well-characterized HLA-B27-restricted epitopes

Allele	Protein	Epitope	Sequence
B27	Gag p24	KK10	KRWIILGLNK
B27	Gag p24	KK10 R264K	KKWIILGLNK
B27	Gag p24	KK10 L268M	KRWIIMGLNK
B27	Gag p24	KK10 R264K L268M	KKWIIMGLNK
B27	Pol Integrase	KY9	KRKGGIGGY
B27	Rev	RR8	RRWRERQR
B27	Nef	KV10 4D	KRQDILDWV
B27	Nef	QV10	QRQDILDLWV
B27	Nef	IW9	IRYPLTFGW
B27	Nef	TFW9	TRFPLTFGW
B27	Nef	TYW10	TRYPLTFGW
B27	Nef	WF9	WRFDSRLAF
B27	Nef	SR10	SRLAFHHMAR

Table 5: Well-characterized HLA-B57/5801-restricted epitopes

Allele	Protein	Epitope	Sequence
B57/5801	Gag p24	ISW9	ISPRTLNAW
B57/5801	Gag p24	LSW9	LSPRTLNAW
B57/5801	Gag p24	KF11 A31G S33N	KGFNPEVIPMF
B57/5801	Gag p24	DW10	DTINEEAAEW
B57/5801	Gag p24	EW10	ETINEEAAEW
B57/5801	Gag p24	TW10 Cwt	TSTLQEQIGW
B57/5801	Gag p24	TW10 Cwt	TSTLQEQIAW
B57/5801	Gag p24	TW10 T110N	TSNLQEQIGW
B57/5801	Gag p24	TW10 T110N G116A	TSNLQEQIAW
B57/5801	Gag p24	QW9 Cwt	QATQDVKNW
B57/5801	Gag p24	QW9 D5E	QATQEVKNW
B57/5801	Gag p24	QW9 T3S	QASQDVKNW
B57/5801	Gag p17	IF11	IVWASRELERF
B57/5801	Gag p17	LF11	LVWASRELERF
B57/5801	Pol RT	IW9	IAMESIVIW
B57/5801	Pol RT	QF10	QATWIPEWEF
B57/5801	Nef	KF9 4V	KAAVDLSHF
B57/5801	Nef	KF9 4F	KAAFDLSHF
B57/5801	Nef	HW9 5F	HTQGFFPDW

HLA-peptide tetrameric complex and phenotype staining

PBMCs were quickly thawed as previously described. PBMCs were stained in 5 mL round-bottom tubes (BD Falcon™, USA) with ACQGVGGPSHK (AK11)-PE tetramer which was provided by NIH Tetramer Core Facility for 30 minutes at 4°C followed by surface staining with PerCP-conjugated anti-human CD3, PE/Cy7-conjugated anti-human CD4, APC/Cy7-conjugated anti-human CD8, PE/Texas Red-conjugated anti-KLRG1, BV421-conjugated anti-PD-1, BV510-conjugated anti-CD45RA and APC-conjugated anti-CCR7 (All antibodies were purchased from Biolegend, USA) for 30 minutes at 4°C. Stained PBMCs were washed with 1X PBS to remove unbound antibodies and were analyzed by BD FACSAria™ II flow cytometer (BD Biosciences, USA).

HIV sequence analysis

HIV viral RNA was extracted from 200 µL of EDTA plasma using High Pure Viral RNA kit (Roche, USA). One step reverse transcription-PCR (Qiagen, Germany) for Gag p24 first round was performed by using 3 individual reactions with 3 pairs of the following primers: ExFwGag1 (5'-GAGGTGCACACAGCAAGAGGCG-3'), ExRvGag1 (5'-CCCCCTATCATTTTTGGTTTCC-3'), ExFwGag2 (5'-AAATCTCT AGCAGTGGCGCCCGAACAG-3'), ExRvGag2 (5'-TAACCCTGCGGGATGTGGT ATTCC-3'), ExFwGag3 (5'-GCGRCTGGTGAGTACGCC-3') and ExRvGag3 (5'-RGGAAGGCCAGATYTTCC-3') (136). The one step RT-PCR conditions included reverse transcription at 50°C for 30 minutes, initial PCR activation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 minutes, and extension at 72°C for 2 minutes 15 seconds, with final extension at 68°C for 20 minutes. Nested PCR was performed by using PIF-24 (5'-GACACCAAGGARG

CTTTRGA-3') and POR-24 (5'-GGGGCCCTGCAATTTCTGGC-3'). The PCR conditions included denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 68°C for 1 minute with final extension at 68°C for 10 minutes. The size of amplified product was approximately 921 base pairs.

Gel electrophoresis (1% agarose, Research organics, USA) was performed to determine nested PCR results and then specific bands were cut and purified by Nucleospin® Gel and PCR Clean-up (Macherey-Nagel, Germany) before sequencing (AITbiotect, Singapore). Sequencing data were analyzed by BioEdit version 7.1.9 (137) and UniPro UGENE version 1.26 (138) then were translated to protein sequences by ExPASy: SIB Swiss Institute of Bioinformatics (https://www.expasy.org/) (139) and Clustal Omega: The European Bioinformatics Institute (http://www.ebi.ac.uk/Tools /msa/clustalo/) (140) for sequence alignment to determine similarity. Nucleotide sequences and protein sequences were compared.

Cell preparation

B lymphoblastoid cells

Five million PBMCs were used to establish B lymphoblastoid cells or B-LCL. PBMCs were depleted CD8 T cells using a magnetic separation kit, Dynabeads® CD8 (Invitrogen, USA). Twenty five microliters of Dynabeads® CD8 was washed with 1 mL of isolation buffer which contained 1X PBS supplemented with 0.1% BSA (Sigma-Aldrich, Germany) and 2 mM EDTA (Sigma-Aldrich, Germany) and placed on a magnetic stand (Invitrogen, USA) for 1 minute. Supernatant was removed and 25 µL of isolation buffer was added to resuspend magnetic beads. One milliliter of 5×10^6 PBMCs in R10 medium was added into washed-magnetic bead tubes and gently rotated in a tube rotator for 30 minutes at 4°C to prevent a phagocytic activity. Tubes were placed on a magnetic stand for 2 minutes and carefully collected supernatant into 1 well of 24-well plates (Corning, USA). CD8 depleted PBMCs were cultured in R10 medium in the presence of 10 ng/mL of interleukin-4 (R&D Systems, USA) at 37 °C, 5% CO_2. The half of culture supernatant was removed and replaced with fresh R10 medium supplemented with 10 ng/mL of interleukin-4 once a week until transformation was observed. Then, transformed cells were transferred from 24-well plates into T25 cm^2 tissue culture flasks (Nunc™, Thermo Scientific, USA).

CD40L-activated B cells

Five million PBMCs were stimulated by 100 Gy irradiated CD40L-expressing K562 cell line (K562 cell line; ATCC CCL-243 was provided by Dr.Koramit Suppapat, department of pediatrics Faculty of Medicine, Chulalongkorn University) in 6-well plates at a ratio of 50:1. The culture was maintained in 5 mL of IMDM medium (Gibco, USA) supplemented with 10% heat-inactivated FBS, 2 ng/mL of interleukin-4 and 1 µg/mL of cyclosporine A (Sigma-Aldrich, Germany). CD40-activated B cells were re-stimulated with CD40L-expressing K562 cell line every 3-5 days. Cells were cultured at 10^6/mL when $CD19^+$ cells reached more than 75%. Phenotype was determined by using Brilliant Violet 421 anti-human CD19 and Alexa Fluor 647 anti-human CD56 and analyzed by flow cytometry.

Cytotoxic T cell line

Two to Five million PBMCs were used to establish cytotoxic T cell line or CTL line. PBMCs were centrifuged at 1,500 rpm for 5 minutes and completely removed supernatant. Two hundred microliters of R10 medium and 10 µM of peptide were added to stimulate PBMCs for 1 hour in 15 mL tubes at 37°C, 5% CO_2. Stimulated PBMCs were topped up with 1 mL of R10 medium supplemented with 5 ng/mL of interleukin-7 (R&D Systems, USA) and transferred into 1 well of 24-well plates (Day 1). At day 3, 1 mL of R10 medium supplemented with 200 IU/mL of interleukin-2 (R&D Systems, USA) was added into the CTL culture. When CTL expanded nearly all the area, CTLs were split from 1 well to 2 wells. CTL culture were performed functional assay at day 14 and cryopreserved for further experiments.

Plasmid construction

Lentiviral expression system (Figure 10) was employed to deliver the gene of interest. Plasmid constructs were designed and visualized by SnapGene software (from GSL Biotech; available at snapgene.com) Tandem HLA-A11 epitopes as showed in Figure 11 were cloned into a lentiviral vector, pWPXLd, called A11-pWPXLd. The pWPXLd was a gift from Prof. David Sabatini (Addgene plasmid #12258) (141).

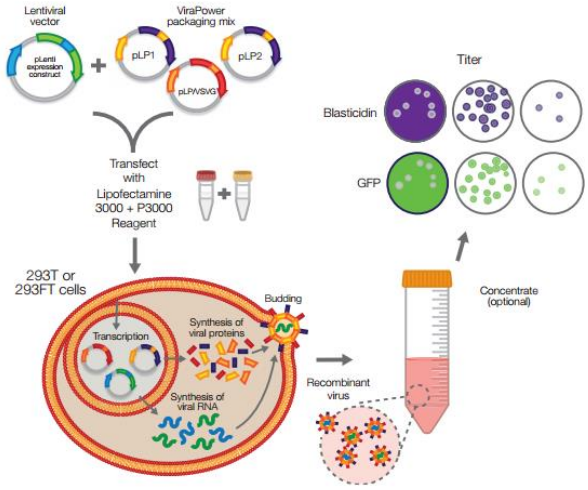


Figure 10: Lentiviral expression system

```
ATGATCGCAACCCTCTGGTGCGTACACCAAAGGGCATGCCAGGGAGTGGG      50
AGGACCTAGCCATAAGGCAGTATTCATTCACAATTTTAAAAGAAAAGGAA     100
TACCGCATCCAGCAGGTTTAAAAAAGTCAGTTCCTTTAGATGAAAGCTTT     150
AGAAAGGCAATATTCCAGAGTAGCATGACAAAACAAATTTATGCAGGGAT     200
TAAGGTAAAGCAAATTTATCAAGAGCCATTTAAAAATCTAAAACAAATAA     250
TAGAGCAGCTAATAAAAAAGTTTGTTAATACCCCTCCTCTAGTAAAAACA     300
CAGATGAATTGGCCAAACTTGTGGAAAGTTACAGTTTATTATGGGGTGCC     350
TGTGTGGAGAATCAGTTTATGGGATCAAAGTCTAAAGATAACTGTGGGAC     400
CAGGACAGGTATTCTATCGCGTTTTAAAACAGGTAACTGAAAAAAGCCTG     450
TGCCTCTTCAGCTACCACCGGCAGGTACCTCTAAGACCAATGACTTATAA     500
GGGAGCTTTTGATCTTAGCTTCTTTTTAAAATACCCGTCGGCGGCCGAGC     550
GCAAACACCGACACCTGCCCGTAGCCGACGCTGTTATTCACGCGCACGAG     600
CTGGTTTGCTCCATGGAGAACACGCGCGCAACCAAGATGCAGGTGATAGG     650
TGACCAGTACGTCAAGGTGTACCTGGAGTCCATAATCAAACCGGGCAAGA     700
TCTCGCACATCATGCTGGATGTGGCTTTTACCTCACACGAGGATTCCGAC     750
AACGAAATCCACAATCCGGCCGTGTTCACCTGGCCGCCCTGGCAGGCCGG     800
CATCCTGGCCCGCAACCTGGTGCCCATGGTGGCTACGGTTCAGGGTCAGA     850
ATCTGAAGTACGGCAACGGCCTGGGCCAGAAGGAGGACAGCAGCGGCCCC     900
GAGGGCAGCGGCGGCAGCGGCCCCCAGCGGCGGGGCGGCGACAACCACGG     950
CTTCGAGTACCTGCAGGAGGGCGGCCCCGACGGCGAGCCCATGGTGTTCC    1000
TGCAGACCCACATCTTCGCCGAGGTGCTGAAGGACGAAGATCTTCCTTGT    1050
ATAGTGTCCAGAGGTGGACCTAAGGTCAAACGACCCCCTATATTTTTTAT    1100
TGAGTTTGTAGGATGGTTATGCAAGAAGGACCACACTCATATACGCGAAT    1150
GGTTCCCAGCTCCCCAGGCTCCATACCAGGGATACCAGGAGCCGCCGGCC    1200
CCCCAGGCTCCATACTATCTACAACAAAACTGGTGGACTCTATTGGTTGA    1250
TCTCCTTTGGCTCCTCGACTATCAACCACTAGGAACCCAAGATCAAAGTC    1300
TGTACTTGGGAGCGGTGTTTGACCGAAAGTCAGATGCAAAATCTTCGTGC    1350
TCTTCATGTCCACTGAGCAAGATCGGTAGCGGCAGCGGTAGC          1392

A11                          3521 .. 4912    1392 bp    CDS

/translation  = MIATLWCVHQRACQGVGGPSHKAVFIHNFKRKGIPHPAGLKKSVPLDESFRKAIFQSSMTKQIYAGIKVKQIYQEPFKNLKQIIEQLIKKFVNTPPLVKTQMNWPNLWKVTVYYGVPVWRISLWDQSLK
              ITVGPGQVFYRVLKQVTEKSLCLFSYHRQVPLRPMTYKGAFDLSFFLKVPSAAERKHRHLPVADAVIHAHELVCSMENTRATKMQVIGDQYVKVYLESIIKPGKISHIMLDVAFTSHEDSDNEIHNPAV
              FTWPPWQAGILARNLVPMVATVQGQNLKYGNGLGQKEDSSGPEGSGGSGPQRRGGDNHGFEYLQEGGPDGEPMVFLQTHIFAEVLKDEDLPCIVSRGGPKVKRPPIFFIEFVGWLCKKDHTHIREWFPA
              PQAPYQGYQEPPAPQAPYYLQQNWWTLLVDLLWLLDYQPLGTQDQSLYLGAVFDRKSDAKSSCSSCPLSKIGSGSGS

              464 amino acids  =  52.4 kDa
```

Figure 11: Tandem HLA-A11 epitopes.

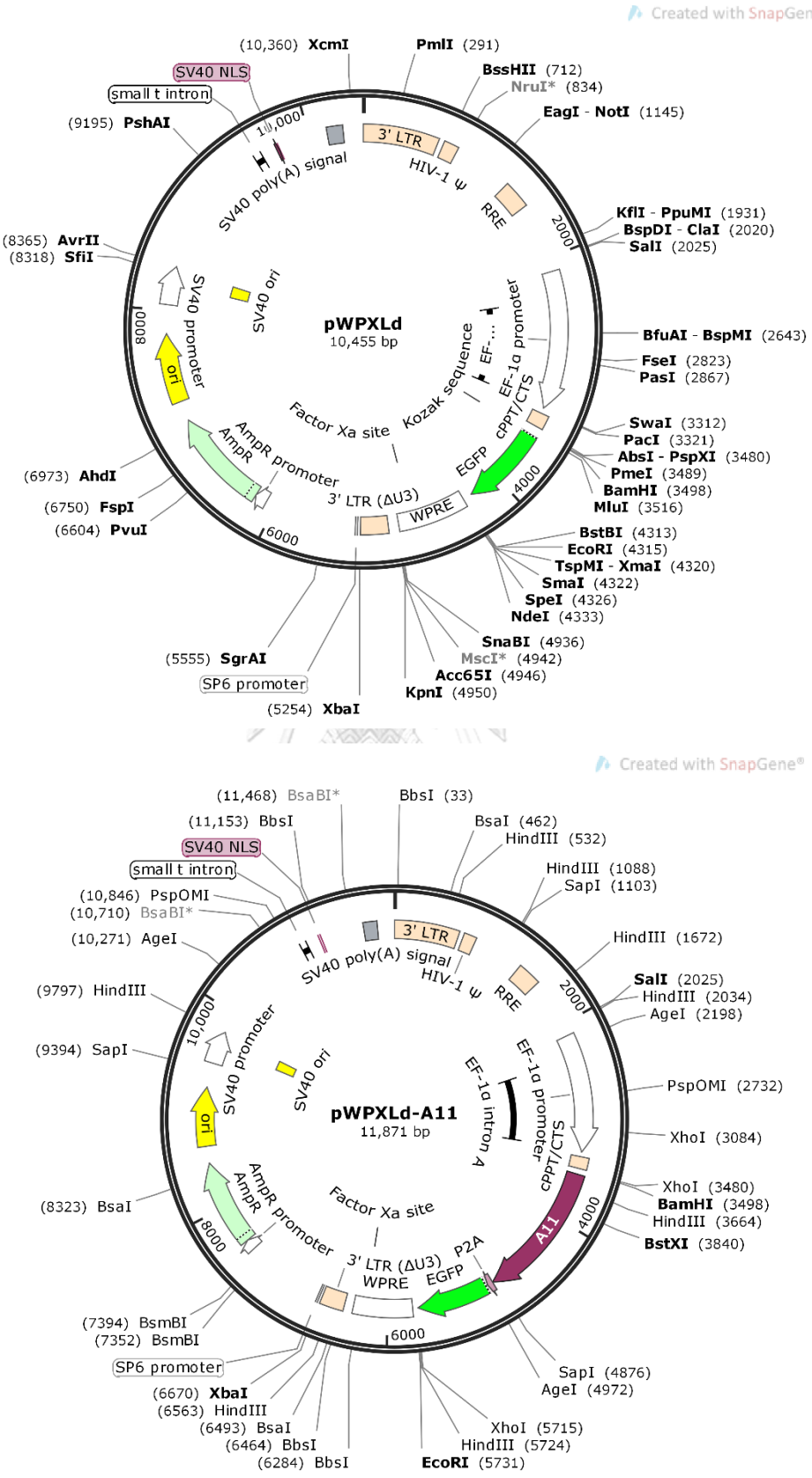


Figure 12: pWPXLd vector (above) and A11-pWPXLd (below).

Gene of interest was inserted between BamHI site and EcoRI site of pWPXLd. There were 2 constructs of A11-pWPXLd 1.) pA11-AAG which lysine (K) codon at C-terminal position of AK11 epitope was translated by AAG codon and 2.) pA11-AAA was translated by AAA codon. pA11-AAG was used as a template for mutagenesis. Primers were synthesized to generate pA11-AAA as followed; A11Mut-F (5'-GAGGACCTAGCCATAAAGCAGTATTCATTCAC-3'), and A11Mut-R (5'A TACTGCTTTATGGCTAGGTCCTCCCAC-3'). Sequencing was performed to confirm mutagenesis result as shown in Figure 13.

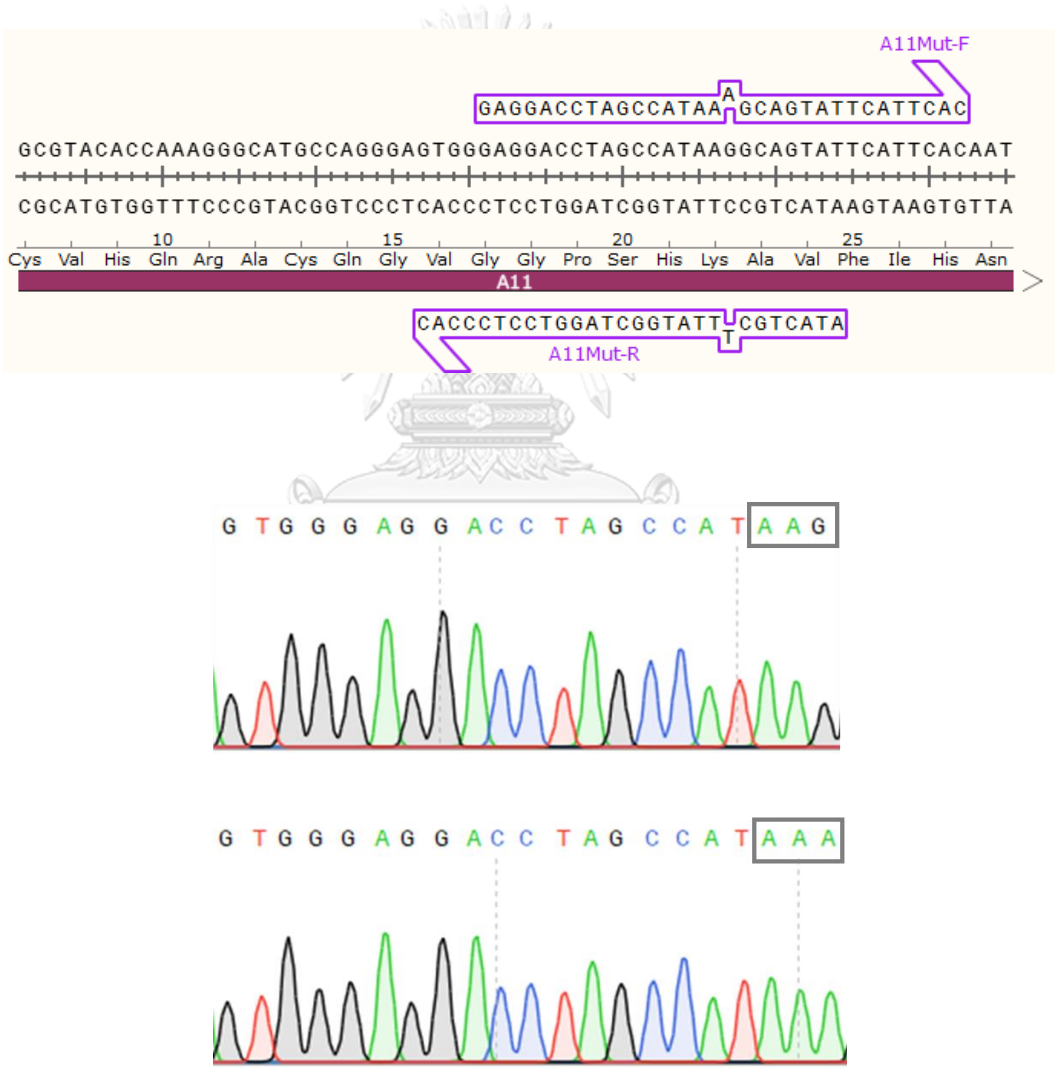


Figure 13: Mutagenesis primers and sequencing results of pA11-AAG and pA11-AAA constructions.

Lentiviral production and titration

The A11-pWPXLd as a transfer vector was transfected into HEK293T cell line (ATCC® CRL-3216™) using lipofectamine 3000 (Invitrogen, USA) together with 2 protein expression vectors for Gag/Pol (psPAX2 was a gift from Prof. Didier Trono, Addgene plasmid #12260) and G protein of vesicular stomatitis virus (pCMV-VSV-G was a gift from Bob Weinberg, Addgene plasmid #8454) as envelop. First, 9.5×10^6 HEK293T cells were seeded on T75 cm^2 tissue culture flasks (Nunc™, Thermo Scientific, USA) in DMEM medium (Gibco, USA) supplemented with 10% heat-inactivated FBS and were incubated at 37°C, 5% CO_2 overnight. The lipo-DNA mixture was prepared as per manufacturer's recommendations. The molar ratio of plasmid concentration was 5:3:2 (Transfer vector: Gag/Pol: Env).

$$\text{Plasmid (µg)} = \text{total plasmids concentration} \times \frac{\text{Molar ratio} \times \text{plasmid size}}{\Sigma\ (\text{Molar ratio} \times \text{plasmid size})}$$

The culture flasks were maintained in Opti-MEM™ I Reduced Serum Media (Gibco, USA) supplemented with 5% heat-inactivated FBS and 1mM of sodium pyruvate (Gibco, USA) as packaging medium and incubated for 6 hours at 37°C, 5% CO_2. After 6 hours post-transfection, medium was carefully removed and replaced with pre-warmed packaging medium and returned into an incubator. After 24 hours post-transfection, culture supernatant which contained viral particles was collected and replaced with packaging medium. After 52 hours post-transfection, culture supernatant was collected and pooled with previous collection. Total culture supernatant was centrifuged at 2,000 rpm for 10 minutes to remove cell debris, filtered by 0.45 µM filter (Merck Millipore, USA), aliquoted and stored at -80°C.

Lentiviral titer was determined by infecting virus at various concentrations into Nalm-6 cell line (ATCC: CRL 3273) which is human leukemic pre-B cell. Transduction units was detected by flow cytometry and mean fluorescence intensity of GFP was further calculation.

