ความแตกต่างทางคุณภาพของการตอบสนองผ่าน T cell ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ระหว่างผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัส และผู้ติดเชื้อที่มีการดำเนินโรคตามปกติ

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DIFFERENT QUALITY OF HIV-1 GAG P24 SPECIFIC T CELL RESPONSE BETWEEN VIRAEMIC CONTROLLERS AND PROGRESSORS

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นายนวพล เตชะเกรียงไกร : ความแตกต่างทางคุณภาพของการตอบสนองผ่านทีเซลล์ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ระหว่างผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัสและผู้ติดเชื้อที่มีการดำเนินโรคตามปกติ. (DIFFERENT QUALITY OF HIV-1 GAG P24 SPECIFIC T CELL RESPONSE BETWEEN VIRAEMIC CONTROLLERS AND PROGRESSORS) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก : ผู้ช่วยศาสตราจารย์ นายแพทย์ ดร. ปกรัฐ หังสสูต, 141 หน้า.

คำสำคัญ: โปรตีน Gag p24 ของเชื้อเอชไอวี-๑, HLA-B*27, -B*57/-B*58, คุณภาพของการตอบสนองผ่านทีเซลล์, ผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัส

ในการที่จะได้มาซึ่งวัคซีนป้องกันการติดเชื้อเอชไอวี-1 ที่มีประสิทธิภาพนั้น ความเข้าใจว่าองค์ประกอบใดในระบบภูมิคุ้มกันที่ทำหน้าที่ในการป้องกันและควบคุมการติดเชื้อตามธรรมชาติเป็นสิ่งที่มีความสำคัญอย่างยิ่งยวด การศึกษาในกลุ่มผู้ติดเชื้อเอชไอวี-๑กลุ่มพิเศษที่สามารถควบคุมปริมาณไวรัสได้อย่างตามธรรมชาติ (นั้นคือมีปริมาณไวรัสในพลาสมาต่ำกว่า 2000 ตัว/มิลลิลิตรโดยปราศจากการได้รับยาต้านไวรัส) ได้จัดให้มีโอกาสในการศึกษาถึงบทบาทที่สำคัญของการตอบสนองทางภูมิคุ้มกันของโฮสต์ในการควบคุมการติดเชื้อเอชไอวี-1 ในการศึกษานี้ คุณภาพของการตอบสนองของ $CD8^+$ T cells ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ถูกประเมินและเปรียบเทียบระหว่างกลุ่มของผู้ติดเชื้อเอชไอวีที่มีลักษณะทางคลินิคที่แตกต่างกัน ผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัส (ปริมาณไวรัสในพลาสมาต่ำกว่า 2000 ตัว/มิลลิลิตร) และผู้ติดเชื้อที่มีการดำเนินโรคตามปกติ (ปริมาณไวรัสในพลาสมาสูงกว่า 2000 ตัว/มิลลิลิตร) เพื่อพิจารณาผลกระทบที่เกิดจากเซลล์เหล่านี้ต่อผลทางคลินิคของการติดเชื้อเอชไอวี-1 คุณภาพของการตอบสนองของ $CD8^+$ T cells นั้นหมายถึงจำนวนของหน้าที่ที่เซลล์สามารถกระทำอย่างพร้อมเพรียงกัน (ตั้งแต่หน้าที่เดี่ยวจนถึงทั้งห้าหน้าที่ของการสร้าง IL-2, TNF-α, IFN-γ, MIP1-β และการแสดงออกของ CD107a) จากการศึกษาพบว่า $CD8^+$ T cells ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ของผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัสได้นั้นประกอบด้วย $CD8^+$ T cells ที่มีคุณภาพของการตอบสนองที่สูงกว่าเซลล์เหล่านั้นของผู้ติดเชื้อที่มีการดำเนินโรคตามปกติ ($p < 0.05$) คุณภาพของการตอบสนองที่สูงของ $CD8^+$ T cells ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ที่สังเกพบได้ในผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัสเหล่านี้นั้นเป็นอิสระจากอิทธิพลของอายุ ระยะเวลาของการติดเชื้อ และแม้แต่การมีอยู่ของ HLA class I อัลลีลที่สัมพันธ์กับการควบคุมเชื้อเอชไอวี-1 (HLA-B*27, -B*57/-B*58) และถูกสังเกตได้ทั้งที่ระดับความจำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 ทั้งหมดและที่ระดับความจำเพาะต่ออิพิโทปเดี่ยว มากไปกว่านั้น $CD8^+$ T cells ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-1 นั้นอยู่ในความสัมพันธ์เชิงลบอย่างมีนัยสำคัญกับปริมาณไวรัสในพลาสมา ($r = -0.6984$, $p = 0.0006$) และอยู่ในความสัมพันธ์เชิงบวกอย่างมีนัยสำคัญกับปริมาณ $CD4^+$ T cells ($r = 0.5648$, $p = 0.0095$) ซึ่งแสดงให้เห็นถึงบทบาทของเซลล์เหล่านี้ในการกำหนดผลทางคลินิคของการติดเชื้อเอชไอวี-1 ในภาพรวม การศึกษานี้บ่งชี้ว่าการมีจำนวนที่เพียงพอของ $CD8^+$ T cells ที่จำเพาะต่อโปรตีน Gag p24 ของเชื้อเอชไอวี-๑ที่มีคุณภาพของการตอบสนองที่สูงนั้นมีความสำคัญในการที่ผู้ติดเชื้อเอชไอวี-1จะกลายเป็นผู้ติดเชื้อที่สามารถควบคุมปริมาณไวรัสและได้แสดงให้เห็นถึงหลักฐานสนับสนุนบทบาทของเซลล์เหล่านี้ในการเป็นองค์ประกอบทางภูมิคุ้มกันที่ทำหน้าที่ในการป้องกันและควบคุมการติดเชื้อเอชไอวี-1 ข้อมูลที่ได้จากการศึกษานี้จะนำไปสู่วัคซีนป้องกันเชื้อเอชไอวี-1 ชนิดกระตุ้นทีเซลล์ที่ประสบความสำเร็จในอนาคตอันใกล้