Lentiviral transduction

Antigen presenting cells were transduced by lentiviral particles at MOI 1, 10 and 100 which contained tandem HLA-A11 epitopes in 6-well plates using a spinoculation method at 1,500xg for 90 minutes at 32°C. The transduction medium composed of R10 medium and 8 µg/mL of polybrene (Sigma-Aldrich, Germany) to promote viral entry. The following day, culture supernatant was taken out and replaced by R10 medium without polybrene. Cultured plates were incubated for 48 hours and subsequently harvested for experiments. Transduction efficiency was determined by flow cytometry (BD FACSAria™ II) to measure mean fluorescence intensity (MFI) of EGFP.

Statistical analysis

Mann-Whitney U test, Fisher's exact test and ANOVA were applied to compare study groups for statistical differences by using GraphPad Prism version 6.00 (GraphPad Software, La Jolla California, USA). If results showed p-value <0.05, they were marked as statistical significance. Mann-Whitney U test was used for nonparametric t-test. Mann-Whitney U test was performed to compare codon usage as contingency tables.

CHAPTER V
RESULTS

Demographic data

Sixty six HIV-infected volunteers were recruited to this study from anonymous clinic, The Thai Red Cross AIDS Research Center. Volunteers were classified into 2 groups based on their HIV plasma viral load (pVL). Viraemic controllers (VC) had pVL less than 2,000 copies/mL and non-controllers (NC) had pVL greater than 2,000 copies/mL.

The demographic data were shown in Table 6. Nineteen VC consisted of 14 males (74%) and 5 females (26%). Forty seven NC composed of 36 males (77%) and 11 females (23%). For sexual preference, most of VC and NC were homosexual 68% and 69% with small numbers of heterosexual 32% and 31%, respectively. Median age of VC was 30 years (range between 21 and 52 years) and NC was 28 years (range between 20 and 58 years). Median time since seroconversion was comparable in both groups (9 years for VC and 6.5 years for NC). The presence of protective alleles in VC and NC such as HLA-B27 (5.7% vs 8.8%), HLA-B57 (8.6% vs 4.4%) and HLA-B58 (5.7% vs 4.4%) was also similar among these two groups. However, none of those parameters was statistically significant which may be suggested that confounding factors were minimized in this study.

Table 6: Demographic data of study population

	Controllers (VL < 2,000 cp/mL)	**Non-controllers (VL > 2,000 cp/mL)**
n	19 (29%)	47 (71%)
• Male	14 (74%)	36 (77%)
• Female	5 (26%)	11 (23%)
Sexual preference		
• Homosexual	13 (68%)	34 (69%)
• Heterosexual	6 (32%)	13 (31%)
Median Age (year)	30 (21-52)	28 (20-58)
Median Time since seroconversion (year)	9 (4-17)	6.5 (4-13)
Protective alleles		
• HLA-B27	2 (5.7%)	8 (8.8%)
• HLA-B57	3 (8.6%)	4 (4.4%)
• HLA-B58	2 (5.7%)	4 (4.4%)

Complete blood count, CD3/CD4/CD8 count and HIV plasma viral load

Complete blood count (CBC) is a laboratory test to evaluate blood components. In this study, CBC was used to determine white blood cell count and total number of lymphocytes. After staining whole blood with Tritest™ CD4/CD8/CD3 antibodies, flow cytometry data and CBC data were further calculated for $CD3^+$ cells which are represented a number of T cells. White blood cell count (Figure 14), was not different among healthy donors (HD, 5,890 cells/µL) and HIV-infected volunteers neither VC (6,420 cells/µL) nor NC (5,890 cells/µL).

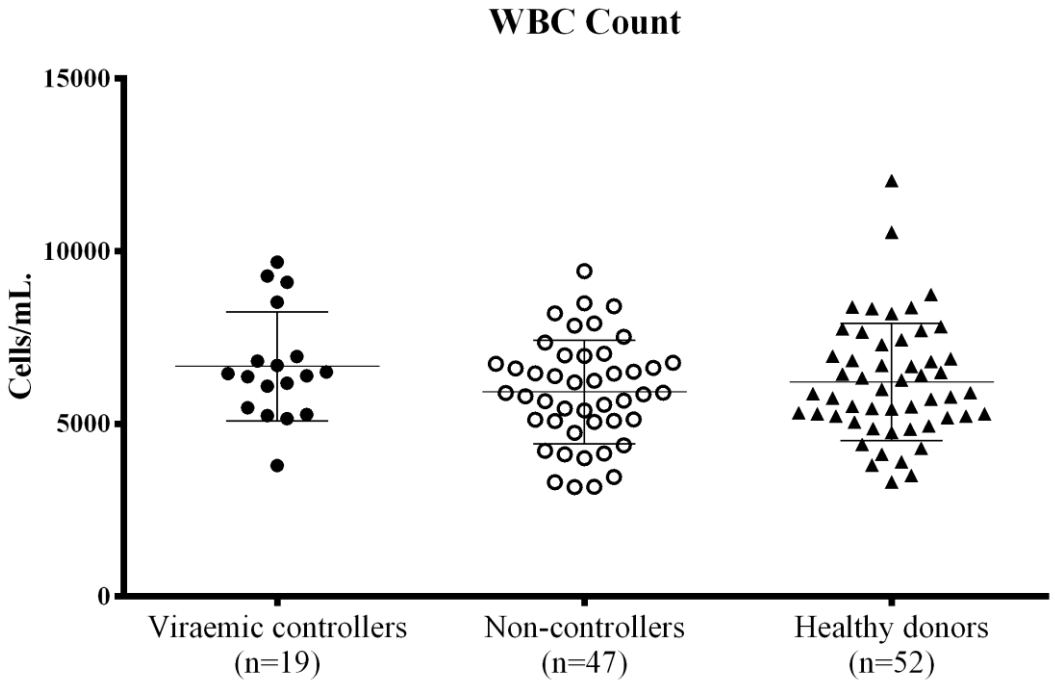


Figure 14: White blood cell count from VC (n=19), NC (n=47) and healthy donors (n=52)

Absolute number and percentage of lymphocytes (Figure 15) were compared between healthy donors, VC and NC. HIV-infected volunteers had higher median absolute number and percentage of lymphocytes than healthy donors significantly. VC had median lymphocytes 2,533 cells/µL (38.5%) while healthy donors had 1,902 cells/µL (32.7%) ($p<0.001$). NC had median lymphocytes 2,440 cells/µL (42.5%) which was statistically significant when compared with healthy donors ($p<0.05$).

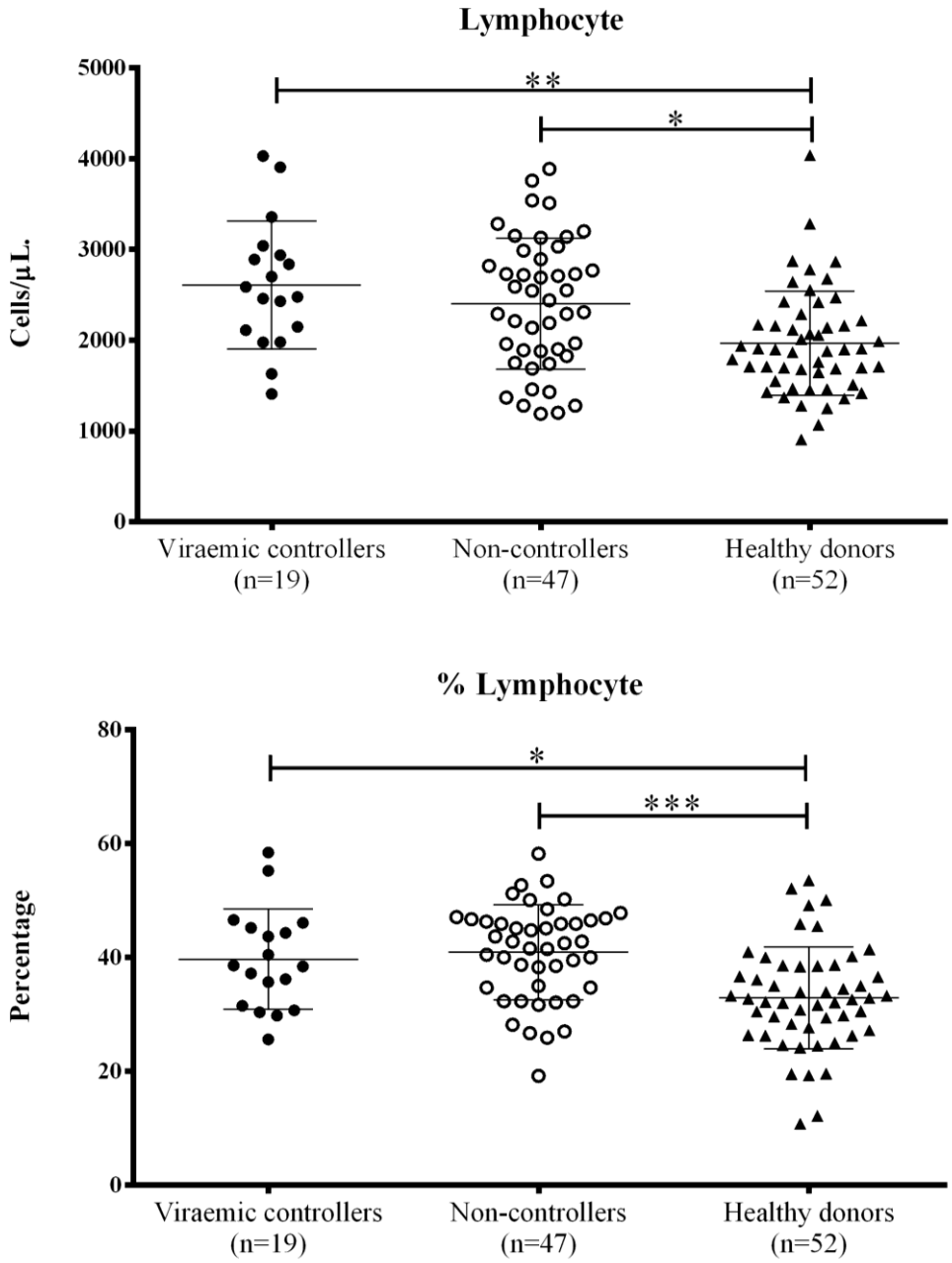


Figure 15: Absolute number and percentage of lymphocytes from VC, NC and HD (* $p<0.05$, ** $p<0.001$, *** $p<0.0001$).

Absolute number and percentage of CD3 (Figure 16) were compared among healthy donors, VC and NC. $CD3^+$ cells are representative of T lymphocytes. HIV-infected volunteers had significantly higher median absolute number and percentage of CD3. VC had median absolute CD3 1,967 cells/µL (78.5%) while healthy donors had 1,328 cells/µL (71%) ($p<0.0001$). NC had median absolute CD3 1,813 cells/µL (79%) which was significant different when compared with healthy donors ($p<0.0001$).

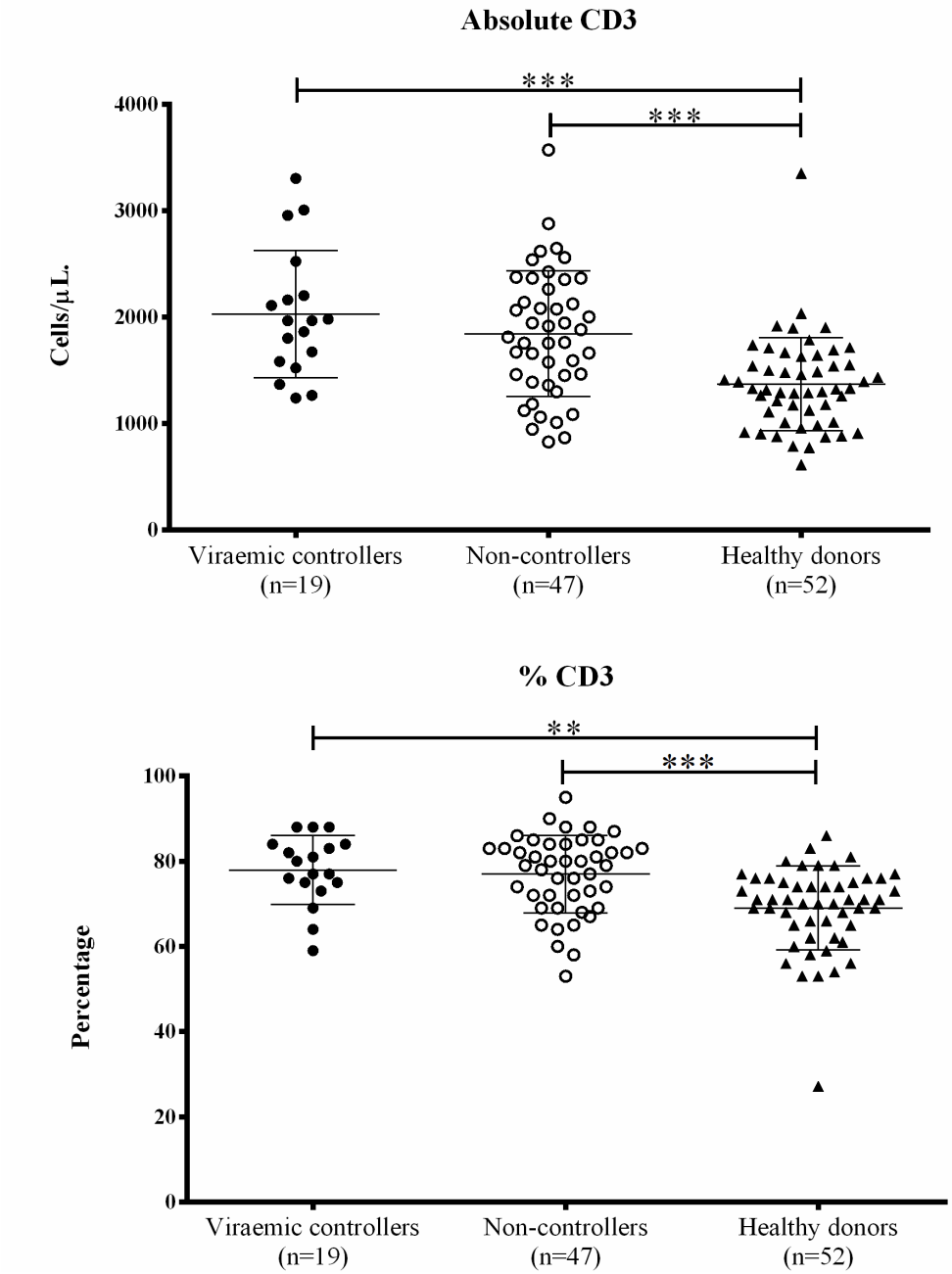


Figure 16: Absolute number and percentage of CD3 from VC, NC and HD (** $p<0.001$, *** $p<0.0001$).

$CD4^+$ T cell is one of the important markers to assess immunological status of HIV-infected patients. Absolute number and percentage of $CD4^+$ T cells as shown in Figure 17, median absolute $CD4^+$ T cell of healthy donors was 605 cells/µL, VC was 639 cells/µL and NC was 575 cells/µL. Nevertheless, there were no difference among these three groups at absolute number count. Percentage of $CD4^+$ T cell was significantly decreased in HIV-infected patients (28% in VC and 27% in NC) when compared to HIV seronegative volunteers (32%). VC and NC had similar CD4 level in addition to our intention to enroll volunteers with the same stage of disease.

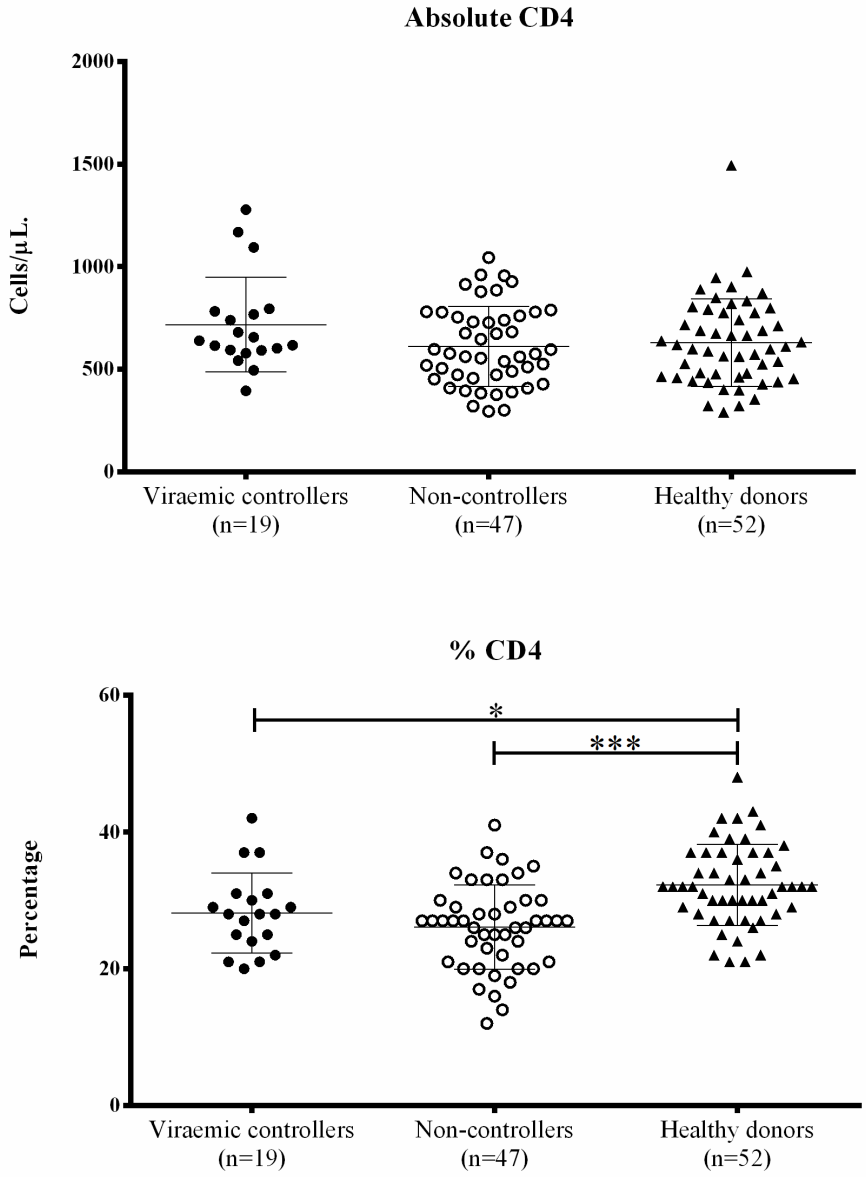


Figure 17: Absolute number and percentage of CD4 of VC, NC and HD (* $p<0.05$, *** $p<0.0001$).

$CD8^+$ T cell or cytotoxic T cell (CTL) has a potential role in HIV control (9, 142). This cell population is subjected to increase during viral infection. Absolute number and percentage of $CD8^+$ T cells were shown in Figure 18. Median absolute $CD8^+$ T cell of healthy donors was 530 cells/µL (29%), VC was 1,078 cells/µL (43%) and NC was 1,070 cells/µL (46%). The results showed that absolute number and percentage of $CD8^+$ T cells of HIV-infected volunteers were significantly greater than healthy donors ($p<0.0001$).

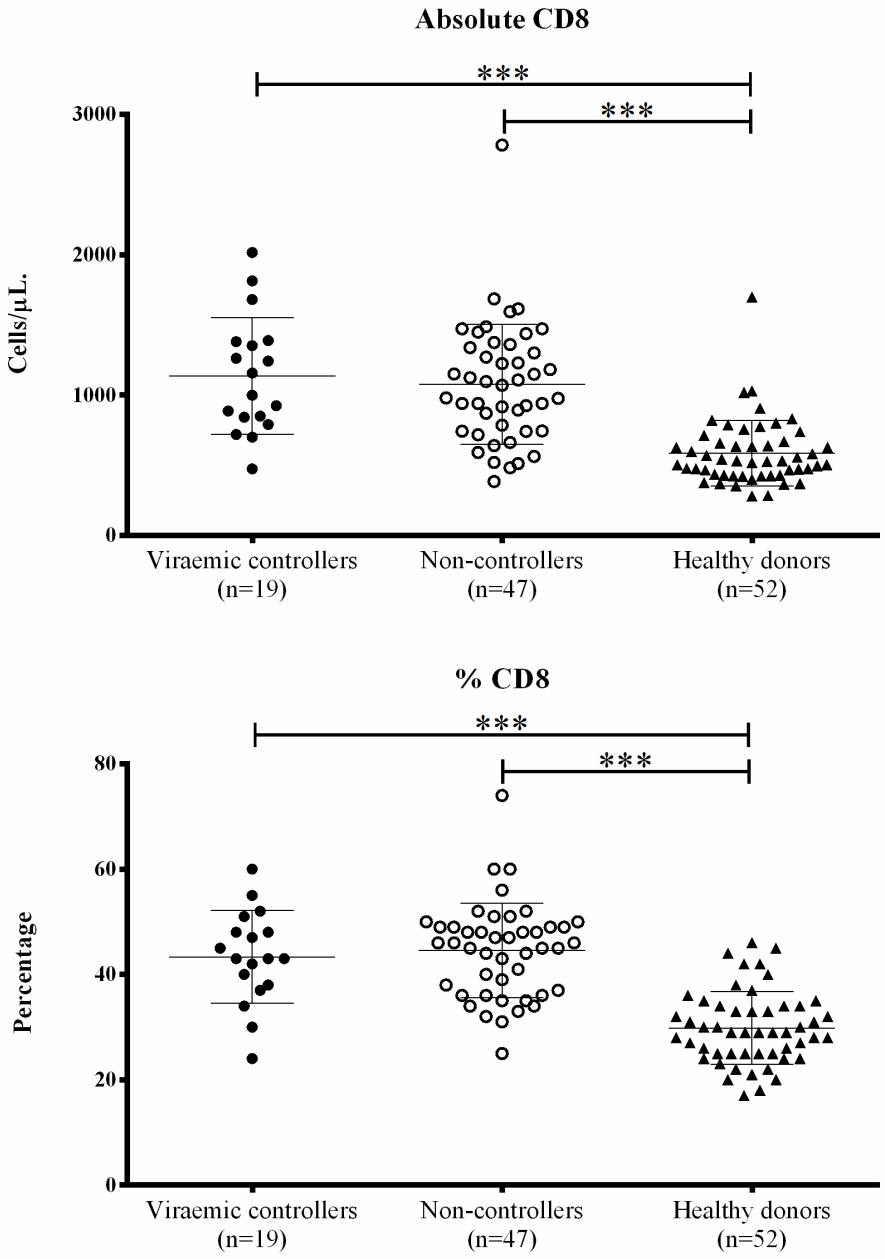


Figure 18: Absolute number and percentage of CD8 of VC, NC and HD (*** $p<0.0001$)

CD4/CD8 ratio is proposed to use as a marker for T cell activation and senescence in HIV-infected patients (143-145). Normally, immunocompetent person has CD4/CD8 ratio >1, on the other hand, HIV-infected patients tend to have inversion of CD4/CD8 ratio (≤1).

We compared CD4/CD8 ratio between healthy donors and HIV-infected volunteers (VC and NC). The results showed that CD4/CD8 ratio in healthy donors, VC and NC were 1.036, 0.6478, 0.5887, correspondingly which was significantly different between healthy donors and HIV-infected patients ($p<0.0001$).

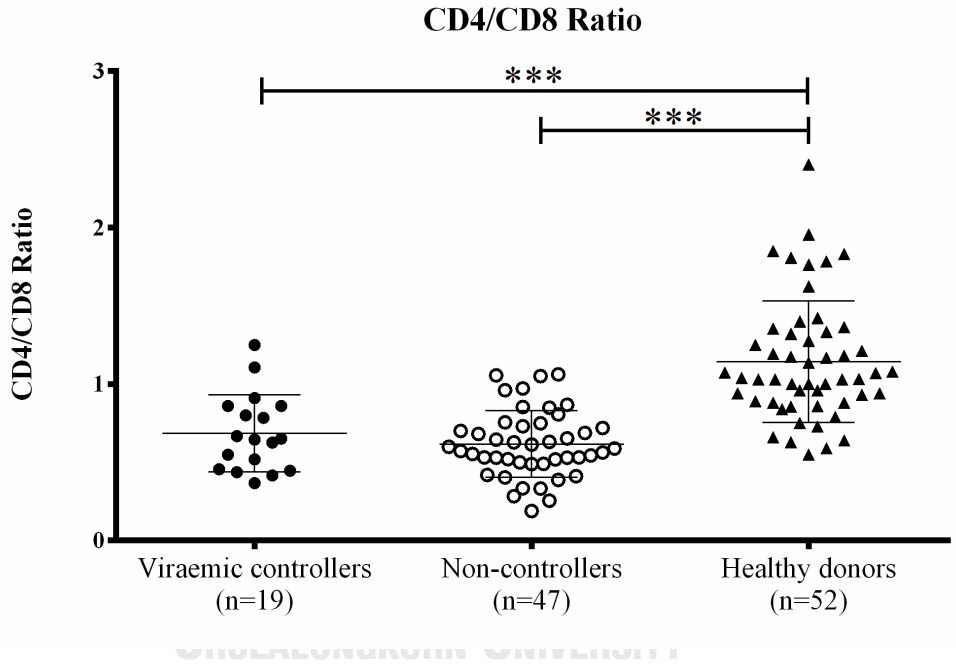


Figure 19: CD4/CD8 ratio among VC, NC and HD

(*** $p<0.0001$)

HIV plasma viral load (pVL) is another marker for HIV disease progression. The median HIV pVL at baseline showed that VC had 419 copies/mL (2.62 log) while NC had 12,231 copies/mL (4.045 log). As we classified volunteers into 2 groups depending on their HIV plasma viral load, we were not surprised that VC had lower level of HIV pVL than NC significantly ($p<0.0001$).

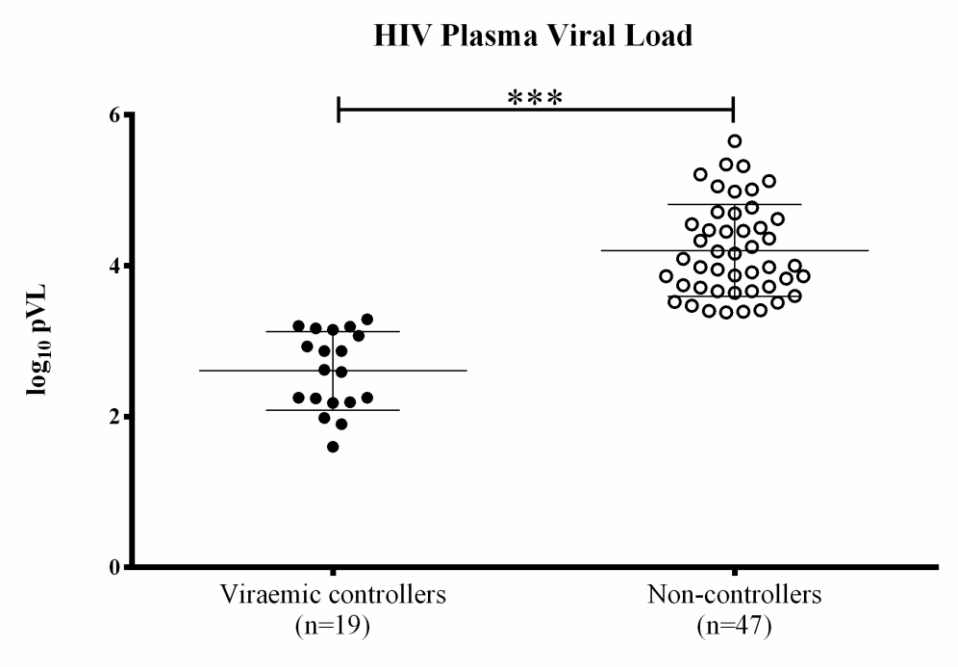


Figure 20: HIV plasma viral load detection in VC (n=19) and NC (n=47). (*** $p<0.0001$)

Table 7 shows the summary of complete blood count including CD3, CD4, CD8 count and CD4/CD8 ratio among viraemic controllers (n=19), non-controllers (n=47) and HIV seronegative healthy donors (n=52).

Table 7: Summary of CBC, CD3/CD4/CD8 count, CD4/CD8 ratio and HIV viral load.

	Viraemic controllers (n=19)	**Non-controllers (n=47)**	**Healthy Donors (n=52)**
WBC count (cells/µL)	6,420	5,890	5,890
Median lymphocytes			
• Absolute number (cells/µL)	2,533	2,440	1,902
• Percentage	38.5	42.5	32.7
Median CD3			
• Absolute number (cells/µL)	1,967	1,813	1,328
• Percentage	78.5	79	71
Median CD4			
• Absolute number (cells/µL)	639	575	605
• Percentage	28	27	32
Median CD8			
• Absolute number (cells/µL)	1,078	1,070	530
• Percentage	43	46	29
CD4/CD8 ratio	0.6478	0.5887	1.036
HIV plasma viral load			
• copies/mL	419	12,231	N.D.
• log	2.62	4.045	N.D.

N.D. = Not determined

T cell activation markers

Activation markers are proposed to determine disease progression (146). CD38 (147) and HLA-DR (148) are frequently used for activation markers in HIV infection. Percentage of $CD38^-$, $HLA\text{-}DR^-$ population (non-activated) either in $CD4^+$ or $CD8^+$ T cells was significantly higher in healthy donors when compared to NC. NC also showed greater percentage of $CD38^+$, $HLA\text{-}DR^+$ $CD8^+$ T cells when compared to VC and healthy donors significantly.

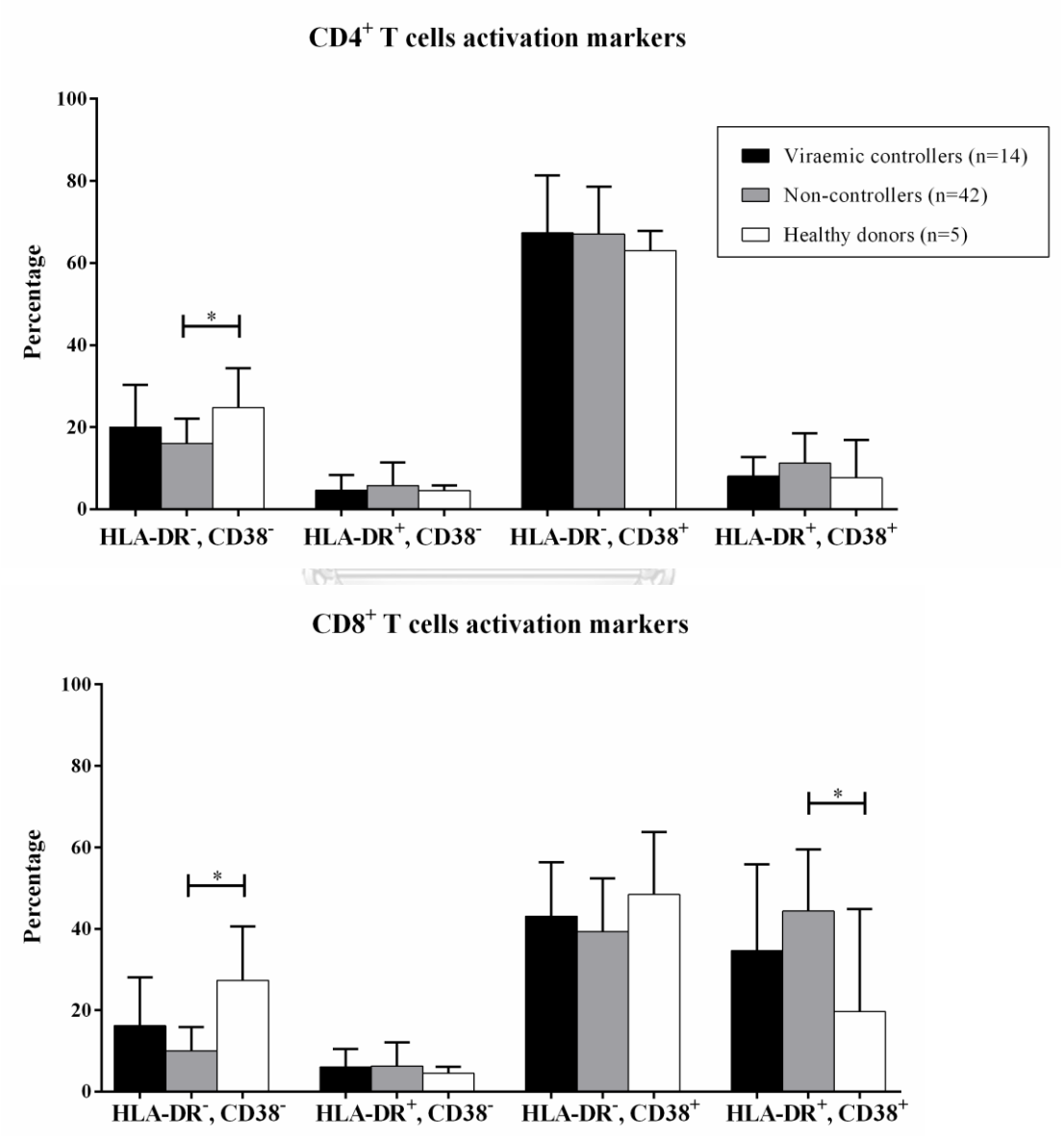


Figure 21: Activation markers (CD38 and HLA-DR) expression on $CD4^+$ T cells and $CD8^+$ T cells in VC (n=14), NC (n=42) and HD (n=5) (* $p < 0.05$).

HLA class I of the study population

Human leukocyte antigen (HLA) class I molecules have shown that these molecules play an important role in presenting antigens to CTL. A number of studies have reported that some HLA alleles such as HLA-B27, HLA-B57 and HLA-B58 are essential for HIV control in Caucasian population (13, 84, 149, 150). Hence, those alleles are called protective alleles.

However, data from our previous study have shown that protective alleles were not different in viraemic controllers and non-controllers (11). In this study, we also had the similar results as shown in Table 6. Thus, we further characterized HLA class I molecules to investigate whether other alleles may have significant effect on viral control rather than HLA-B27, HLA-B57 and HLA-B58.

For HLA-A locus, HLA-A11 was the most frequency among study population (n=38 alleles, 29.2% of total HLA-A alleles) followed by HLA-A02 (n=26, 20%), HLA-A24 (n=21, 16.2%) and HLA-A33 (n=16, 12.3%).

For HLA-B locus which is the most diverse HLA locus, HLA-B15 was the most frequency among study population (n=23 alleles, 17.7% of total HLA-B alleles) followed by HLA-B40 (n=17, 13.1%), HLA-B46 (n=11, 8.5%) and HLA-B27 (n=10, 7.7%)

For HLA-C locus, HLA-C07 was the most frequency among study population (n=36 alleles, 27.9% of total HLA-C alleles) followed by HLA-C08 (n=22, 17.1%), HLA-C03 (n=17, 13.2%) and HLA-C01,-C06 (n=13, 10.1%).

We also compared HLA class I frequency and percentage as showed in Figure ***23*** between viraemic controllers and non-controllers to see whether some dominant alleles are higher in VC or not. However, none of those alleles was not significantly different and associated control or progression in our study population.

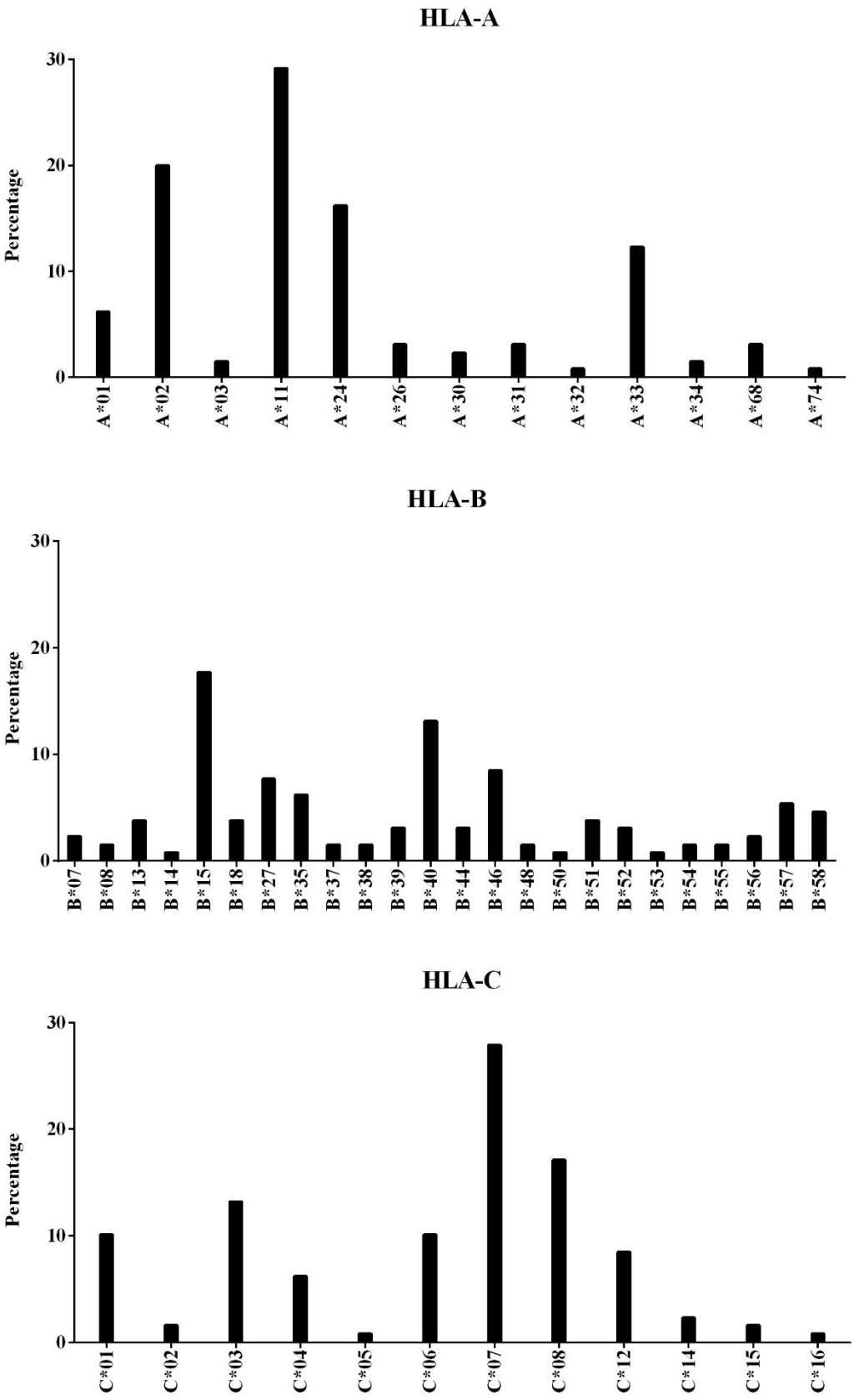


Figure 22: Total HLA class I locus A, B and C frequency in our study.

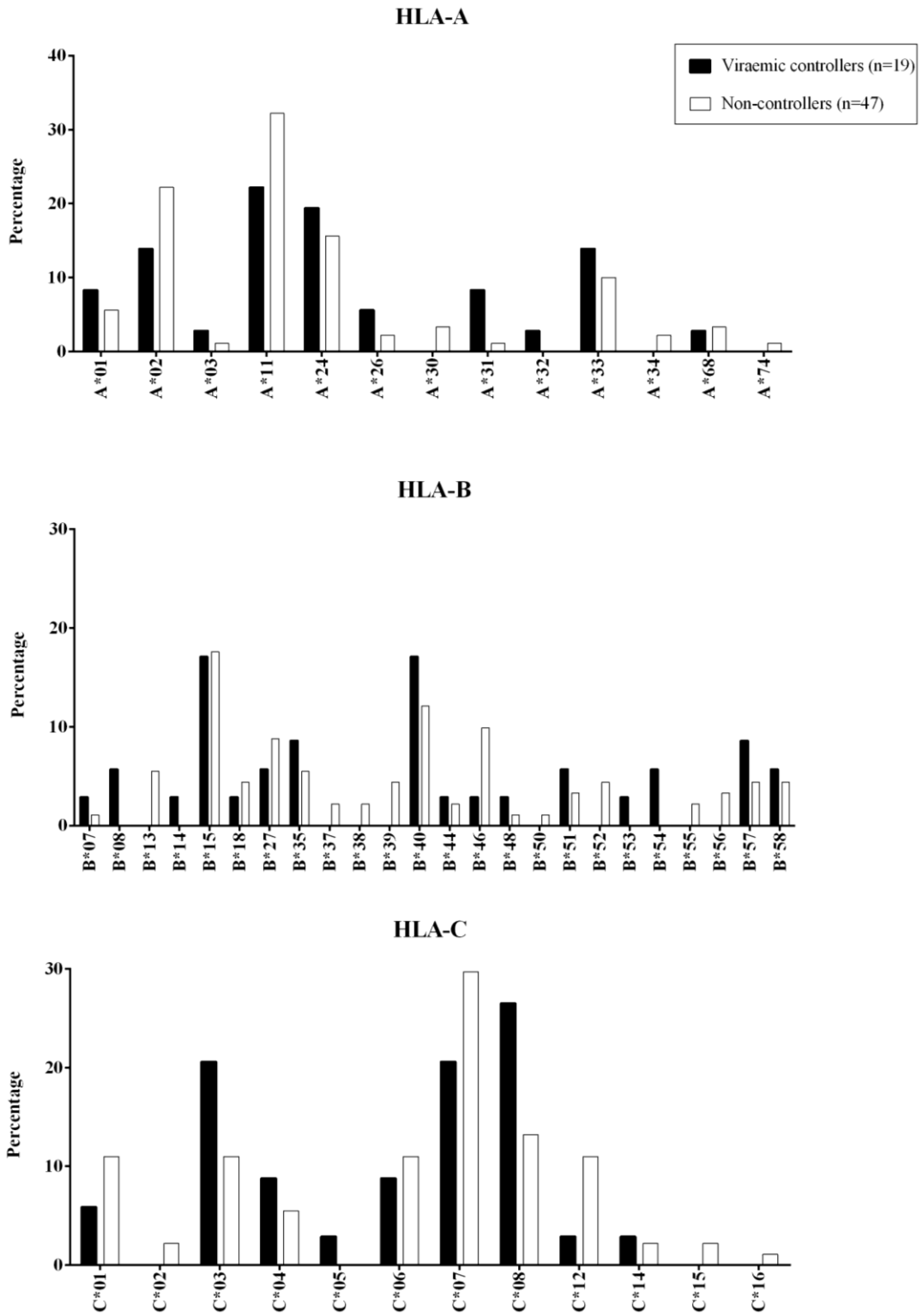


Figure 23: Comparison of HLA class I loci A, B and C percentage between VC (n=19, black bar) and NC (n=47, grey bar).

HLA class I polymorphisms

In the past, we identified HLA types by serological technique (151). This procedure uses a variety of antibodies to identify allele family which are the first 2 digits. Molecular technique called PCR-SSOP has been developed to replace a previous laborious one (152). PCR-SSOP is a combination technique between PCR amplification and hybridization with specific probes. A number of probes provide better resolution results. As shown in

Figure ***24***, HLA nomenclature begins with HLA locus then followed by allele family and allele number. Colon sign is applied to distinguish levels of resolution.

Figure 24: HLA nomenclature at high resolution (4 digits)

According to reports, HLA class I molecules that share the same allele family but differ in allele number may have different properties of a peptide binding groove. As several studies on ankylosing spondylitis, HLA-B2704 and HLA-B2705 are associated with disease while HLA-B2706 and HLA-B2709 are not (153). High resolution HLA typing was performed to determine HLA polymorphisms. The

percentage of those alleles were compared between VC and NC as shown in

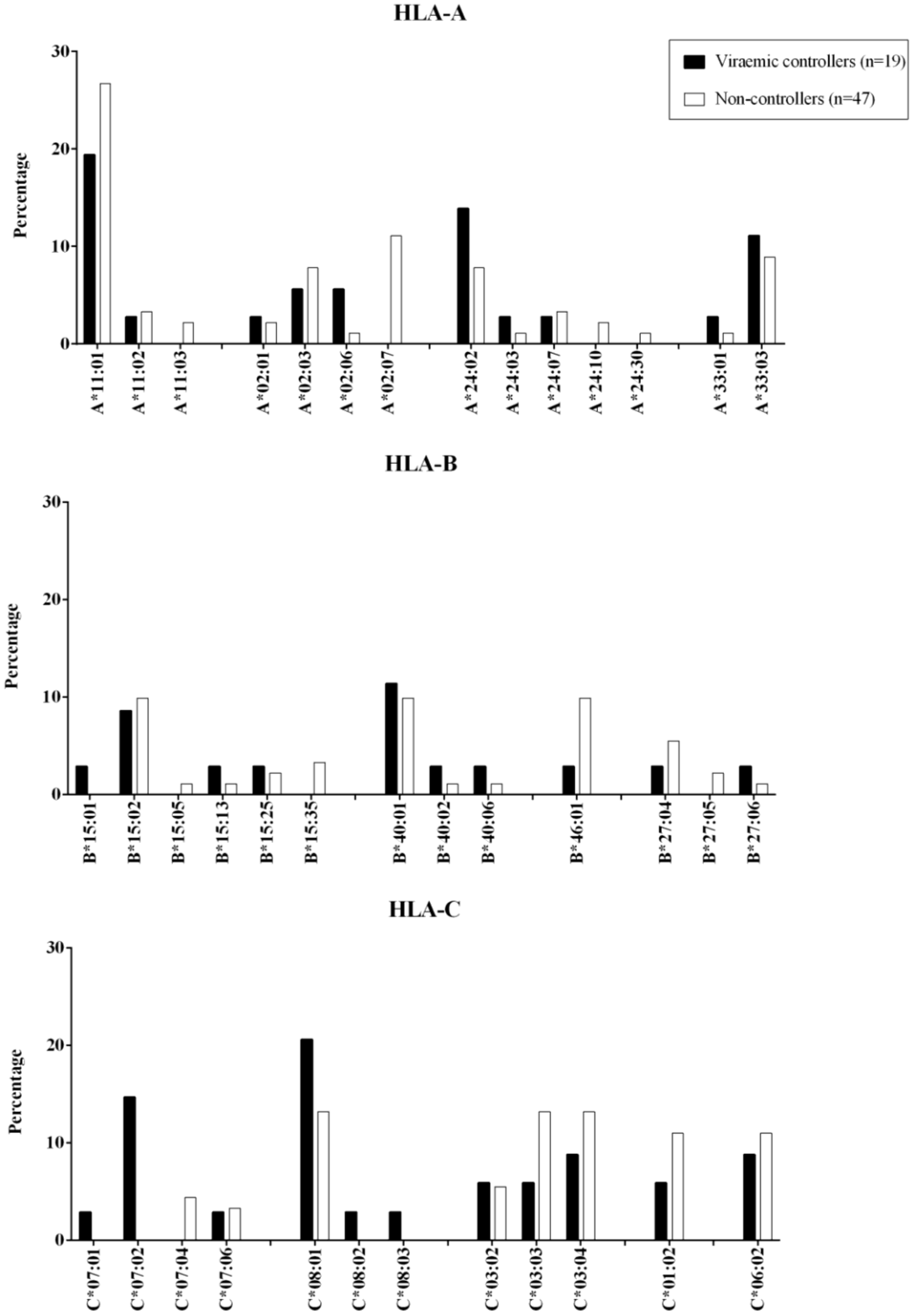
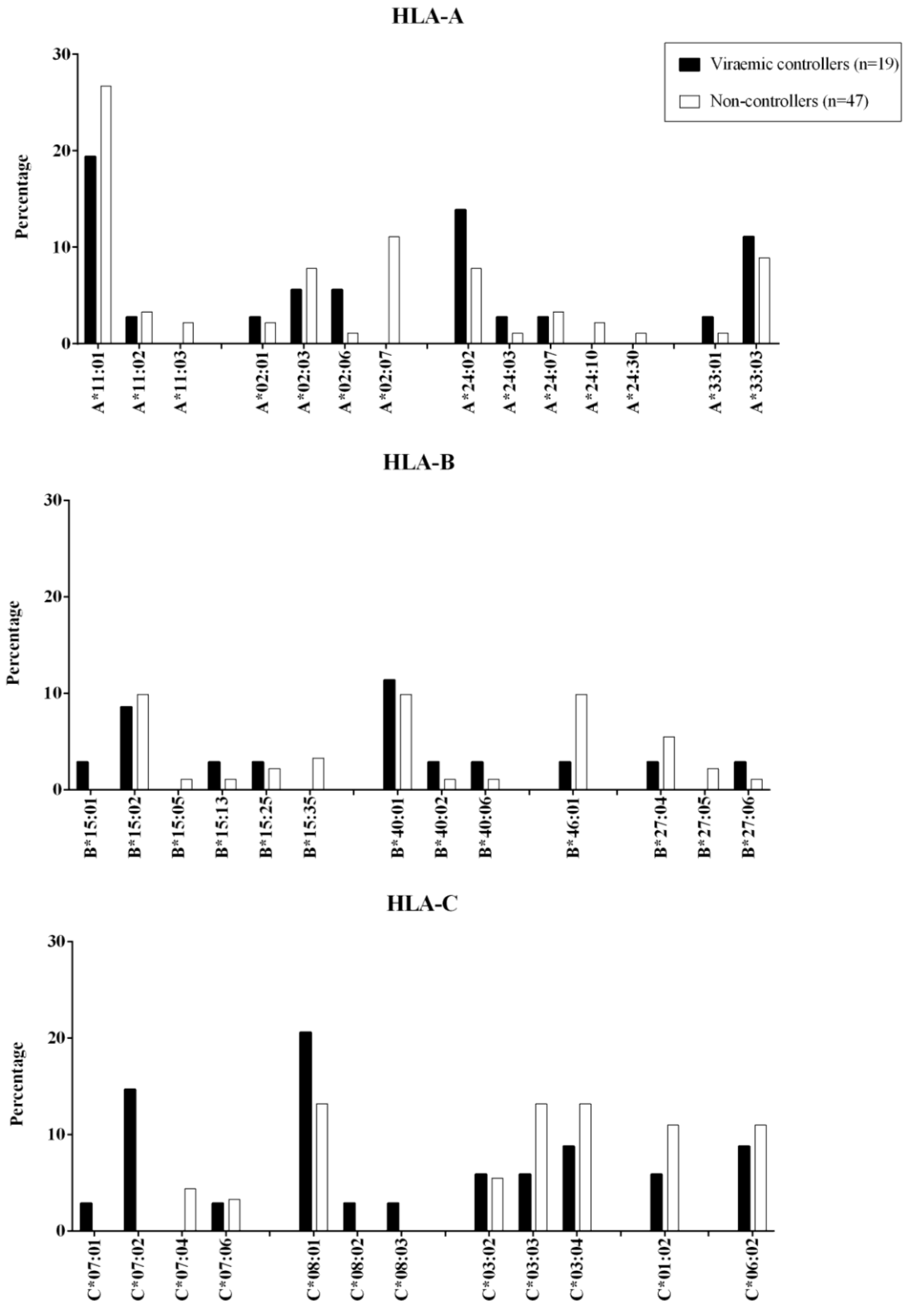


Figure ***25***. However, there was no significant different among HIV-infected volunteers.


HLA-A
Viraemic controllers (n=19)
Non-controllers (n=47)
Percentage
30
20
10
0
A*11:01
A*11:02
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A*02:01
A*02:03
A*02:06
A*02:07
A*24:02
A*24:03
A*24:07
A*24:10
A*24:30
A*33:01
A*33:03
HLA-B
Percentage
30
20
10
0
B*15:01
B*15:02
B*15:05
B*15:13
B*15:25
B*15:35
B*40:01
B*40:02
B*40:06
B*46:01
B*27:04
B*27:05
B*27:06
HLA-C
Percentage
30
20
10
0
C*07:01
C*07:02
C*07:04
C*07:06
C*08:01
C*08:02
C*08:03
C*03:02
C*03:03
C*03:04
C*01:02
C*06:02

Figure 25: Comparison of polymorphisms of dominant HLA class I alleles in this study between VC (n=19) and NC (n=47)

IFNγ-secreting cell response by ELISpot assay

HIV Gag p24 overlapping peptides

To determine IFNγ-secreting cell response against HIV peptides, PBMCs were stimulated with 23 Gag p24 overlapping peptides (OLPs; 20 mers overlapped by 10 mers) as previously described in Table 3 (11). Spot forming units (SFU) were measured and subtracted by negative control before calculation.

All of PBMCs from VC (n=19) could respond at least one Gag p24 OLP while 95.7% (45 out of 47) of NC showed respond.

Number of OLPs responders and magnitude of response were shown in Figure 26 and Figure 27, consecutively. IFNγ-secreting cell response was observed in all across the whole Gag p24. The highest responsive OLP was found in OLP21 (n=35) followed by OLP17 (n=29) and OLP14, OLP19 (n=22).

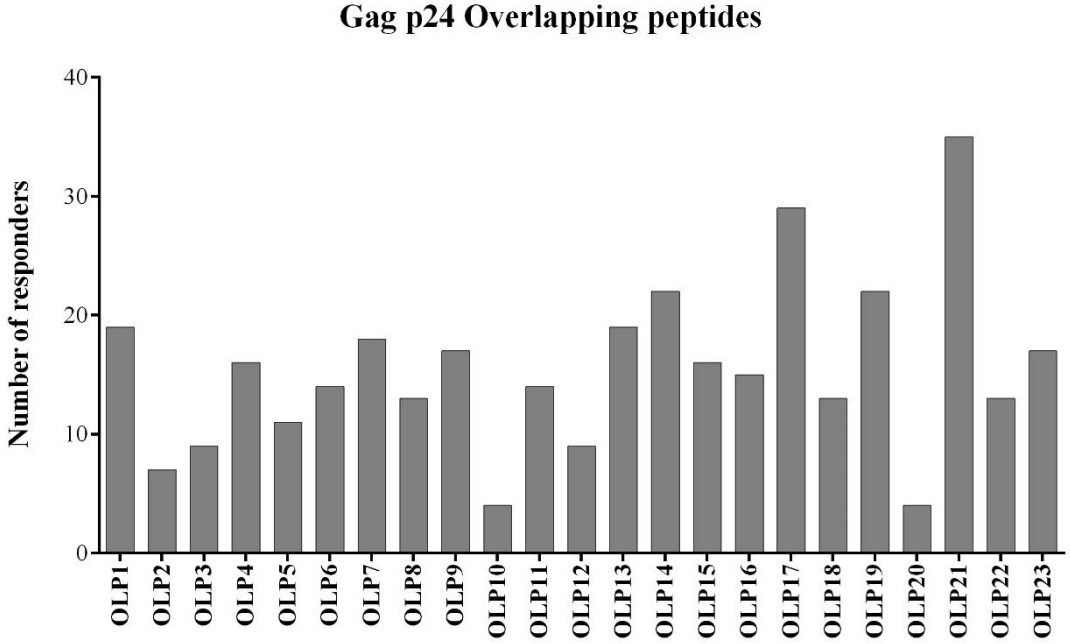


Figure 26: Number of responders against HIV Gag p24 OLPs.

The semi-quantitative measurement from IFNγ ELISpot was presented as magnitude of response against each overlapping peptide. HIV Gag p24 OLP15 had the highest median magnitude (829 SFU/10^6 PBMCs) followed by OLP7 (717 SFU/10^6 PBMCs) and OLP20 (662 SFU/10^6 PBMCs), respectively.

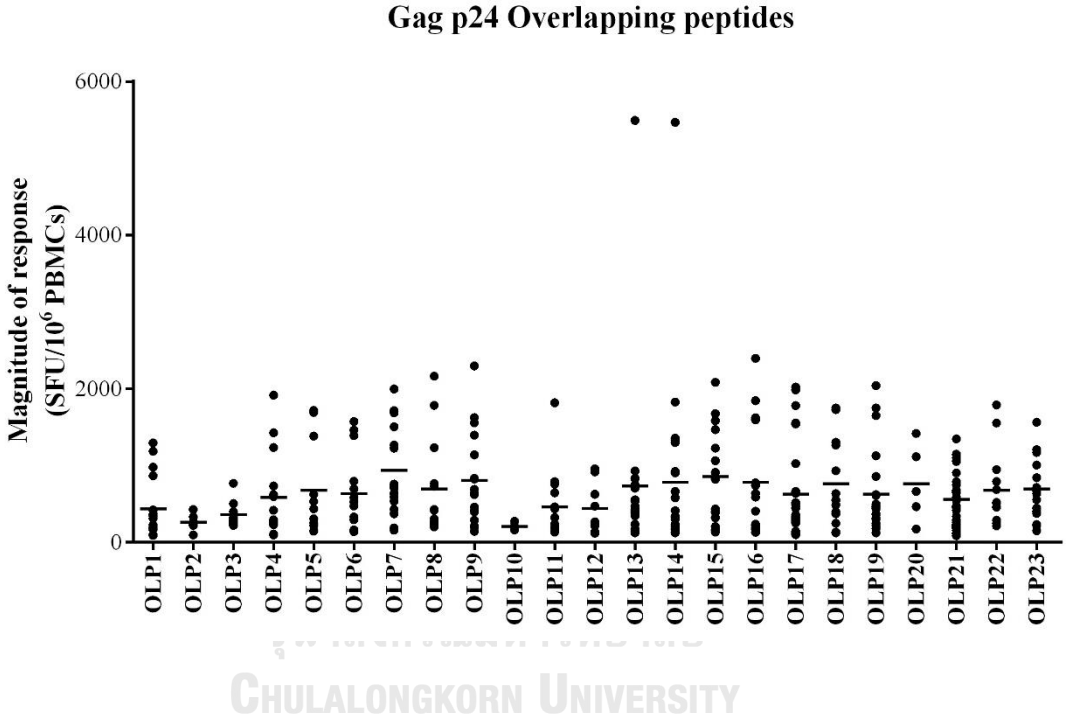


Figure 27: Magnitude of response against HIV Gag p24.

Well-characterized epitopes restricted by protective alleles

Protective alleles for HIV progression are HLA class I molecules which have been reported that associated with HIV control either in long-term non-progressor or HIV controllers. HLA-B27, HLA-B57 and HLA-B58 are considered to be protective alleles. However, the certain mechanism is still unclear. Some studies revealed that those alleles can recognize and present high functional constraint of HIV.

To determine T cell response against well-characterized epitopes, HLA-B27 restricted epitopes and HLA-B57/58 restricted epitopes (as described in Table 4 and Table 5, respectively) were used to stimulate patients' PBMCs. The peptides were not specific only Gag p24 but also others proteins such as Gag p17, Nef, Rev and Pol.

For HLA-B27, PBMCs from HIV infected volunteers who present HLA-B27 allele (n=9) were co-cultured with HLA-B27 restricted epitopes and determined response by ELISpot. The results showed that Gag p24 KK10 epitopes and also its variants were well recognized compared to Nef while there was no response against Rev-RR8 epitope. (Figure 28)

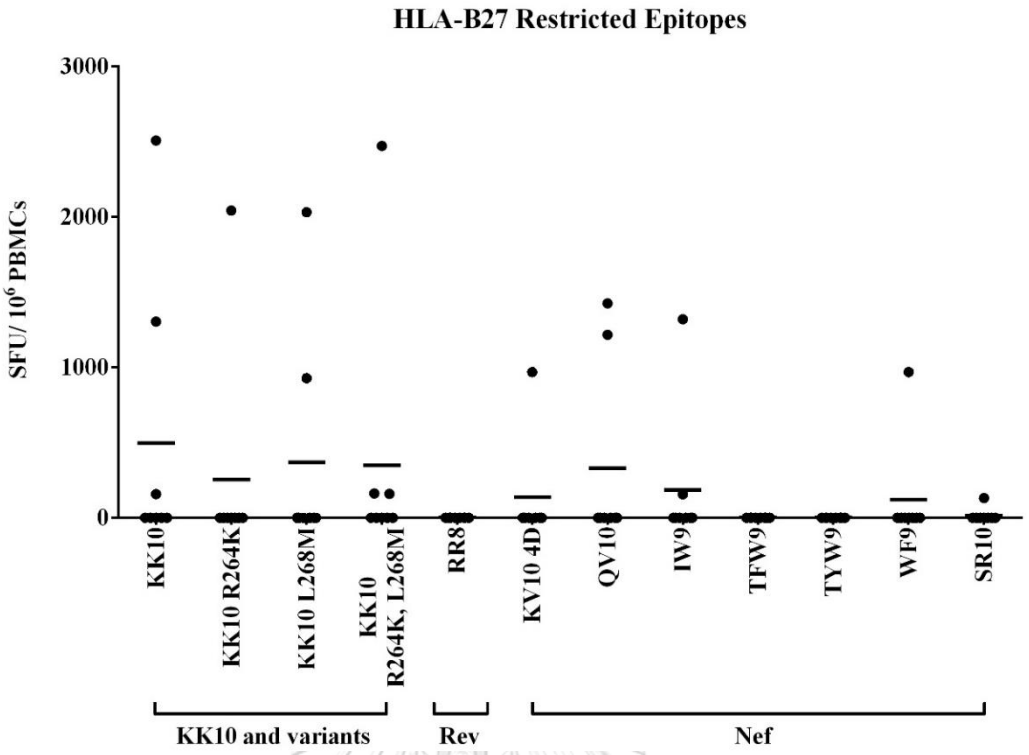


Figure 28: HLA-B27 restricted epitopes (n=12) response in HLA-B27^{+} patients (n=9) as determined by IFNγ ELISpot.

KK10 (Gag position 263 to 270) is important for HIV capsid dimerization. The mutation in this epitope is usually related to viral fitness cost (14). Arginine (R) to lysine (K) at position 264 (R264K) and leucine (L) to methionine (M) at position 268 (L268M) mutations are commonly found. According to our ELISpot results, HN52 showed response against KK10 wild type and also its variants. To determine peptide avidity among KK10 and variants, peptide titration assay was employed. PBMCs from HN52 were stimulated with various peptide concentrations. KK10 avidity was comparable with L268M while R264K showed lower avidity than KK10 wild type.

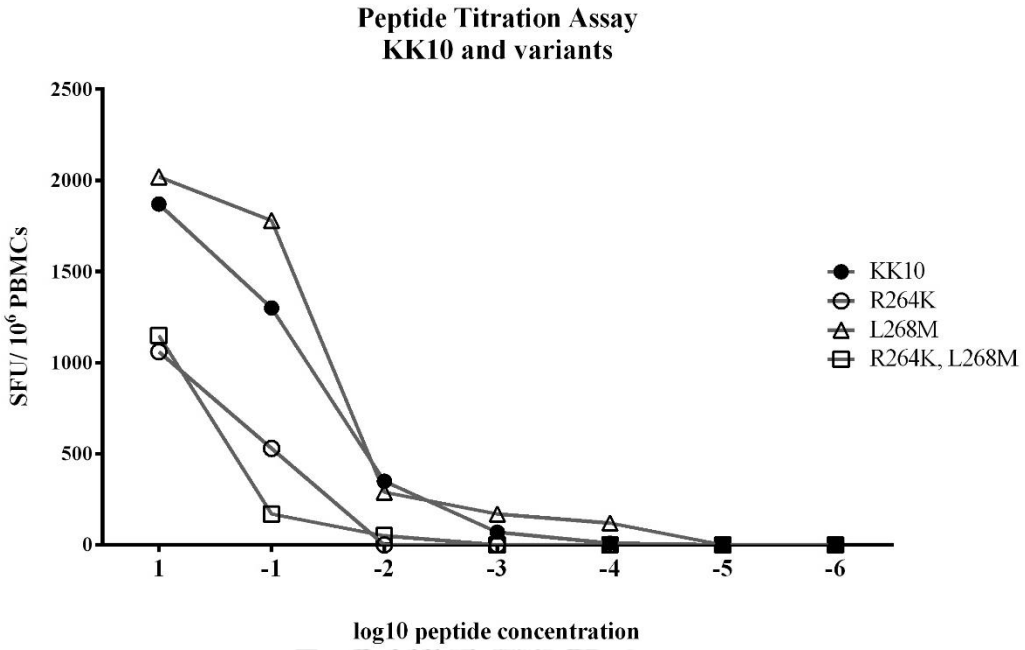


Figure 29: KK10 and variants (R264K, L268M and R264K, L268M) avidity in HN52 determined by peptide titration assay.

For HLA-B57 epitopes, HIV-infected volunteers who carry HLA-B57 allele (n=7) were determined response by HLA-B57/5801 restricted epitopes against Gag p24, Gag p17, RT and Nef in Table 5. ELISpot results revealed that PBMCs from HIV-infected volunteers were much higher recognized Gag p24 epitopes (9/11 epitopes) and RT (1/2 epitopes) than Nef (1/3 epitopes). Unfortunately, there were no response against Gag p17 epitopes.

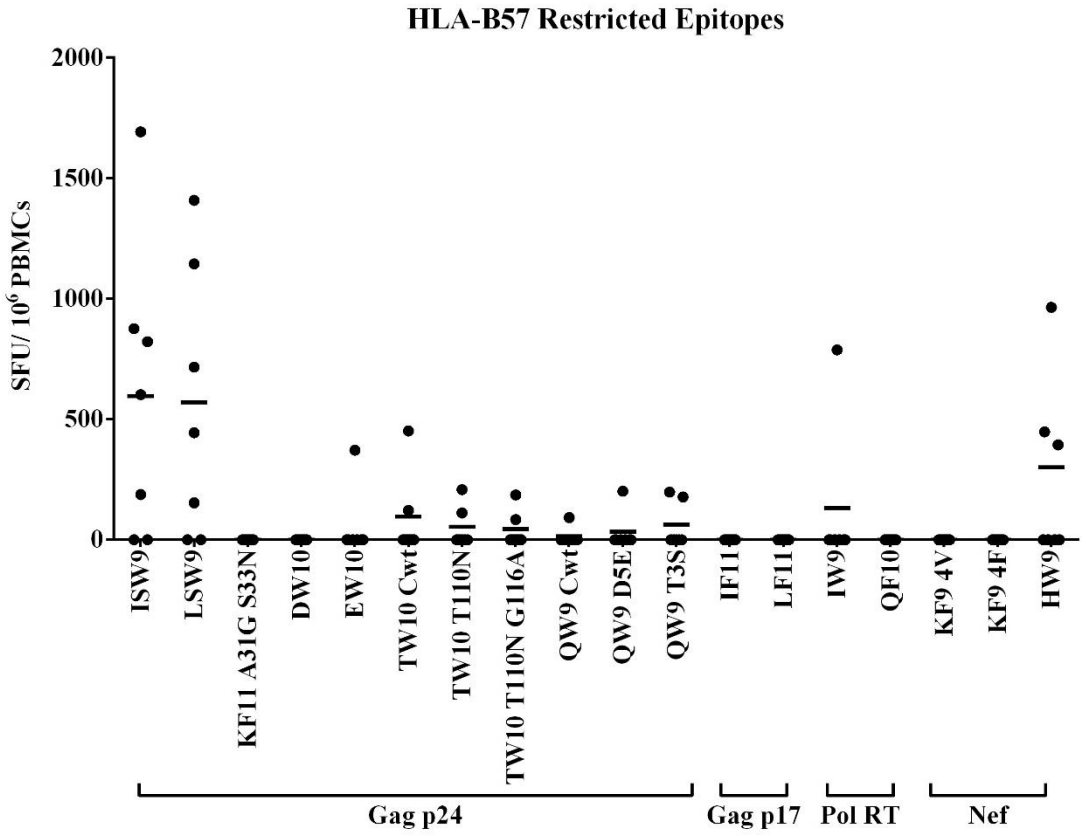


Figure 30: HLA-B57 restricted epitopes (n=18) response in HLA-B57^{+} patients (n=9) by IFNγ ELISpot assay.

For HLA-B58 epitopes, HIV-infected volunteers who positive for HLA-B5801 (n=6) were determined response by HLA-B57/5801 restricted epitopes against Gag p24 and Nef as shown in Table 5. ELISpot results showed that PBMCs from HIV-infected volunteers could recognize both Gag p24 and Nef epitopes as presented in

Figure ***31***.

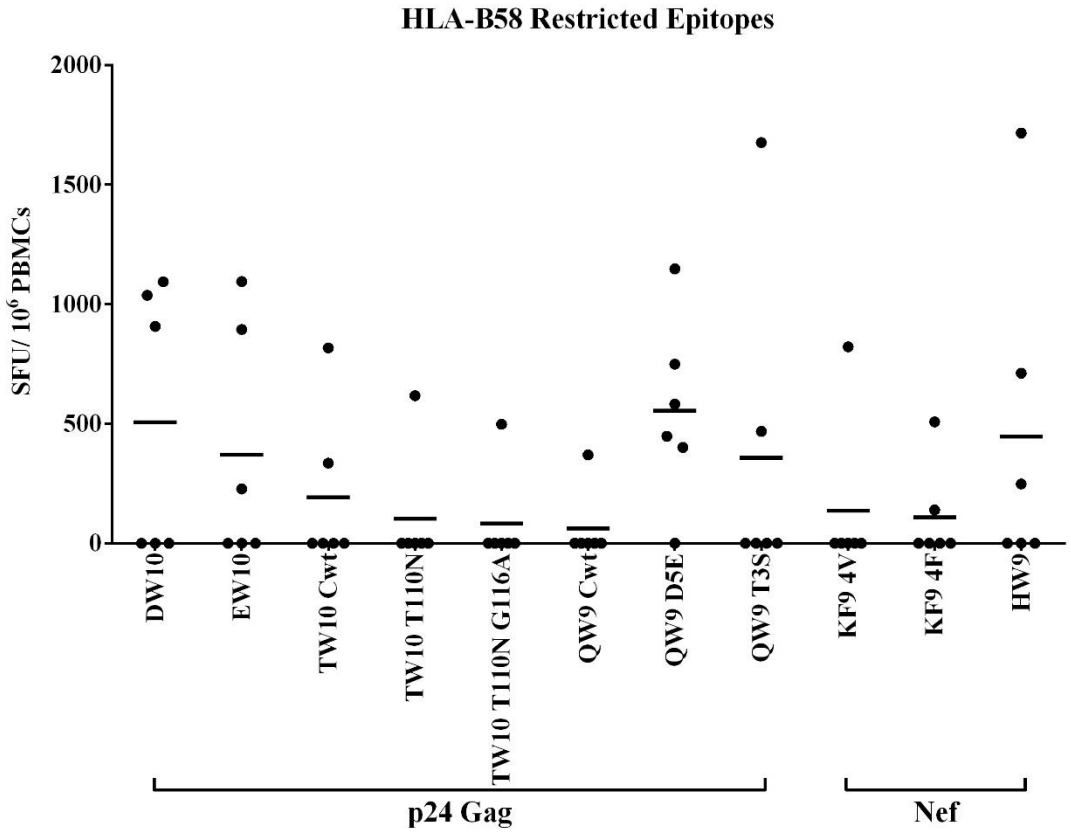


Figure 31: HLA-B5801 restricted epitopes (n=11) response in HLA-B5801^{+} patients (n=9) by IFNγ ELISpot assay.

IFNγ-secreting cell response: HLA-A11 restricted epitopes

According to HLA allele frequency data obtained from stem cell donors at Thai Red Cross (154), HLA-A11 has reported as the most prevalent allele among Thai population. In our study, HLA-A11 has also shown the highest allele (29.2%) similar

to the previous report. Moreover, the response of HLA-A11 restricted epitope named AK11 (ACQGVGGPSHK) was associated with low plasma viral load in Thai HIV-infected patients (142).

To determine HLA-A11 restricted epitopes response, patients' PBMCs (n=29) were stimulated with the following epitopes as shown in Table 8. The response was observed in all HLA-A11 epitopes with different degree of breadth and magnitude. The highest breadth was shown in Nef-GK10 epitope (n=21). Nef-GK10 was also presented the highest magnitude of response which was significantly different from Nef-QK10 (p=0.001), Pol-QIYQ (p=0.0113) and Pol-QIIE (p=0.016). Magnitude of Gag p24-AK11 epitope, which has previous report on viral control association, was greater than Nef-QK10 significantly (p=0.0379). (Figure 32)

Table 8: List of HLA-A11 restricted epitopes

Protein	**Abbreviation**	**Sequences**
Gag p24	AK11	ACQGVGGPSHK
Nef	GK10	GAFDLSFFLK
Nef	QK10	QVPLRPMTYK
Pol	AK9	AIFQSSMTK
Pol	QIYQ	QIYQEPFKNLK
Pol	QIIE	QIIEQLIKK
Pol	QIYA	QIYAGIKVK

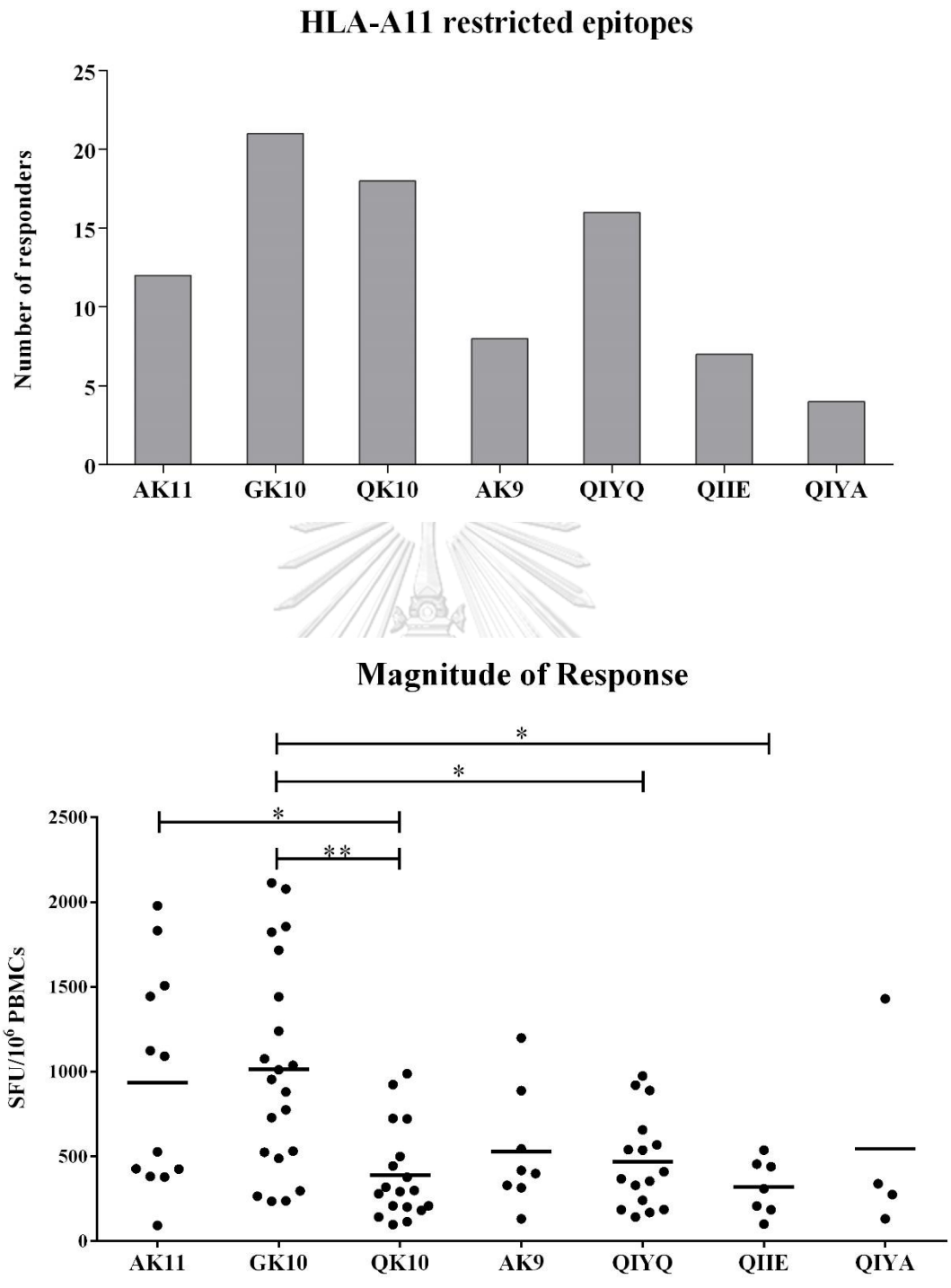


Figure 32: HLA-A11^{+} volunteers (n=29) response against HLA-A11 restricted epitopes by IFNγ ELISpot assay. (* $p<0.05$, ** $p=0.001$)

Table 9: Summary of ELISpot results

HLA allele	Protein	Epitope	Number of responders	Number of non-responders
HLA-A11 (n=29)	Gag p24	AK11 (ACQGVGGPSHK)	12	17
	Nef	GK10 (GAFDLSFFLK)	21	8
	Nef	QK10 (QVPLRPMTYK)	18	11
	Pol	AK9 (AIFQSSMTK)	8	21
	Pol	QIYQ (QIYQEPFKNLK)	16	13
	Pol	QIIE (QIIEQLIKK)	7	22
	Pol	QIYA (QIYAGIKVK)	4	25
HLA-B27 (n=9)	Gag p24	KK10 (KRWIILGLNK)	6	3
	Gag p24	KK10 R264K (KKWIILGLNK)	2	7
	Gag p24	KK10 L268M (KRWIIMGLNK)	3	6
	Gag p24	KK10 R264K L268M (KKWIIMGLNK)	4	5
HLA-B57 (n=7)	Gag p24	LW9 (LSPRTLNAW)	6	1
	Gag p24	KF11 (KAFSPEVIPMF)	7	0
	Gag p24	QW9 (QATQEVKNW)	4	3
	Gag p24	TW10 (TSTLQEQIGW)	4	3
HLA-B58 (n=6)	Gag p24	LW9 (LSPRTLNAW)	2	4
	Gag p24	KF11 (KAFSPEVIPMF)	4	2
	Gag p24	QW9 (QATQEVKNW)	5	1
	Gag p24	TW10 (TSTLQEQIGW)	4	2

As shown in Table 9, a summary of ELISpot results was compared between number of responders and non-responders which determined by IFNγ ELISpot assay. In fact, if patients present the same HLA allele (HLA-matched), they may respond to the same particular HLA-restricted epitope. Nevertheless, our findings have shown that not all of patients could recognize epitopes even if they carry the same HLA allele. For example, HLA-A11 AK11-restricted epitope response, there were 29 volunteers who positive for HLA-A11 but only 12 out of 29 volunteers could respond to this epitope. The similar patterns were observed in most of HLA-restricted epitopes that we tested as shown in Table 9. Viral sequencing was further investigated to identify mechanisms of non-responders.

CTL target characterization by HIV Sequencing

To characterize CTL targets, viral RNA was extracted from EDTA-plasma of HIV infected volunteers (n=114 EDTA-plasma samples). Regarding to patients were longitudinal follow up every 3-6 months, each patients may presented more than one viral sequence. HIV RNA was amplified by nested reverse transcription PCR and sequenced for Gag p24 region. Sequencing data were translated to amino acid by ExPaSy and compared sequence similarity by Clustal Omega.

The consensus Gag p24 from 114 plasma samples, 231 amino acids length, was shown in a following figure.

Gag p24 consensus

PIVQNAQGQMVHQPLSPRTLNAWVKVVEEKGFNPEVIPMFSALSEGATPQDLNMMLNIVGGHQ
AAMQMLKETINEEAAEWDRVHPVHAGPIPPGQMREPRGSDIAGTTSTLQEQIGWMTNNPPIPV
GDIYKRWIILGLNKIVRMYSPVSILDIRQGPKEPFRDYVDRFYKTLRAEQATQEVKNWMTETL
LVQNANPDCKSILKALGTGATLEEMMTACQGVGGPSHKARVLAEAMSQ

Figure 33: Gag p24 consensus in this study.

The amino acid of Gag p24 of our study and CRF01_AE strain CM240 from Thailand (155) GenBank U54771 were compared. Similarity was 99.134% (229 amino acids out of 231 amino acids). Mutations were presented in two positions; A11V and P77A.

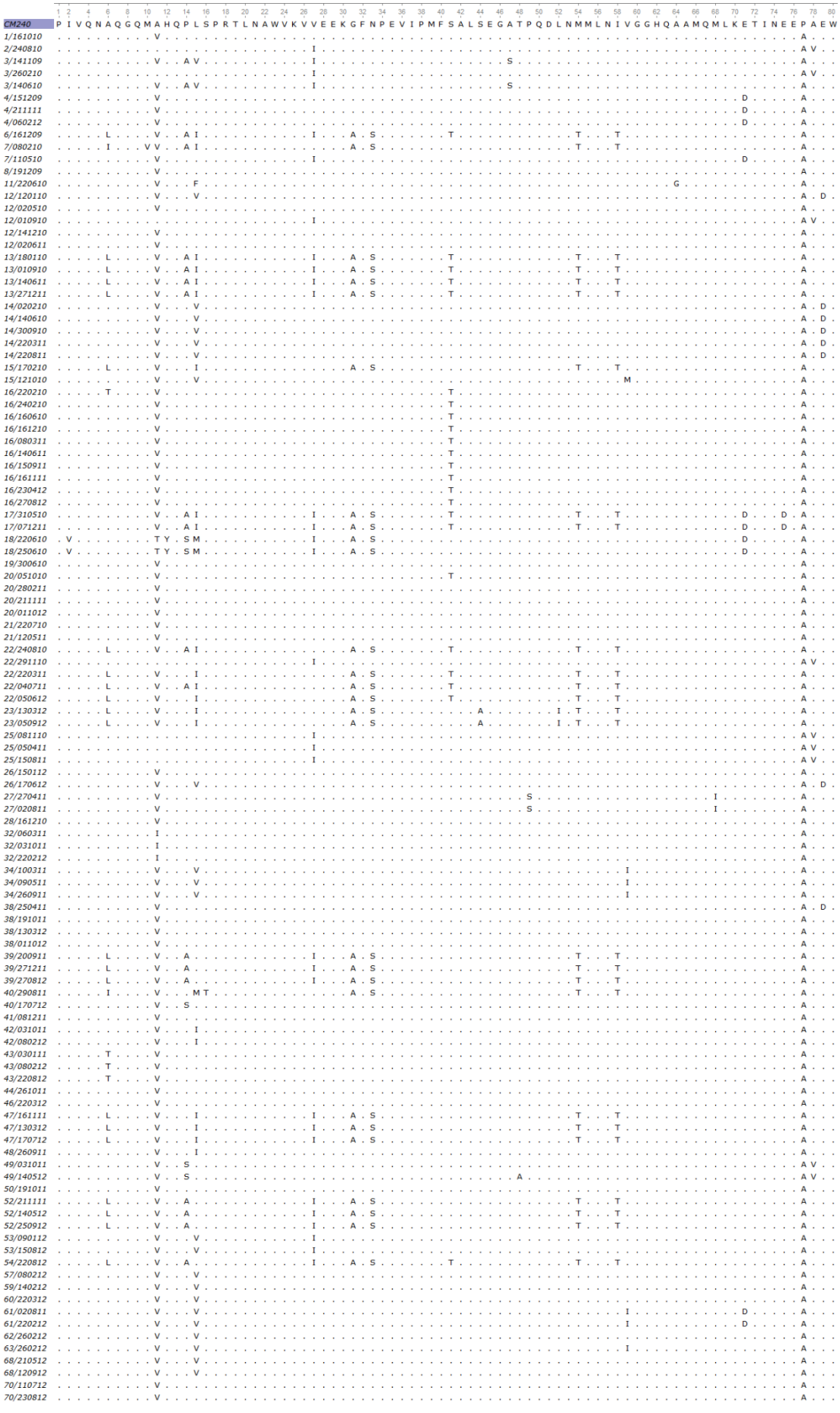

```
HIV-CM240-(U54771)      PIVQNAQGQMAHQPLSPRTLNAWVKVVEEKGFNPEVIPMFSALSEGATPQDLNMMLNIVG    60
Gag-p24-consensus       PIVQNAQGQMVHQPLSPRTLNAWVKVVEEKGFNPEVIPMFSALSEGATPQDLNMMLNIVG    60
                        **********.**************************************************

HIV-CM240-(U54771)      GHQAAMQMLKETINEEPAEWDRVHPVHAGPIPPGQMREPRGSDIAGTTSTLQEQIGWMTN    120
Gag-p24-consensus       GHQAAMQMLKETINEEAAEWDRVHPVHAGPIPPGQMREPRGSDIAGTTSTLQEQIGWMTN    120
                        *************** ****************************************

HIV-CM240-(U54771)      NPPIPVGDIYKRWIILGLNKIVRMYSPVSILDIRQGPKEPFRDYVDRFYKTLRAEQATQE    180
Gag-p24-consensus       NPPIPVGDIYKRWIILGLNKIVRMYSPVSILDIRQGPKEPFRDYVDRFYKTLRAEQATQE    180
                        ************************************************************

HIV-CM240-(U54771)      VKNWMTETLLVQNANPDCKSILKALGTGATLEEMMTACQGVGGPSHKARVL     231
Gag-p24-consensus       VKNWMTETLLVQNANPDCKSILKALGTGATLEEMMTACQGVGGPSHKARVL     231
                        ********************************************
```

We also compared whole Gag p24 (231 amino acids) in all 112 plasma samples as following alignment results in Patient code/Time point format. Sequences were varied in each volunteers or each time point which similarity percentage was 68.83.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1/161010 V A
2/240810 I A V
3/141109 V A V I S A
3/260210 I A V
3/140610 V A V I S A
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4/060212 V D A
6/161209 L V A I I A S T T T A
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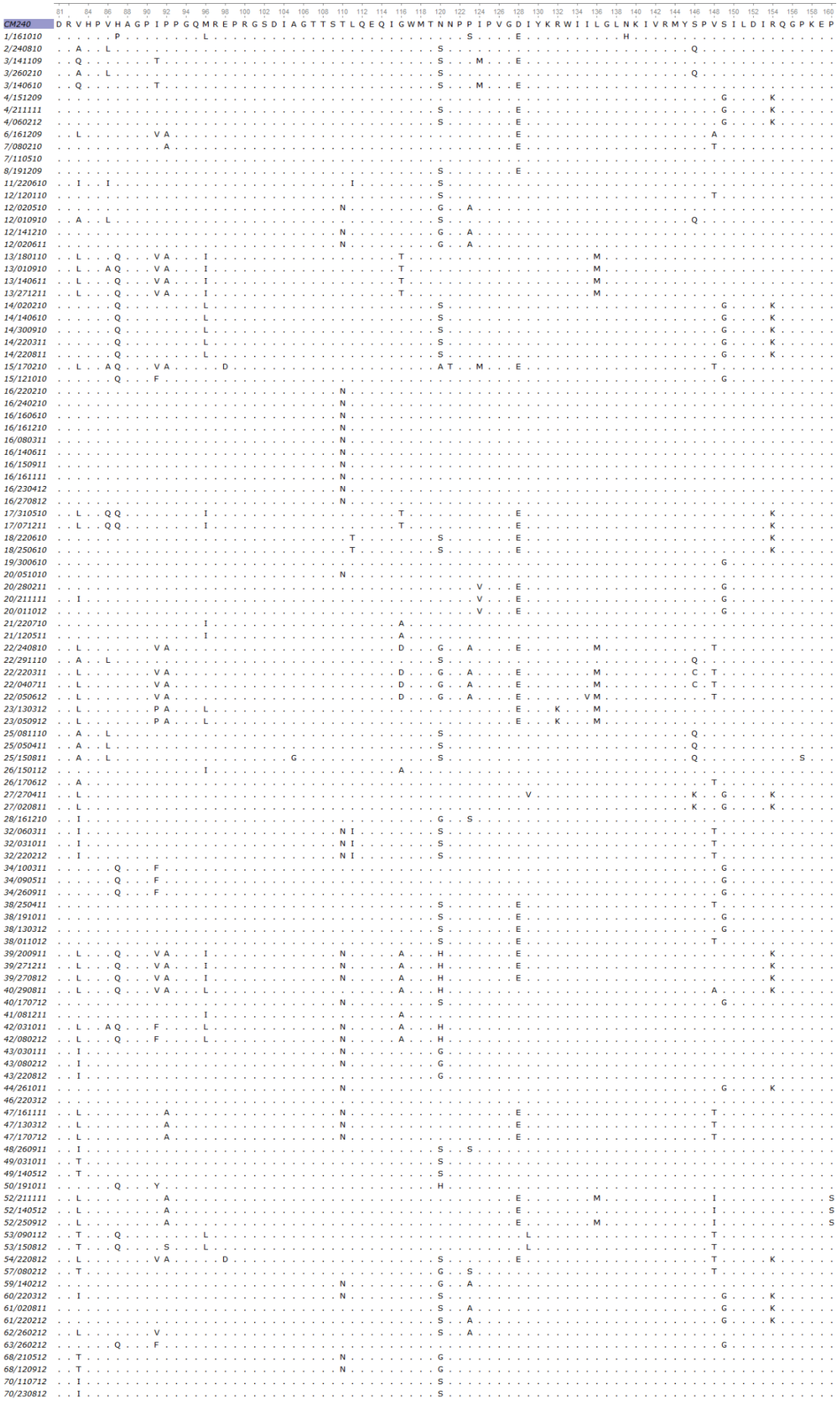
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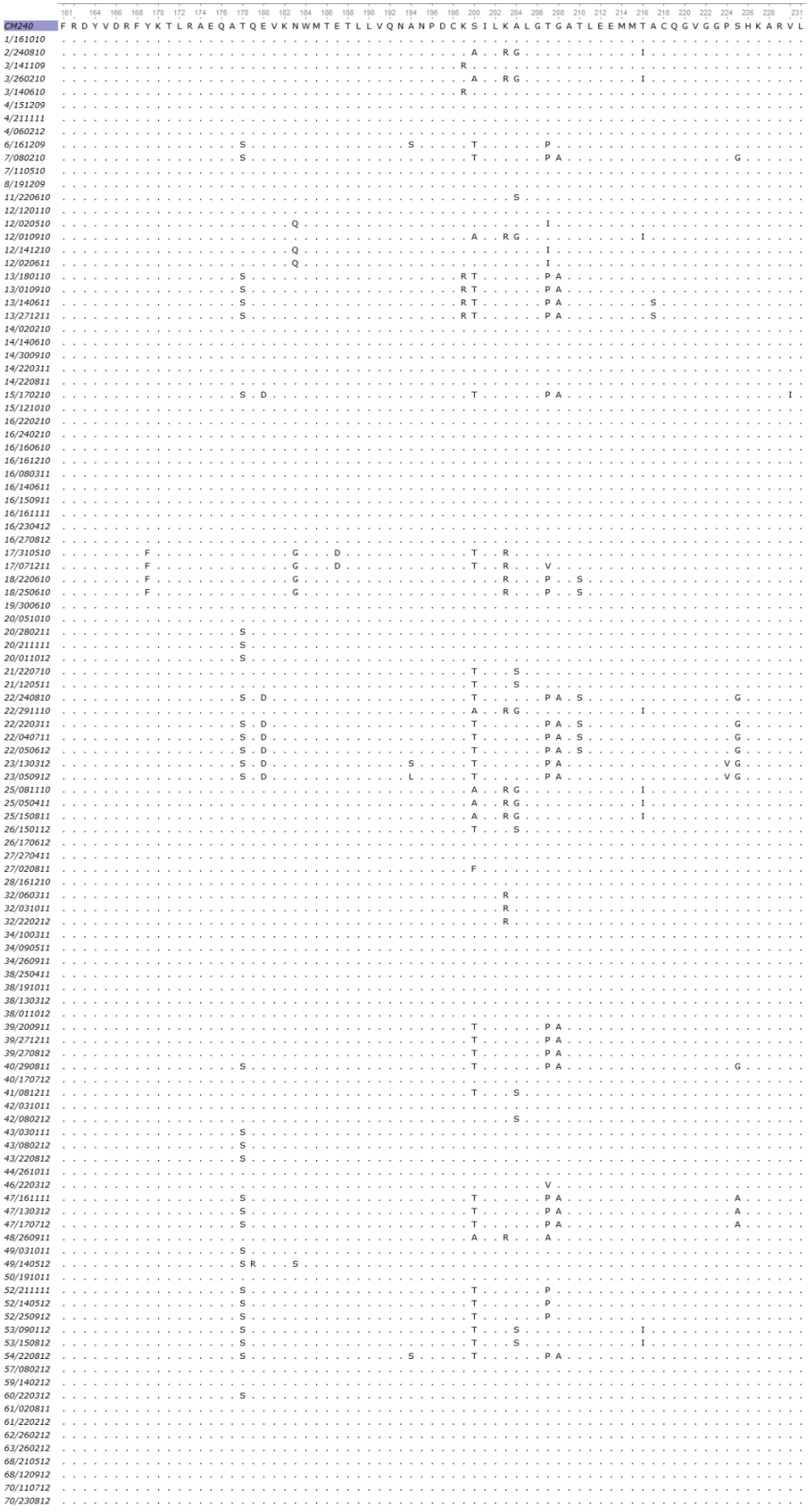
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3/260210
3/140610
4/151209
4/211111
4/060212
6/161209
7/080210
7/110510
8/191209
11/220610
12/120110
12/020510
12/010910
12/141210
12/020611
13/180110
13/010910
13/140611
13/271211
14/020210
14/140610
14/300910
14/220311
14/220811
15/170210
15/121010
16/220210
16/240210
16/160610
16/161210
16/080311
16/140611
16/150911
16/161111
16/230412
16/270812
17/310510
17/071211
18/220610
18/250610
19/300610
20/051010
20/280211
20/211111
20/011012
21/220710
21/120511
22/240810
22/291110
22/220311
22/040711
22/050612
23/130312
23/050912
25/081110
25/050411
25/150811
26/150112
26/170612
27/270411
27/020811
28/161210
32/060311
32/031011
32/220212
34/100311
34/090511
34/260911
38/250411
38/191011
38/130312
38/011012
39/200911
39/271211
39/270812
40/290811
40/170712
41/081211
42/031011
42/080212
43/030111
43/080212
43/220812
44/261011
46/220312
47/161111
47/130312
47/170712
48/260911
49/031011
49/140512
50/191011
52/211111
52/140512
52/250912
53/090112
53/150812
54/220812
57/080212
59/140212
60/220312
61/020811
61/220212
62/260212
63/260212
68/210512
68/120912
70/110712
70/230812

Specific HLA restricted CTL epitopes

Specific HLA class I molecule targets particularly protective alleles (HLA-B27, HLA-B57 and HLA-B58) and HLA-A11 in which the most prevalent in Thai population were included. Epitope sequences from IFNγ ELISpot-responders and IFNγ ELISpot-non-responders were compared within intraepitope and flanking regions at N-terminal (10 amino acids) and C-terminal (10 amino acids) (156).

a) HLA-B27-KK10 restricted epitope

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
KK10	N P P I P V G D I Y	K R W I I L G L N K	I V R M Y S P V S I
15/121010	. . . . .	. . . . .	. . . . . G .
20/051010	. . . . .	. . . . .	. . . . .
20/280211	. . . V . . . E . .	. . . . .	. . . . . G .
20/211111	. . . V . . . E . .	. . . . .	. . . . . G .
20/011012	. . . V . . . E . .	. . . . .	. . . . . G .
21/220710	. . . . .	. . . . .	. . . . .
21/120511	. . . . .	. . . . .	. . . . .
38/250411	. . . . . E . .	. . . . .	. . . . . T . .
38/191011	. . . . . E . .	. . . . .	. . . . . G .
38/130312	. . . . . E . .	. . . . .	. . . . . G .
52/211111	. . . . .	. . . . .	. . . . .
52/140512	. . . . . E . .	. . . . .	. . . . . I . .
52/250912	. . . . . E . .	. . . . . M	. . . . . I . .
54/220812	. . . . . E . .	. . . . .	. . . . . T . .
70/110712	. . . . .	. . . . .	. . . . .
70/230812	. . . . .	. . . . .	. . . . .
Non-responder			
1/161009	. . S . . . . E . .	. . . . . H .	. . . . .
2/240810	. . . . .	. . . . .	. . . . . Q . . . .
2/011210	. . . . .	. . . . .	. . . . . T . .
2/291111	. . . . .	. . . . .	. . . . . T . .
15/170210	T . . M . . . E . .	. . . . .	. . . . . T . .
38/011012	. . . . . E . .	. . . . .	. . . . . T . .
62/260212	. . A	. . . . .	. . . . .
62/091012	. . A	. . . . .	. . . . .
Non-B27 Consensus	. . . . .	. . . . .	. . . . .

HLA-B27-KK10 restricted epitope (Continued)

KRWIILGLNK, so called KK10, is restricted by HLA-B27. At amino acid level, KK10 sequences derived from plasma were analyzed and compared using Unipro UGENE (138). As shown in Figure 34, percentage of amino acid sequence identity among IFNγ ELISpot-responders and IFNγ ELISpot-non-responders was compared.

% conserved = [No. of conserved sequence(s) / No. of total sequences] × 100

Either intraepitope region [94% (16 out of 17) vs 87.5% (7 out of 8), respectively] or flanking regions [35.3% (6 out of 17) vs 0%, respectively] were not different by Mann-Whitney test (p=0.6667).

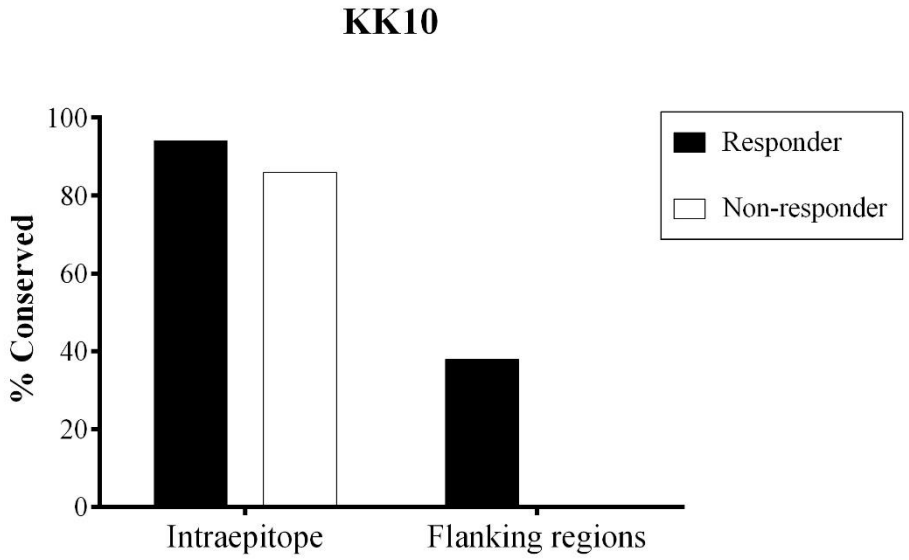


Figure 34: Percent amino acid residues conserved of HLA-B27 restricted KK10 epitope compared between IFNγ ELISpot-responders (n=16, black) and IFNγ ELISpot-non-responders (n=8, white)

b) HLA-B57-restricted epitopes

There are four well-characterized epitopes within p24 Gag which restricted by HLA-B57. LSPRTLNAW (LW9, Gag 147 to 155), KGFNPEVIPMF (KF11, Gag 162 to 172), TSTLQEQIGW (TW10, Gag 240 to 249) and QATQEVKNW (QW9, Gag 308 to 316) have been shown that the responses were correlated to HIV control (157). According to the fact that LW9 has found in CRF_01AE instead of ISW9 (**I**SPRTLNAW), LW9 was used to stimulate PBMCs. Escape mutations are normally found in those 4 epitopes within first few years after infection. Amino acid residues were compared as followed.

a. HLA-B57-LW9 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
LW9	N A Q G Q M I H Q S	L S P R T L N A W	V K V V E E K G F S
22/240810	. L . . . . V . . A	I	. . . . . A . .
22/291110	. . . . . A . . P	. . . . .	. . . I . . . . . N
22/220311	. L . . . . V . . P	I	. . . . . A . .
22/040711	. L . . . . V . . A	I	. . . . . A . .
22/090112	. L . . . . V . . P	I	. . . . . A . .
22/050612	. L . . . . V . . P	I	. . . . . A . .
39/200911	. L . . . . V . . A	. . . . .	. . . I . . . A . .
39/271211	. L . . . . V . . A	. . . . .	. . . I . . . A . .
39/270812	. L . . . . V . . A	. . . . .	. . . I . . . A . .
68/210512	. . . . . V . . P	V	. . . . . N
68/120912	. . . . . V . . P	V	. . . . . N
Non-responder			
12/120110	. . . . . V . . P	V	. . . . . N
12/010910	. . . . . A . . P	. . . . .	. . . I . . . . . N
12/141210	. . . . . V . . P	. . . . .	. . . . . N
12/020511	. . . . . V . . P	. . . . .	. . . . . N
Non-B57 Consensus	. . . . . V . . P	. . . . .	. . . . . N

b. HLA-B57-KF11 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
KF11	L N A W V K V V E E	K G F N P E V I P M F	S A L S E G A V P Q
12/020511	. . . . .	. . . . .	. . . . . T . .
39/271211	. . . . . I . .	. A . S	. . . . . T . .
39/270812	. . . . . I . .	. A . S	. . . . . T . .
68/210512	. . . . .	. . . . .	. . . . . T . .
Non-responder			
12/141210	. . . . .	. . . . .	. . . . . T . .
12/120110	. . . . .	. . . . .	. . . . . T . .
12/010910	. . . . . I . .	. . . . .	. . . . . T . .
22/240810	. . . . .	. A . S	T . . . . . T . .
22/291110	. . . . . I . .	. . . . .	. . . . . T . .
22/220311	. . . . .	. A . S	T . . . . . T . .
22/040711	. . . . .	. A . S	T . . . . . T . .
22/090112	. . . . .	. A . S	T . . . . . T . .
22/050612	. . . . .	. A . S	T . . . . . T . .
39/200911	. . . . . I . .	. A . S	. . . . . T . .
68/120912	. . . . .	. . . . .	. . . . . T . .
Non-B57 Consensus	. . . . .	. . . . .	. . . . . T . .

c. HLA-B57-TW10 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
TW10	E P R G S D I A G T	T S T L Q E Q I G W	M T S N P P I P V G
12/120110	. . . . .	. . . . .	. . . . .
12/010910	. . . . .	. . . . .	. . . . .
12/141210	. . . . .	. . N	. . G . . A . . . .
12/020511	. . . . .	. . N	. . G . . A . . . .
22/240810	. . . . .	. . . . . D .	. . G . . A . . . .
22/291110	. . . . .	. . . . .	. . . . .
22/220311	. . . . .	. . . . . D .	. . G . . A . . . .
22/040711	. . . . .	. . . . . D .	. . G . . A . . . .
22/090112	. . . . .	. . . . . D .	. . G . . A . . . .
22/050612	. . . . .	. . . . . D .	. . G . . A . . . .
Non-responder			
39/200911	. . . . .	. . N . . . . A .	. . H . . . . .
39/271211	. . . . .	. . N . . . . A .	. . H . . . . .
39/270812	. . . . .	. . N . . . . A .	. . H . . . . .
68/210512	. . . . .	. . N	. . G . . . . .
68/120912	. . . . .	. . N	. . G . . . . .
Non-B57 Consensus	. . . . .	. . . . .	. . N . . . . .

d. HLA-B57-QW9 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
QW9	D R F Y K T L R A E	Q A T Q E V K N W	M T E T L L I Q N A
12/120110	. . . . .	. . . . .	. . . . . V . . .
12/010910	. . . . .	. . . . .	. . . . . V . . .
12/141210	. . . . .	. . . . . Q .	. . . . . V . . .
12/020511	. . . . .	. . . . . Q .	. . . . . V . . .
68/120912	. . . . .	. . . . .	. . . . . V . . .
Non-responder			
22/240810	. . . . .	. . S . D	. . . . . V . . .
22/291110	. . . . .	. . . . .	. . . . . V . . .
22/220311	. . . . .	. . S . D	. . . . . V . . .
22/040711	. . . . .	. . S . D	. . . . . V . . .
22/090112	. . . . .	. . S . D	. . . . . V . . .
22/050612	. . . . .	. . S . D	. . . . . V . . .
39/200911	. . . . .	. . . . .	. . . . . V . . .
39/271211	. . . . .	. . . . .	. . . . . V . . .
39/270812	. . . . .	. . . . .	. . . . . V . . .
68/210512	. . . . .	. . . . .	. . . . . V . . .
Non-B57 Consensus	. . . . .	. . . . .	. . . . . V . . .

HLA-B57-restricted epitopes

Man-Whitney test was performed and revealed no difference as shown in Figure 35. Percentage of conserved amino acid in the intraepitope and flanking regions among responders and non-responders in which LW9, KF11, TW10 and QW9 in HLA-B57^{+} volunteers was comparable.

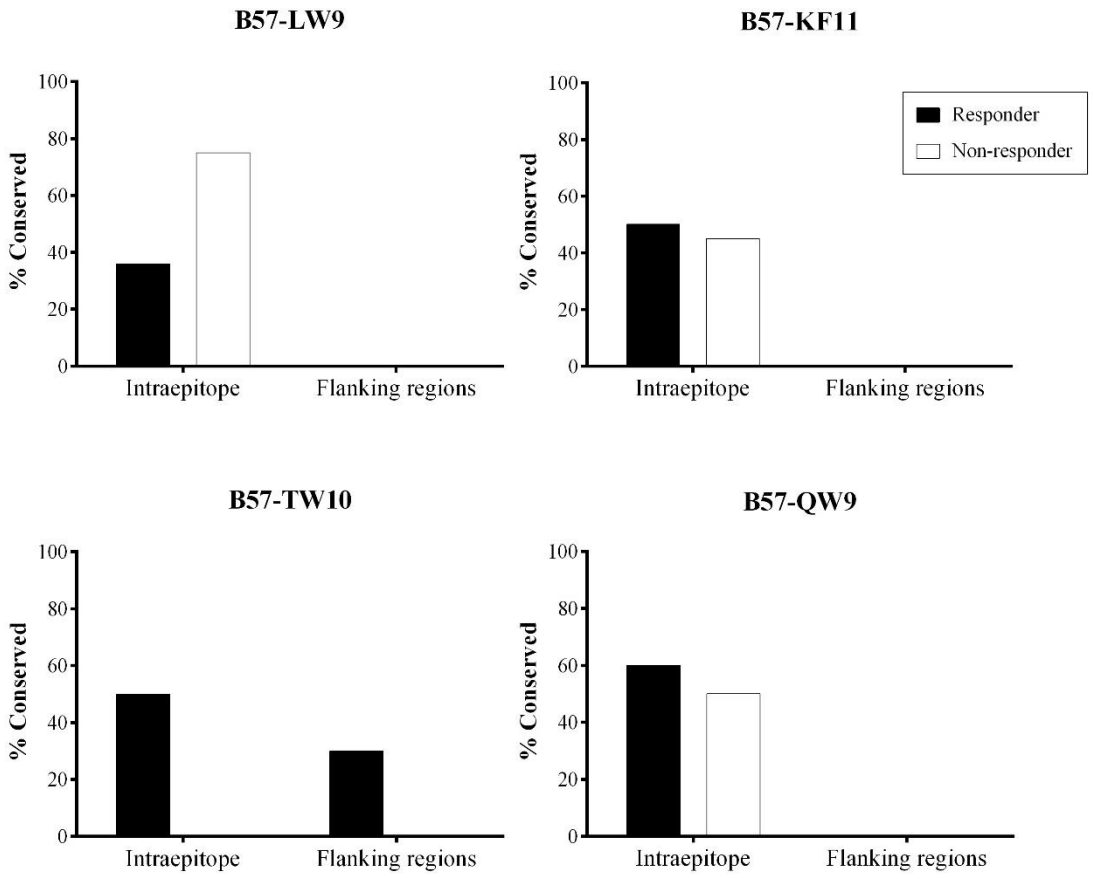


Figure 35: Percent amino acid residues conserved of HLA-B57 restricted epitopes compared between responders (black) and non-responders (white)

c) HLA-B58-restricted epitopes

HLA-B57 and HLA-58 have similar peptide biding properties so called HLA B58 supertype (158). These four epitopes; LSPRTLNAW (LW9, Gag 147 to 155), KGFNPEVIPMF (KF11, Gag 162 to 172), TSTLQEQIGW (TW10, Gag 240 to 249) and QATQEVKNW (QW9, Gag 308 to 316) were also tested in HLA-B58 positive volunteers. Amino acid residues were compared as previously mentioned.

a. HLA-B58-LW9 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Non-responder			
LW9	N A Q G Q M I H Q S	L S P R T L N A W	V K V V E E K G F S
16/240210	. . . . . V . . P	. . . . .	. . . . . N
16/160610	. . . . . V . . P	. . . . .	. . . . . N
16/150910	. . . . . V . . P	. . . . .	. . . . . N
16/161210	. . . . . V . . P	. . . . .	. . . . . N
16/080311	. . . . . V . . P	. . . . .	. . . . . N
16/140611	. . . . . V . . P	. . . . .	. . . . . N
16/161111	. . . . . V . . P	. . . . .	. . . . . N
16/230412	. . . . . V . . P	. . . . .	. . . . . N
16/270812	. . . . . V . . P	. . . . .	. . . . . N
40/290811	. I . . . . V . . P	M T	. . . . . A . .
40/170712	. . . . . V . . .	. . . . .	. . . . . N
47/161111	. L . . . . V . . P	I	. . . I . . . A . .
47/130312	. L . . . . V . . P	I	. . . I . . . A . .
47/170712	. L . . . . V . . P	I	. . . I . . . A . .
59/140212	. . . . . V . . P	V	. . . . . N
60/220312	. . . . . V . . P	V	. . . . . N
Non-B58 Consensus	. . . . . V . . P	. . . . .	. . . . . N

b. HLA-B58-KF11 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
KF11	L N A W V K V V E E	K G F N P E V I P M F	S A L S E G A V P Q
16/161210	. . . . .	. . . . .	T . . . . . T . .
16/080311	. . . . .	. . . . .	T . . . . . T . .
16/140611	. . . . .	. . . . .	T . . . . . T . .
16/161111	. . . . .	. . . . .	T . . . . . T . .
16/230412	. . . . .	. . . . .	T . . . . . T . .
16/270812	. . . . .	. . . . .	T . . . . . T . .
40/170712	. . . . .	. . . . .	. . . . . T . .
59/140212	. . . . .	. . . . .	. . . . . T . .
Non-responder			
16/240210	. . . . .	. . . . .	T . . . . . T . .
16/160610	. . . . .	. . . . .	T . . . . . T . .
16/150910	. . . . .	. . . . .	T . . . . . T . .
40/290811	. . . . .	. A . S	. . . . . T . .
47/161111	. . . . . I . .	. A . S	. . . . . T . .
47/130312	. . . . . I . .	. A . S	. . . . . T . .
47/170712	. . . . . I . .	. A . S	. . . . . T . .
60/220312	. . . . .	. . . . .	. . . . . T . .
Non-B58 Consensus	. . . . .	. . . . .	. . . . . T . .

c. HLA-B58-TW10 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
TW10	E P R G S D I A G T	T S T L Q E Q I G W	M T S N P P I P V G
47/161111	. . . . .	. . N	. . N . . . . .
Non-responder			
16/240210	. . . . .	. . N	. . N . . . . .
16/160610	. . . . .	. . N	. . N . . . . .
16/150910	. . . . .	. . N	. . N . . . . .
16/161210	. . . . .	. . N	. . N . . . . .
16/080311	. . . . .	. . N	. . N . . . . .
16/140611	. . . . .	. . N	. . N . . . . .
16/161111	. . . . .	. . N	. . N . . . . .
16/230412	. . . . .	. . N	. . N . . . . .
16/270812	. . . . .	. . N	. . N . . . . .
40/290811	. . . . .	. . . . . A .	. . H . . . . .
40/170712	. . . . .	. . N	. . . . .
47/130312	. . . . .	. . N	. . N . . . . .
47/170712	. . . . .	. . N	. . N . . . . .
59/140212	. . . . .	. . N	. . G . . A . . .
60/220312	. . . . .	. . N	. . . . .
Non-B58 Consensus	. . . . .	. . . . .	. . N . . . . .

d. HLA-B58-QW9 epitope alignment

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
QW9	D R F Y K T L R A E	Q A T Q E V K N W	M T E T L L I Q N A
16/240210	. . . . .	. . . . .	. . . . . V . . .
16/160610	. . . . .	. . . . .	. . . . . V . . .
16/150910	. . . . .	. . . . .	. . . . . V . . .
16/161210	. . . . .	. . . . .	. . . . . V . . .
16/080311	. . . . .	. . . . .	. . . . . V . . .
16/140611	. . . . .	. . . . .	. . . . . V . . .
16/161111	. . . . .	. . . . .	. . . . . V . . .
16/230412	. . . . .	. . . . .	. . . . . V . . .
40/290811	. . . . .	. . S	. . . . . V . . .
40/170712	. . . . .	. . . . .	. . . . . V . . .
59/140212	. . . . .	. . . . .	. . . . . V . . .
60/220312	. . . . .	. . S	. . . . . V . . .
Non-responder			
16/270812	. . . . .	. . . . .	. . . . . V . . .
47/161111	. . . . .	. . S	. . . . . V . . .
47/130312	. . . . .	. . S	. . . . . V . . .
47/170712	. . . . .	. . S	. . . . . V . . .
Non-B58 Consensus	. . . . .	. . . . .	. . . . . V . . .

HLA-B58-restricted epitopes

There were no differences as shown in Figure 36. Percentage of conserved amino acid in the intraepitope and flanking regions of which KF11, TW10 and QW9 were similar among IFNγ ELISpot-responders and IFNγ ELISpot-non-responders. For LW9, we could not compare similarity due to the lack of IFNγ ELISpot-responders' sequences.

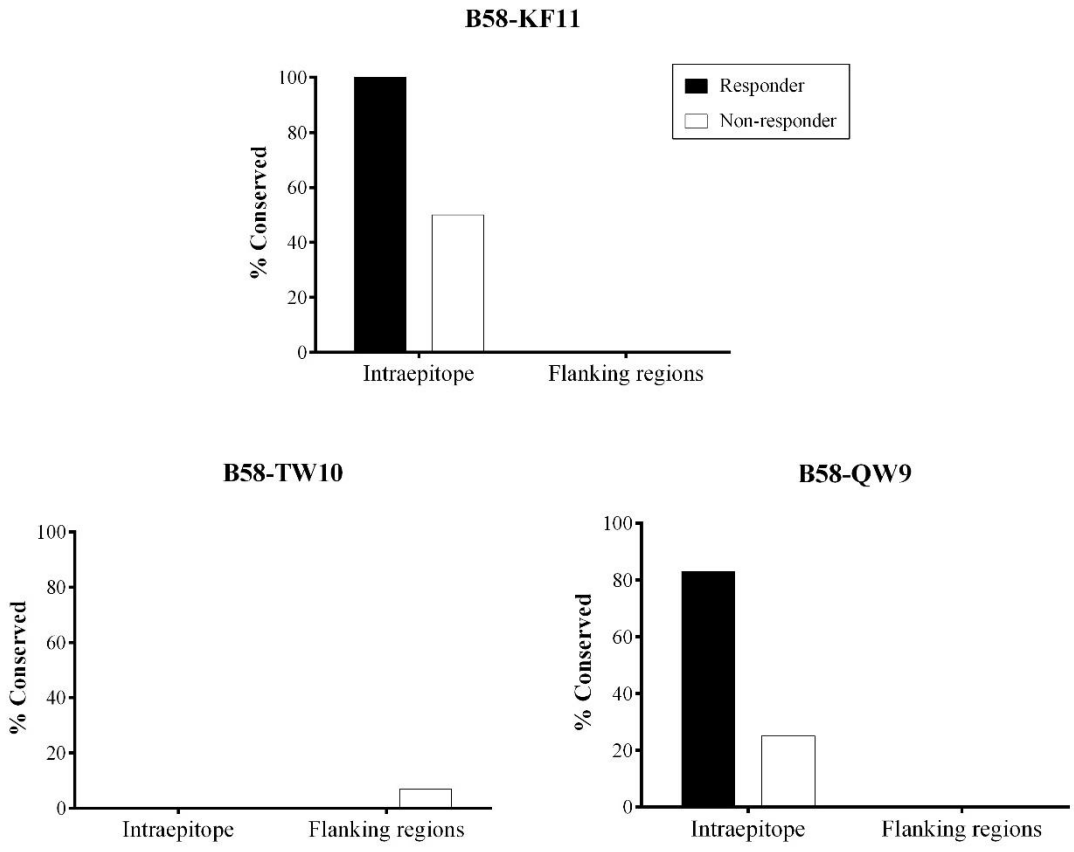


Figure 36: Percentage amino acid residues conserved of HLA-B5801 restricted epitopes compared between responders (black) and non-responders (white).

d) HLA-A11-AK11 epitope alignment

(IFNγ ELISpot-responders' sequences)

	N-terminal flanking	Intraepitope	C-terminal flanking
Responder			
AK11	T G A T L E E M M T	A C Q G V G G P S H K	A R V L A E A M S H
2/240810	 I	. . . . .	. . . . . Q
12/020510	I	. . . . .	. . . . . Q
12/141210	I	. . . . .	. . . . . Q
13/180110	P A	. . . . .	. . . . . Q
13/010910	P A	. . . . .	. . . . . Q
13/140611	P A	S	. . . . . Q
13/271211	P A	S	. . . . . Q
20/051010	. . . . .	. . . . .	. . . . . S
20/280211	. . . . .	. . . . .	. . . . . Q
20/211111	. . . . .	. . . . .	. . . . . Q
20/011012	. . . . .	. . . . .	. . . . . Q
25/081110	 I	. . . . .	. . . . . Q
25/050411	 I	. . . . .	. . . . . Q
25/150811	 I	. . . . .	. . . . . Q
28/161210	. . . . .	. . . . .	. . . . . Q
32/060311	. . . . .	. . . . .	. . . . . .
32/031011	. . . . .	. . . . .	. . . . . .
32/220212	. . . . .	. . . . .	. . . . . .
41/081211	. . . . .	. . . . .	. . . . . Q
59/140212	. . . . .	. . . . .	. . . . . Q
62/260212	. . . . .	. . . . .	. . . . . S
63/260212	. . . . .	. . . . .	. . . . . N

HLA-A11-AK11 epitope alignment

(IFNγ ELISpot-non-responders' sequences)

	N-terminal flanking	Intraepitope	C-terminal flanking
Non-responder			
AK11	T G A T L E E M M T	A C Q G V G G P S H K	A R V L A E A M S H
14/020210	. . . . .	. . . . .	. . . . .
14/140610	. . . . .	. . . . .	. . . . .
14/300910	. . . . .	. . . . .	. . . . .
14/220311	. . . . .	. . . . .	. . . . .
14/220811	. . . . .	. . . . .	. . . . .
16/220210	. . . . .	. . . . .	. . . . . S
16/240210	. . . . .	. . . . .	. . . . . S
16/160610	. . . . .	. . . . .	. . . . . S
16/161210	. . . . .	. . . . .	. . . . . S
16/080311	. . . . .	. . . . .	. . . . . S
16/140611	. . . . .	. . . . .	. . . . . S
16/150911	. . . . .	. . . . .	. . . . . S
16/161111	. . . . .	. . . . .	. . . . . S
16/230412	. . . . .	. . . . .	. . . . . I A A
16/270812	. . . . .	. . . . .	. . . . . N E Q
21/220710	. . . . .	. . . . .	. . . . . Q
21/120511	. . . . .	. . . . .	. . . . . Q
22/220311	P A . S	. . . . . G . .	. . . . . Q
22/040711	P A . S	. . . . . G . .	. . . . . Q
22/090112	P A . S	. . . . . G . .	. . . . . Q
22/050612	P A . S	. . . . . G . .	. . . . . Q
26/150112	. . . . .	. . . . .	. . . . . Q
26/170612	. . . . .	. . . . .	. . . . . Q
34/100311	. . . . .	. . . . .	. . . . . N
34/090511	. . . . .	. . . . .	. . . . . N
34/260911	. . . . .	. . . . .	. . . . . N
34/050612	. . . . .	. . . . .	. . . . . N
39/200911	P A	. . . . .	. . . . . N
39/271211	P A	. . . . .	. . . . . N
39/270812	P A	. . . . .	. . . . . N
49/031011	. . . . .	. . . . .	. . . . . Q
49/140512	. . . . .	. . . . .	. . . . . Q
54/220812	P A	. . . . .	. . . . . Q
61/220212	. . . . .	. . . . .	. . . . . N
70/110712	. . . . .	. . . . .	. . . . . Q
70/230812	. . . . .	. . . . .	. . . . . Q
Non-A11 Consensus	. . . . .	. . . . .	. . . . . Q

HLA-A11-AK11 restricted epitope (Continued)

ACQGVGGPSHK, called AK11, is restricted by HLA-A11. The response against AK11 was associated with low HIV plasma viral load (136). As shown in Figure 37, percentage conserved amino acid in both intraepitope and flanking regions among responders and non-responders (100% vs 91% and 9% vs 9%, respectively) were comparable. Statistics were performed by using Mann-Whitney test.

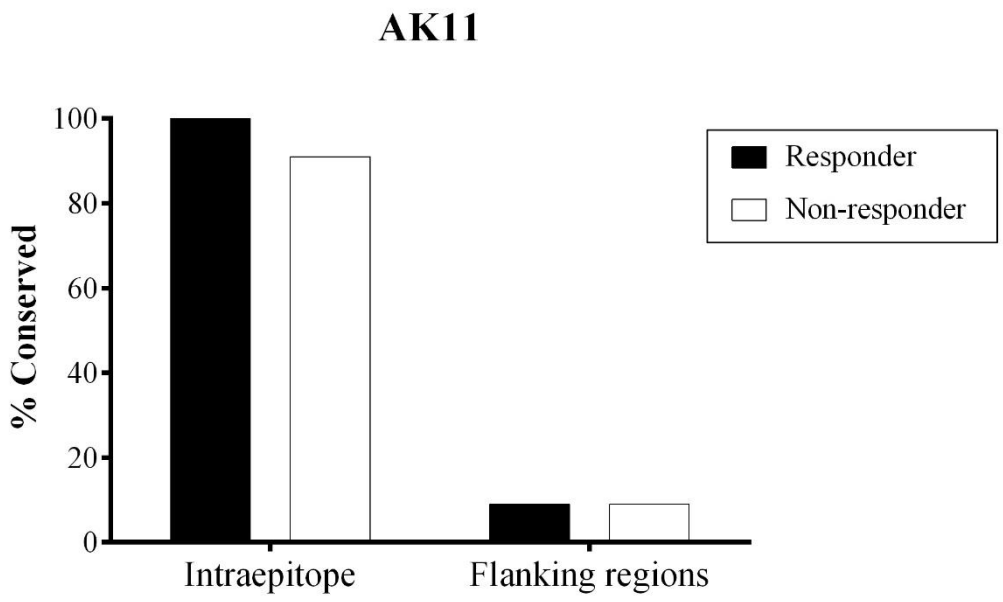


Figure 37: Percent amino acid residues conserved of AK11epitope compared between responders (black, n=12) and non-responders (white, n=17)

Mechanisms of escape in HIV-infected individuals

As the summary results shown in Table 9, not all volunteers were able to recognize stimulated peptides which determined by IFNγ ELISpot assay. There were some HIV-infected volunteers that could not respond to particular HLA class I restricted epitopes. To investigate mechanisms of escape, HIV consensus sequence of each volunteer were generated using Simple Consensus Maker (https://www.hiv.lanl-.gov) (159) and compared by Unipro UGENE.

1. **HLA allele polymorphism: HLA-B27 KK10**

In our study, the difference between KK10-responders and KK10-non-responders could not distinguish by mutations either in the intraepitope or flanking regions as shown in Figure 34. Naturally, KK10 has identified binding with HLA-B2705 (85). Even though HLA-B27 subtypes are classified into the same allelic family (First two digits), but not all allele numbers (Third and fourth digits) can bind to similar peptides (153). HLA-B2704 and HLA-B2705 are associated with ankylosing spondylitis (AS) while HLA-B2706 is not (160). We compared HLA-B27 subtypes among volunteers and found that KK10-non-responders (HN2 and HN62) carried HLA-B2706. In fact, HLA-B2706 cannot incorporate KK10 into its binding groove because of difference in F-pocket residue; aspartic acid (D) in HLA-B2705 and tyrosine (Y) in HLA-B2706.

Table 10: HLA-B27 subtypes in this study

B27 subtypes	**HIV-infected volunteers**
B2704	HN15, HN20, HN21, HN38, HN54, HN70
B2705	HN1, HN52
B2706	**HN2, HN62**

2. Mutational escape

a. Non-synonymous mutation: HLA-B57/58 TW10

Position		240 242 249
Ref. Seq.	CM240	T S T L Q E Q I G W
Responder	HN12	. . . . .
	HN22	. . . . . D .
Non-Responder	HN39	. . N . . . . . A .
	HN68	. . N . . . . .

Figure 38: TW10 epitope mutation analyzed by UGENE.

TW10 epitope in non-responders had a T242N mutation which this position functions as T cell receptor residue. Our findings were equivalent to previous reports. Changing from T to N affects binding to alpha helix of T cell receptor (161).

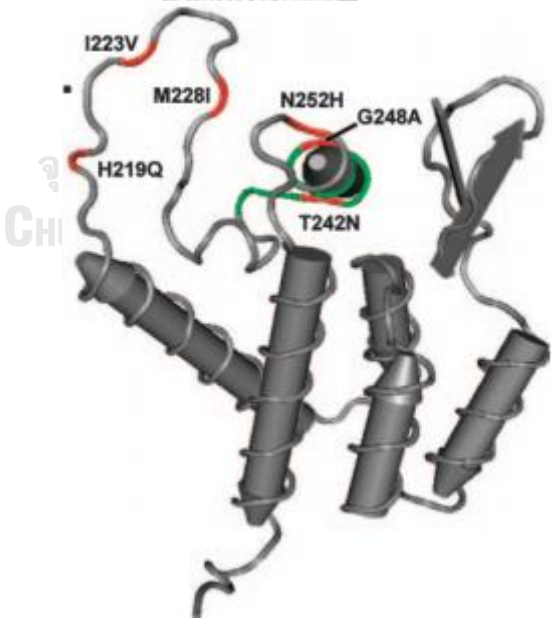


Figure 39: Structure diagram of TW10 contains T242N mutation (161).

b. Non-synonymous mutation: HLA-B57/58 QW9

Position	308 310 316
Ref. Seq.	CM240 Q A T Q E V K N W
Responder	HN16 . . . . HN40 . . . . HN59 . . . .
Non- Responder	HN47 . . S . . . . . .

Figure 40: QW9 epitope mutation analyzed by UGENE.

As shown in Figure *40*, there was a mutation presenting in the intraepitope of QW9-non-responder. Since threonine (T) changed to serine (S) at position 310 (T310S), peptide avidity was decreased when compared to QW9 wild type (Figure 41) (157).

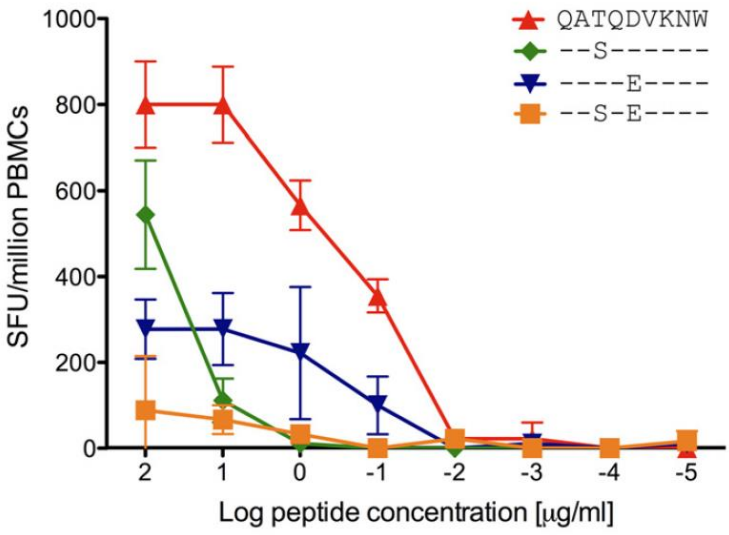


Figure 41: Peptide titration assay for QW9 wild type and variants (157).

c. Synonymous mutation: HLA-A11 AK11

Since there were not different in any amino acid residues among AK11-responders and AK11-non-responders as shown in Figure 37. Unlike HLA-B27, HLA-A11 Polymorphisms had less evidences on the difference in peptide accommodation. In fact, there are 61 triplet codons for 20 amino acids which means that some amino acid can be translated from more than one codons. Codon usage has been shown to affect translational efficiency even when translating into the same amino acid (30). To explore a mechanism beyond AK11- IFNγ ELISpot-non-responder, codon usages were compared in the intraepitope and flanking regions. Surprisingly, codon usages for lysine (K) at C-terminal position which function as anchor residue was statistically significant among AK11-responders and AK11-non-responders ($p<0.05$ by Fisher's exact test). From our results, AAG codon which is predominant in human was used to translate to lysine in AK11-responders while AK11-non-responders, AAA was more preferable.

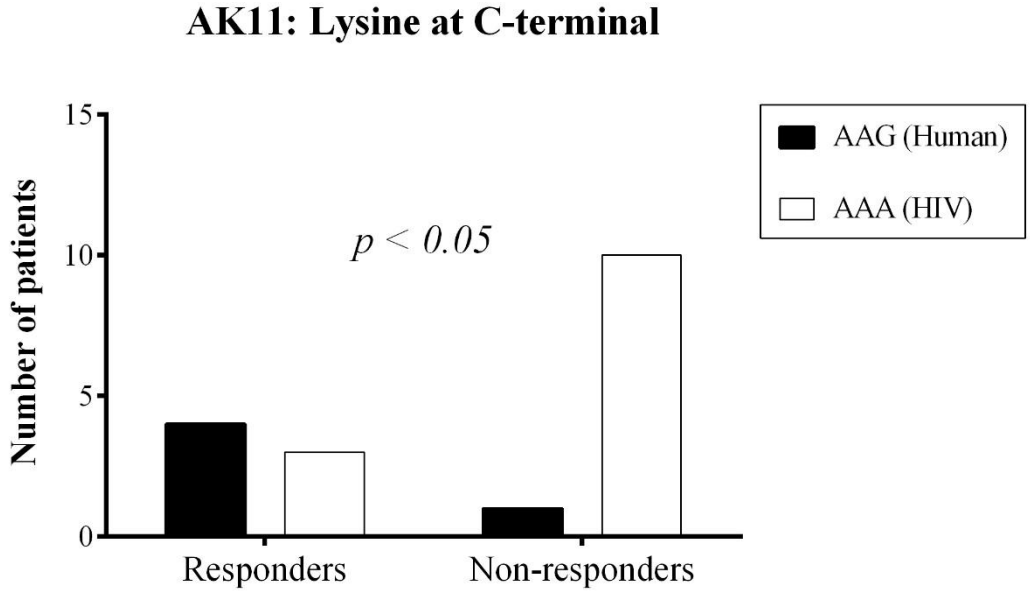


Figure 42: Codon usage analysis in AK11 epitope between AK11 responders (n=12) and non-responders (n=17).

Exploring 'AK11 non-responder' affecting factors

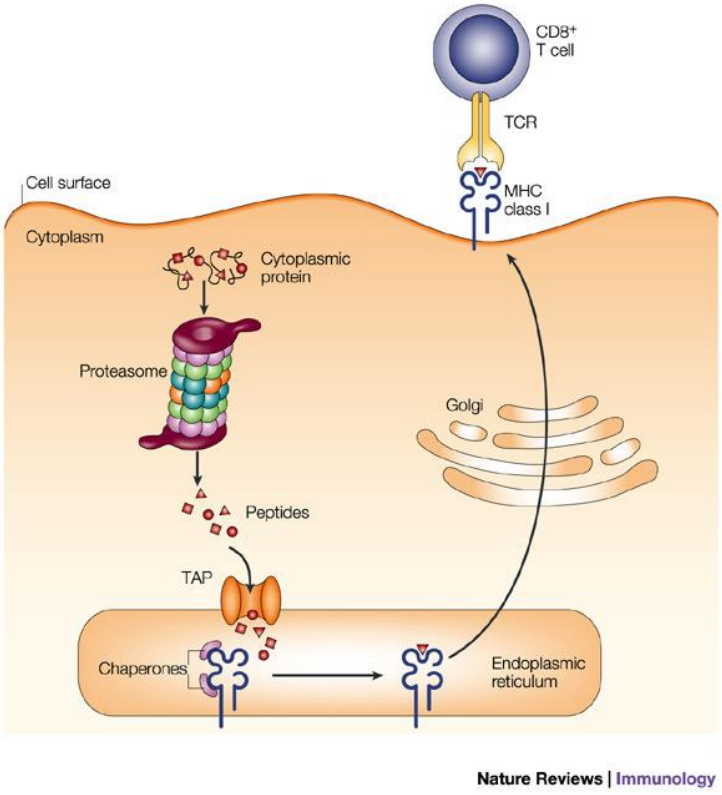


Figure 43: T cell recognition by Yewdell *et al*. (162)

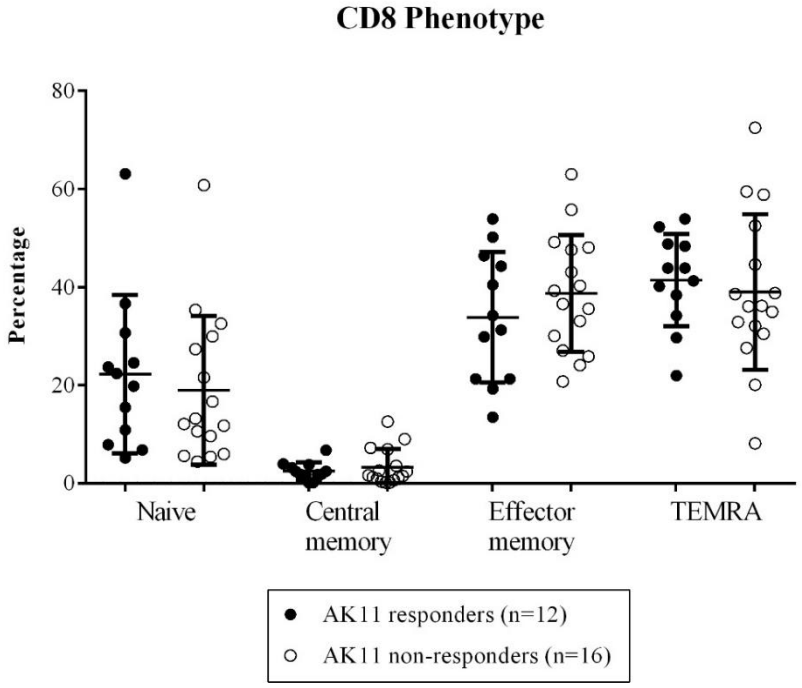
Affecting factors for CTL recognition as revealed in Figure 43 could be

a) Cytotoxic T lymphocytes
b) HLA expression
c) Antigen presentation

To investigate differences among AK11 responders and non-responders, those 3 possible issues were further investigated in HLA-A11 AK11 epitope.

a. Cytotoxic T lymphocytes

CTL memory phenotype and exhaustion markers were performed to compare between HLA-A11 positive volunteers. All CTL population naïve ($CD45RA^+$, $CCR7^+$), Tcm ($CD45RA^-$, $CCR7^+$), Tem ($CD45RA^-$, $CCR7^-$) and TEMRA ($CD45RA^+$, $CCR7^-$) were comparable between AK11 responders and non-responders as similar to exhaustion markers as shown in Figure 44.



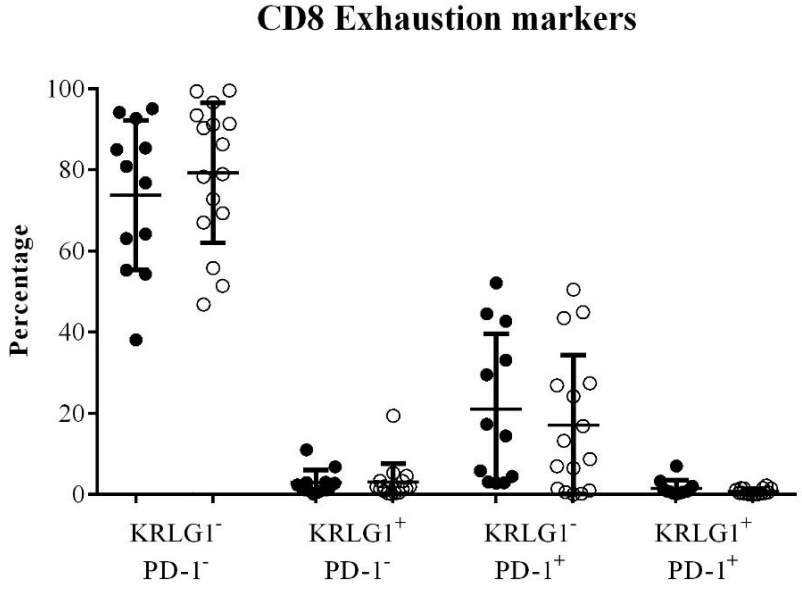


Figure 44: CTL memory phenotype and exhaustion markers among AK11 responders (n=12) and non-responders (n=16).

Moreover, to test the presence of AK11 specific TCR, PBMCs from HLA-A11 positive patients were stained with HLA-peptide tetrameric complex or tetramer for HLA-A11 AK11 conjugated with PE. Percentage of AK11 tetramer positive cells was gated on $CD3^+$ $CD8^+$ population. Patients were classified into responder and non-responder which previously determined by IFNγ ELISpot assay. AK11 responders showed significant greater AK11 tetramer positive cells than AK11 non-responders (1.51% vs 0.28%, respectively).

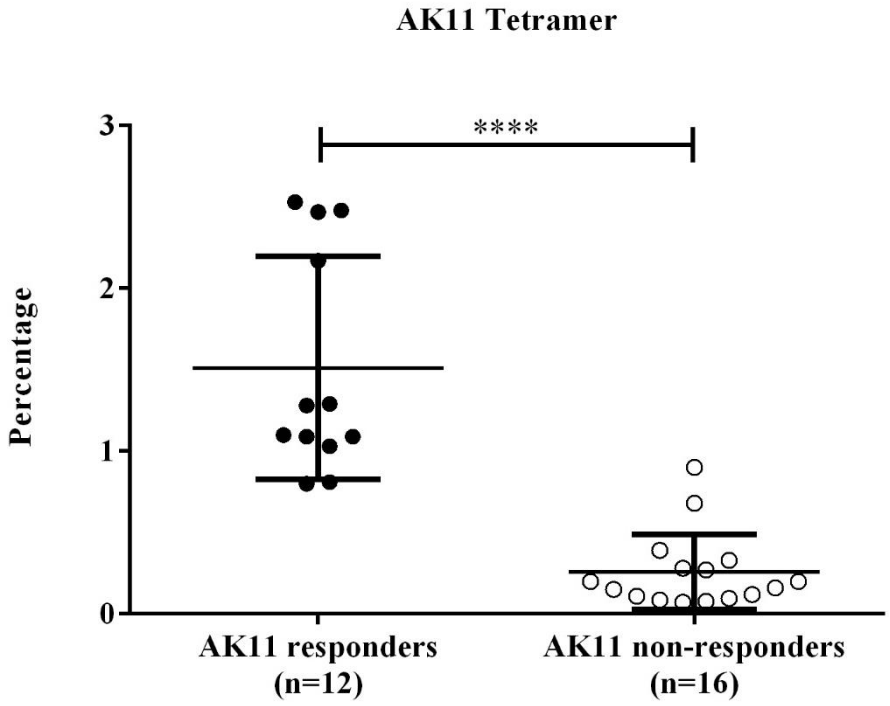


Figure 45: Percentage of AK11 $tetramer^+$ cells gated on $CD3^+CD8^+$ cells (**** $p < 0.0001$).

b. Level of HLA expression

HLA-A11 expression on various cells which are $CD3^+$ cell, $CD3^+$ $CD4^+$ cell and $CD19^+$ B cell were determined using specific antibody against this particular alleles. Mean fluorescence intensity (MFI) was analyzed on FlowJo® and compared among HLA-A11 positive volunteers. When compared HLA-A11 MFI of AK11 IFNγ ELISpot-responders with AK11 IFNγ ELISpot-non-responders, level of HLA-A11 expression was significant higher in AK11 IFNγ ELISpot-responders on $CD3^+$ cell and $CD19^+$ B cell. Even though $CD3^+$ $CD4^+$ cell showed no statistical difference, there was a higher trend in AK11 responders.

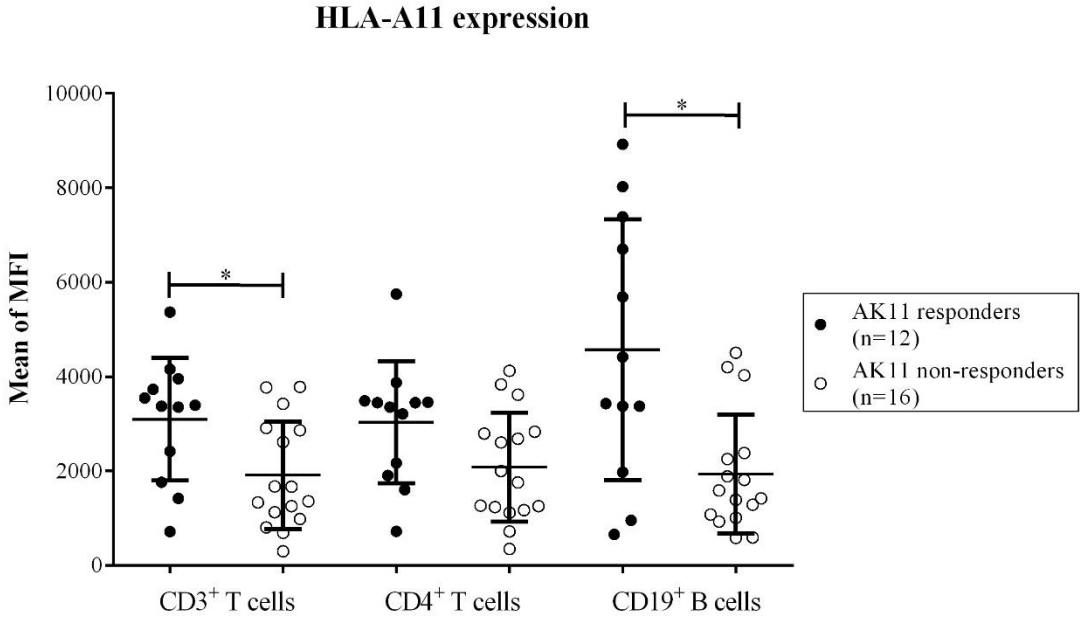


Figure 46: HLA-A11 expression on various cells (* $p < 0.05$).

c. Antigen presentation

The last possible factor that we hypothesized on being 'responder' may cause by level of antigen expression. To determine whether codon usage may have a potential role on translational efficiency in which representing antigen expression, 2nd generation lentiviral expression system was employed. Tandem HLA-A11 epitopes were cloned into lentiviral vector. Briefly, lentivirus transfer vector, pWPXLd, was transfected into HEK293T cells together with two lentiviral packaging plasmids; psPAX2 and pVSV-G to generate lentiviral particles for 52 hours as shown in

Figure ***47***.

Subsequently, lentiviral titer was determined by transducing virus into Nalm-6 cell for 72 hours. Percentage of EGFP positive cells was measured by flow cytometry. From Table 11, only dilutions 1:1250 which presenting 1-20% EGFP positive cells were further calculated for transduction units per milliliter (TU/mL) as below formula.

Formula for transduction unit calculation

$$\text{TU/mL} = \frac{\%\text{GFP}^{+}\text{cell}}{100} \times \frac{\text{Starting cell number}}{\text{Transduction volume (mL)}} \times \text{viral dilution}$$

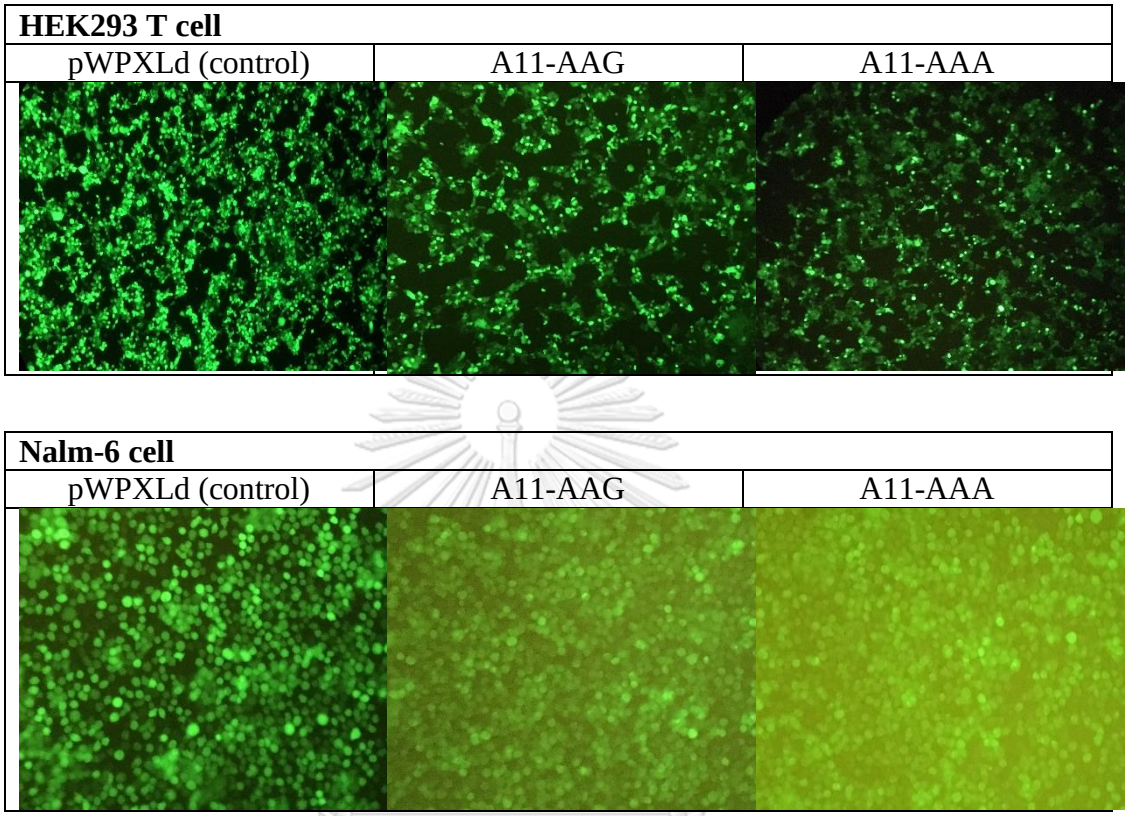
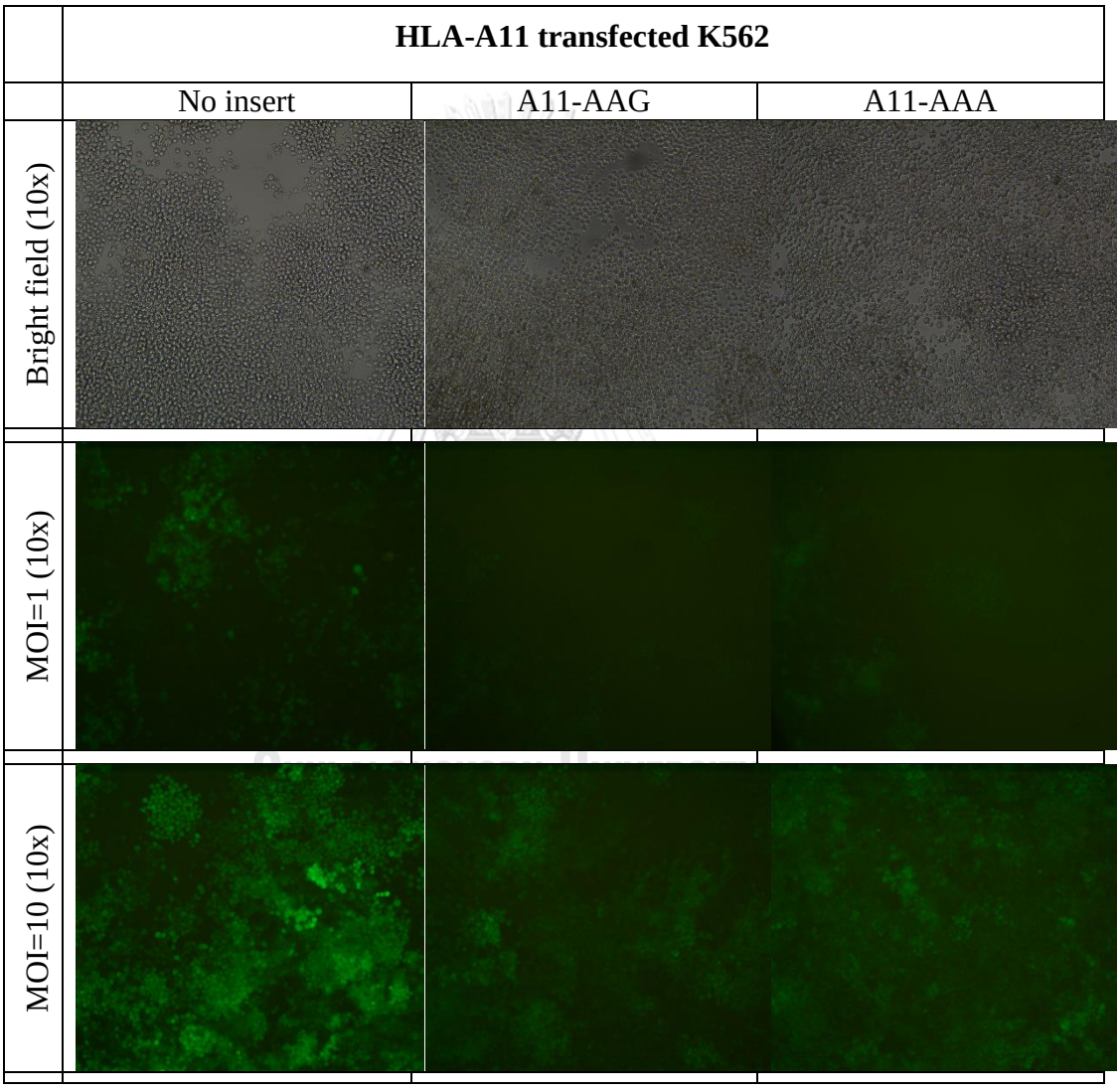


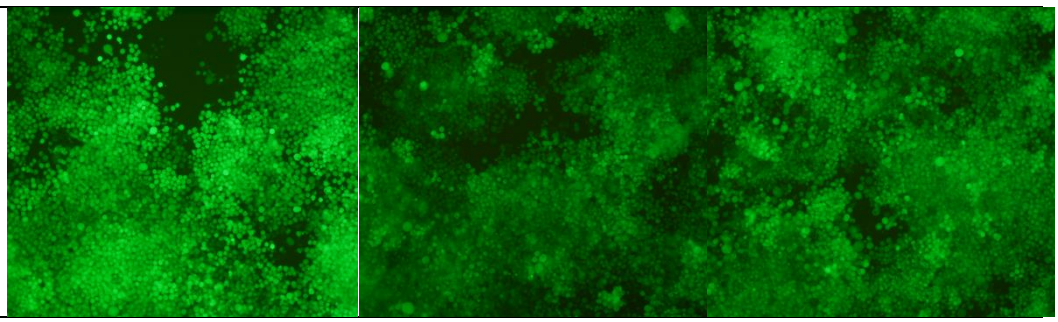
Figure 47: Lentiviral packaging in HEK293T cell (Upper panel) and lentiviral transduction in Nalm-6 cell (Lower panel).

Table 11: Lentivirus titration using GFP as a marker.

Dilution	**pWPXLd**	**A11-AAG**	**A11-AAA**
0	0.013	0.034	0.03
1:10	92.2	91.8	88.6
1:20	93.5	92.6	85.0
1:50	92.2	86.4	73.1
1:250	56.7	42.4	33.6
1:1250	**19.7**	**13.5**	**8.02**
TU/mL	2.71×10^7	1.86×10^7	1.10×10^7

Lentiviral particles were transduced into HLA-A11 expressing K562 cell at MOI 1, 10 and 100. Lentiviral infection in K562 cell line was successful and could be suggested that our lentiviral stock is effective for further experiments.



MOI=100 (10x)	

CHAPTER VI
DISCUSSION

HIV infection is one of the most global health concerns for decades. Although, combination antiretroviral therapy (HARRT) is highly successful to suppress viraemia, there are some drawbacks such as side effective and lifelong treatment. Phase III HIV vaccine trial, RV-144, set in Thailand has shown only 31.2% efficacy for protection (163). The main purpose of all researchers on HIV immunology is to explore which immune components play an important role in viral control.

The aim of this study is to determine the role of CTL on HIV control and identify mechanisms that HIV uses to escape from CTL response. Study subjects were 64 HIV-infected patients enrolled from the anonymous clinic, Thai Red Cross Society. Volunteers were classified into 2 groups based on their HIV viral load; VC, viraemic controller, (VL < 2,000 copies/mL) and NC, non-controller, (VL > 2,000 copies/mL). Percentage of VC in this study was 36 which slightly higher than other studies (164, 165) due to our inclusion criteria.

Complete blood count was performed to compare quantity of lymphocytes between HIV-infected patients and healthy controls. Total lymphocyte and $CD3^+$ lymphocyte were higher in HIV-infected group compared to healthy controls. CD4/CD8 ratio which is suggested to be a possible disease progression marker (166) has shown at normal ratio (1.036) in healthy donors in which consistent with previous report in Thai population on CD4/CD8 ratio 1.5 ± 0.6 (167) while VC and NC have an inverted ratio (<1). However, VC, NC and healthy controls had no difference in CD4 count which may not be consistent with previous reports (168). According to the inclusion criteria, only patients with CD4 counts greater than 450 cells/μL were intentionally enrolled into study to minimize confounding factors. Previously, patients were included regardless of their CD4 count and therefore it might not be able to clarify that the difference between VC and NC resulting from a variety of clinical stages in the first place. Demographic data including gender, age, sexual preference and time since seroconversion were compared among patients. None of these parameters was significantly different including the presence of HLA protective alleles. HLA-B27, -

B57 and -B58 have been suggested to correlate with good clinical outcome on HIV infection in Caucasians (12). As presented in our previous study (n=30) (11) frequency of these protective alleles was comparable between VC and NC. We also got similar results even when sample size was increased twice (n=64) in this study. However, the proportion of protective alleles is limited in Thai population when compared with Caucasians; HLA-B27 3.3% vs 4%, HLA-B57 1.3% vs 3.6% and HLA-B58 0.7% vs 8.5%, respectively (169, 170). Regarding to a previous HLA report from 16,807 healthy stem cell donors at Thai Red Cross (154), we also found that HLA-A11 is one of the most prevalent alleles in Thai population (27.7% in report vs 29.2% in this study). It could be implied that nearly one third of total Thai population possess this allele.

Rather than innate immunity and neutralizing antibodies, CTL has been largely proposed to be a possible mechanism on HIV control as shown in natural history (6) and *in vivo* evidences in humanized mice (171) and non-human primate model (9, 113, 172). However, precise mechanisms of CTL on viral control have not been clearly identified. Targeting to high structural or functional constraint region such as Gag p24 (173, 174) and cross-reactivity (175) are parts of possible hypotheses. Entire HIV proteins can be targeted by CTL but not all responses to each protein lead to viral control. Gag p24 responders presented lower HIV viral load than Gag p24 non-responders while Vif and glycoprotein41 responses had correlated with higher HIV viral load (119). Moreover, HIV Gag-specific CTL has a crucial impact on HIV control (118, 120). In order to compare CTL responses among HIV-infected volunteers, IFNγ-ELISpot assay against whole Gag p24 overlapping peptides (n=23) was performed. CTL responses could be detected throughout the whole Gag p24 protein. The most responsive peptide has found in OLP21 (ILKALGTGATLEEMMTACQG) by 35 out of 64 volunteers. The highest magnitude of response (829 SFU/10^6 PBMCs) has shown in OLP15 (IVRMYSPVSILDIRQGPKEP). Not only Gag 24 overlapping peptides but we subsequently performed IFNγ-ELISpot assay against well-characterized epitopes in Gag p24 and other proteins (Gag17, Pol, Nef and Rev) which are restricted by HLA protective alleles such as KK10 (KRWIILGLNK) and its variants (R264K, L268M and R264K L268M) for HLA-B27 positive patients (n=9), LSW9 (LSPRTLNAW), KF11 (KGFNPEVIPMF), TW10 (TSTLQEQIGW) and QW9 (QATQEVKNW) for HLA-B57/5801 positive patients (n=13). Those listed epitopes have shown positive

correlation with control (176). When CTL recognition to these particular epitopes has lost, plasma viral load suddenly increased afterwards (14).

CTL response against Gag p24 has presented more than other proteins in term of breadth and magnitude. KK10 (456 SFU/10^6 PBMCs) and LSW9 (531 SFU/10^6 PBMCs) have shown the highest magnitude of response among HLA-B27 and HLA-B57/5801, respectively. Interestingly, one of our volunteers called HN70, who carries HLA-B27, can respond to KK10 and its variants similarly when using peptide at concentration of 10 µg/mL for ELISpot stimulation. Peptide titration assay was employed to compare the avidity of peptides. KK10 and KK10 L268M show similar results while KK10 R264K and KK10 R264K L268M magnitude of response dramatically drop compared to wild type when peptide concentration is decreased. These findings are consistent with previous reports (173, 177). KK10 amino acid at position 264 (P2 of HLA molecule) is an anchor residue and it is important for binding to HLA-B27 when position 268 is a T cell receptor residue (178). Regarding to the fact that mutation in an anchor residue may abrogate peptide binding to HLA molecule, KK10 R264K shows much lower avidity when compared to wild type. Nevertheless, HLA-B57/5801 epitopes, LSW9 and KF11, used in this study are different from the Caucasians' sequences. In the CRF01_AE reference strain (CM240) which is predominant in the Thai population (155), K**G**F**N**PEVIPMF was identified rather than K**A**F**S**PEVIPMF and **L**SPRTLNAW (LSW9) was identified instead of **I**SPRTLNAW (IW9) (155). In fact, leucine (L) and isoleucine (I) are isomeric molecules and usually interchangeable between clades (179) and cause cross-reactivity (180).

We not only studied epitopes restricted to protective alleles, we also explored HLA-A11-restricted epitopes which are Gag-p24-AK11, Nef-GK10 and Nef-QK10, Pol-AK9, Pol-QIYQ, Pol-QIIE and Pol-QIYA for HLA-A11 positive patients (n=29). GK10 is the most responsive epitope (21 out of 29) and give the highest magnitude of response. When AK11 which has been reported that response to this epitope has related to low viral load (142), AK11 IFNγ ELISpot-responders had lower viral loads compared to AK11 non-responders (median viral load 3,529 copies/mL (log 3.55) vs 24,483 copies/mL (log4.39), respectively) but not statistically significant. We grouped volunteers based on the presence of certain HLA alleles as shown in ELISpot summary results (Table 9), not all of patients could respond against particular epitopes as they

should. According to ELISpot results, patients who could respond to epitope stimulation are termed as 'IFNγ ELISpot-responder' while who could not are termed as 'IFNγ ELISpot-non-responder'. In some cases such as CTL exhaustion no IFNγ is secreted (10, 181, 182) or empty function, using IFNγ-ELISpot alone might not be a good choice to classify responder and non-responder. Recent study showed that ELISpot detecting granzyme B was more correlated to control than IFNγ (183). Nonetheless, monofunctionality of CTL response is not always associated with control. As seen in previous studies, quality of CTL response such as polyfunctionality (10, 11) has beneficial effect over a single function.

To investigate escape mechanisms behind becoming a non-responder, we further analyzed viral population sequence in plasma by Sanger sequencing (184, 185) and compared sequences of intraepitope and flanking regions at amino acid level. The limitation of this method were 1) we could only amplified viral sequences when plasma viral load is greater than 700 copies/mL and 2) population sequencing can detect only the major population while some population which less than 20-25% of total cannot be detected (186). Next-generation sequencing (187, 188) may be applied to solve these problems for further experiments. HLA-B27 polymorphism is the reason for HLA-B27-KK10 IFNγ ELISpot-non-responders. KK10 is one of high functional constraint epitope which HIV uses for capsid dimerization (189). There was no difference in KK10 sequences among IFNγ ELISpot-responders and non-responders. KK10 IFNγ ELISpot-non-responders are possess HLA-B2706 which has 2 amino acid residues at position 114 (D; Aspartic acid) and 116 (Y; Tyrosine) differs from HLA-B2704 and HLA-B2705 (Position 114 is H; Histidine and position 116 is D; Aspartic acid (190). Moreover, HLA-B2706 also shows less correlation on ankylosing spondylitis (191). Mechanisms of escape from CTL response that we discovered are non-synonymous and synonymous mutation. Mutational escape with 'non-synonymous' change was found in HLA-B57/5801 restricted epitopes; TW10 and QW9. T242N is well-defined mutation for TW10 which promotes CTL escape by interrupting TCR binding (16, 161). A recent study revealed that T242N docks into HLA molecule by a different arrangement compared to wild type epitope. This mutation does not only interfere CTL, but also the interaction with KIR3DL1 on NK cell (192). For QW9, changing from threonine to serine in IFNγ ELISpot-non-responders decreases peptide avidity and leads

to CTL escape (135, 157). Another type of mutational escape called ‘synonymous mutation’ which is found in HLA-A11-AK11 epitope in this study. There was no difference in either flanking regions or intraepitope region at amino acid level among volunteers. HLA polymorphisms as seen in HLA-B27-KK10 is not the reason behind AK11-IFNγ ELISpot-non-responders. We further hypothesized that codon usage may play a role on this synonymous change which contributes to CTL escape (193). To prove our hypothesis, nucleotide sequences were compared. Interestingly, lysine (K) at C-terminal anchor residue of AK11 showed a difference in codon usage. AAG was more preferable in AK11-IFNγ ELISpot-responders when non-responders were AAA codon. As seen in codon optimization in DNA vaccine (194) or protein expression (28, 195), codon usage affects the translational efficiency of particular protein (196, 197).

To confirm the role of codon usage on AK11 expression, lentiviral expression system was employed to transfer AK11 with different codon for lysine. Lentivirus was tested in Nalm-6 cell line (Human leukemic pre-B cell) and HLA-A11 transfected-K562 cell which given effective infectivity as shown in Figure 47. Subsequently, lentiviral particles were used to infect autologous B-LCL which was established from two separated methods; using exogenous B95.8 supernatant (198) or CD8 depletion technique. Unfortunately, we were not successfully transduced B-LCL even when MOI was increased to 100. However, there is another technique to generate B-LCL called CD40L-activated B-LCL, this cell is claimed to be as effective antigen presenting cell as mature dendritic cell (199). Our further plan is to establish CD40L-activated B-LCL and will transduced with AK11-containing lentivirus to see whether different codon usage affects level of antigen expression on the surface of target cell or not.

In conclusion, this study revealed the role of CTL on viral control longitudinally and possible ways of HIV to escape from CTL response. The most important finding was we found another mechanism of HIV escape by codon usage. These results may contribute to better knowledge on HIV pathogenesis and the way to improve vaccine efficacy in the future.

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APPENDIX A
REAGENT PREPARATIONS

1. PBMCs isolation

a. R10 medium

RPMI-1640 medium	90 mL
Heat-inactivated fetal bovine serum	10 mL

b. Trypan blue solution

Trypan blue stock solution	7 mL
1X phosphate buffered saline	3 mL

c. Freezing medium

Heat-inactivated fetal bovine serum	8 mL
DMSO	2 mL

d. Thawing medium

R10 medium	10 mL
1 mg/mL of DNase I solution	100 µL

2. B cell line establishment

a. IL-4 containing medium

R10 medium	2 mL
10 µg/mL of IL-4	2 µL

3. HIV specific T cell line establishment

a. IL-7 containing medium

R10 medium	2 mL
5 µg/mL of IL-7	2 µL

b. IL-2 containing medium

R10 medium	2 mL
200,000 IU/mL of IL-2	2 µL

4. Lentiviral production and transduction

a. Complete culture medium for HEK293T cell

DMEM medium	90 mL
Heat-inactivated fetal bovine serum	10 mL

b. Packaging medium

Opti-MEM™ I Reduced Serum Media	19 mL
Heat-inactivated fetal bovine serum	1 mL
100 mM sodium pyruvate	200 µL

c. Transduction medium

R10 medium	20 mL
8 mg/mL of polybrene	2 µL

APPENDIX B

REAGENTS, MATERIALS, INSTRUMENTS AND COMMERCIAL KITS

1. Reagents

Absolute ethanol	(Merck, Germany)
Agarose	(Research organics, USA)
AK11-tetramer PE conjugated	(NIH, USA)
Alkaline phosphate substrate	(Bio-Rad, USA)
Anti-human IFN-γ mAb 1-D1K	(Mabtech, Sweden)
Anti-human IFN-γ mAb 7-B6-1	(Mabtech, Sweden)
APC-conjugated anti-CCR7	(Biolegend, USA)
APC/Cy7-conjugated anti-human CD8	(Biolegend, USA)
Bovine serum albumin (BSA)	(Sigma-Aldrich, Germany)
BV421-conjugated anti-PD-1	(Biolegend, USA)
BV510-conjugated anti-CD45RA	(Biolegend, USA)
Cyclosporine A	(Sigma-Aldrich, Germany)
Dimethyl sulfoxide (DMSO)	(Sigma-Aldrich, Germany)
DMEM medium	(Gibco, USA)
DNase I	(Sigma-Aldrich, Germany)
DNase/RNase-free water	(Apsalagen, Thailand)
dNTP	(Fermentas, Canada)
Ethylenediaminetetraacetic acid (EDTA)	(Sigma-Aldrich, Germany)
Fetal bovine serum (US certified)	(Gibco, USA)
Ficoll-Hypaque® solution	(Robbins Scientific Corporation, USA)
GM-CSF	(R&D Systems, USA)
IMDM medium	(Gibco, USA)
Interleukin-2	(R&D Systems, USA)
Interleukin-4	(R&D Systems, USA)
Isopropanol	(Merck, Germany)
Opti-MEM™ I Reduced Serum Media	(Gibco, USA)

PE-Cy7-conjugated anti-human CD4	(Biolegend, USA)
PE-Cy7-conjugated anti-human HLA-DR	(Biolegend, USA)
PE/Texas Red-conjugated anti-KLRG1	(Biolegend, USA)
Penicillin/Streptomycin	(Gibco, USA)
Peptides	(GenScript, USA)
Peptides	(Mimotopes, USA)
PerCP-conjugated anti-human CD3	(Biolegend, USA)
Phosphate buffered saline	(Sigma-Aldrich, Germany)
Phytohemagglutinin (PHA)	(Sigma-Aldrich, Germany)
Plasmids	(Addgene, USA)
Polybrene	(Sigma-Aldrich, Germany)
Primers	(Macrogen, Korea)
Recombinant CMV pp65	(Miltenyi Biotec, Germany)
RPMI-1640 medium	(Gibco, USA)
Sodium pyruvate	(Gibco, USA)
Streptavidin-conjugated ALP	(Mabtech, Sweden)
SYBR® Green gel stain	(Invitrogen, USA)
Tris-boric acid EDTA buffer	(Research organics, USA)
Trypan blue	(Sigma-Aldrich, Germany)
Tween20	(Sigma-Aldrich, Germany)

2. Materials

Barrier tips	(Sorenson, USA)
Cryotubes (2 mL)	(Sarstedt, Germany)
DNase/RNase free clear tubes (1.5 mL)	(Axygen, USA)
EDTA blood collection tubes	(Greiner bio-one, Austria)
Filters (0.22 µM and 0.45 µM)	(Merck Millipore, USA)
Pipette tips	(Axygen, USA)
PVDF 96-well plates	(Merck Millipore, USA)
Round-bottom polystyrene tubes (5 mL)	(BD Falcon™, USA)
Sterile serological pipettes	(Eppendorf, Germany)
Sterile tubes (15 mL and 50 mL)	(Becton Dickinson, USA)

Tissue culture flasks	(Nunc™, Thermo Scientific, USA)
Tissue culture plates	(Corning, USA)

3. Instruments

Automated COBAS® Taqman® analyzer	(Roche, USA)
Automated ELISpot reader	(Carl Zeiss, Germany)
Autopipettes	(Eppendorf, Germany)
BD FACSAria™ II flow cytometer	(BD Biosciences, USA)
BD FACSCalibur™ flow cytometer	(BD Biosciences, USA)
Biosafety cabinet	(Labconco, USA)
Centrifuge (5804)	(Eppendorf, Germany)
Electrophoresis chamber	(MiniRun GE100, China)
FlowJo® version 10	(FlowJo, LLC, USA)
Freezer (-80°C)	(Thermo scientific, USA)
Incubator	(Thermo scientific, USA)
Inverted fluorescence microscope (IX81)	(Olympus, Japan)
Inverted light microscope	(Nikon, Japan)
Light microscope	(Nikon, Japan)
Liquid nitrogen tank	(Chart/MVE, USA)
Magnetic stand for LS/MS column	(Miltenyi Biotec, Germany)
Magnetic stand for 1.5mL tube	(Invitrogen, USA)
Multichannel pipette	(Brand, Germany)
Nalgene® Mr. Frosty cryopreserve box	(Sigma-Aldrich, Germany)
NanoDrop™ Spectrophotometer	(Thermo Scientifi c, USA)
Neubauer hemocytometer	(Boeco, Germany)
Pipette controller	(Accu-jet, USA)
Refrigerated centrifuge (Allegra X-15R)	(Beckman Coulter, USA)
Shaking incubator	(Zhicheng, China)
Thermal cycler (EP gradient S)	(Eppendorf, Germany)
Vortex mixer	(Brand, Germany)
Water bath incubator	(Grant, UK)

4. Commercial kits

BCA protein assay	(Thermo Scientific, USA)
BD Tritest™ CD4/CD8/CD3	(BD Biosciences, USA)
COBAS® AmpliPrep/COBAS®	
Taqman® HIV-1 test	(Roche, USA)
Dynabeads® CD8	(Invitrogen, USA)
Gentra Puregene Blood Kit	(Qiagen, Germany)
High Pure Viral RNA kit	(Roche, USA)
Lipofectamine 3000	(Invitrogen, USA)
Nucleospin® Gel and PCR Clean-up	(Macherey-Nagel, Germany)
Nucleospin® Plasmid EasyPure	(Macherey-Nagel, Germany)
One step RT-PCR kit	(Qiagen, Germany)

VITA

Ms. Prathanporn Kaewpreedee was born and raised in Bangkok, Thailand. She received her Bacherlor of Science (Medical Technology) with First-Honour from Chulalongkorn University. She specializes in HIV immunology. More specifically, her work focuses on CTL response in HIV and mechanisms of immune evasion. Apart from her thesis, researches cellular immunotherapy in virus-associated cancer is one of her interests.