สหสาขาวิชา..สหสาขาวิชาจุลชีววิทยาทางการแพทย์..ลายมือชื่อนิสิต.....

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NAVAPON TECHAKRIENGKRAI: DIFFERENT QUALITY OF HIV-1 GAG P24 SPECIFIC T CELL RESPONSE BETWEEN VIRAEMIC CONTROLLERS AND PROGRESSORS. THESIS ADVISOR: POKRATH HANSASUTA, M.D., D.Phil (Oxon), 141 pp.

Immune correlates of protection against HIV infection are crucial for a successful HIV-1 vaccine development. Study in a unique group of infected individuals who are able to control HIV naturally (HIV controllers; HIV load less than 2,000 copies/ml without antiretroviral therapy) have provided us a chance to investigate the role of host immune responses in HIV protection. In this study, the functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses on HIV-1 control were assessed and compared between the groups of clinically distinct HIV-1 infected individuals, viraemic controllers (VC, pVL < 2,000 copies/ml) and typical progressors (TP, pVL > 2,000 copies/ml) to determine their impacts on natural HIV-1 clinical outcome. The functional quality of specific-$CD8^+$ T cell responses was defined as the number of function simultaneously performed (from single to full 5 functions of IL-2, TNF-α, IFN-γ, MIP1-β and CD107a expression). In agreement with previous study, HIV-1 Gag p24-specific-$CD8^+$ T cell responses of VC were composed of higher functional quality $CD8^+$ T cells than that of TP ($p < 0.05$). This high functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses observed in VC were independent of age, duration of HIV-1 infection or even presence of protective HLA-I alleles (HLA-B*27, -B*57 and –B*58) and were observed at both a whole p24 protein specific and a single epitope specific level. Moreover, the absolute number of high functional quality HIV-1 Gag p24-specific-$CD8^+$ T cells was significantly in a negative correlation with pVL ($r = -0.6984$, $p = 0.0006$) and also in a positive correlation with $CD4^+$ T cell count ($r = 0.5648$, $p = 0.0095$), hence clearly illustrated their roles in determining HIV-1 clinical outcome. In conclusion, this study indicated that possession of an adequate numbers of high functional quality HIV-1 p24-specific-$CD8^+$ T cells is important in order to become a natural HIV controller and provided a solid evidence supporting their roles as an immune correlate of HIV-1 protection. The information obtained from this study will pave the way toward a successful HIV-1 T-cell based vaccine in the near future.

Field of Study : MEDICAL MICROBIOLOGY Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

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LIST OF ABBREVIATIONS

A	Absorbance
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APC-H7	Allophycocyanin-H7
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
AP3	Adaptor protein complex 3
ART	Antiretroviral therapy
BCA	Bicinchoninic acid
BFA	Brefeldin A
Bp	Base pair
°C	Celsius degree
CA	Capsid
CBC	Complete blood count
CCR	Chemokine receptor
CD	Cluster of differentiation
CHI	Chronic phase
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRF_01AE	Circulating Recombinant Form_01AE
CTD	C-terminal domain
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DDW	Deionized distilled water
dsDNA	Double-strand deoxyribonucleic acid
EBV	Epstein-Barr virus

Abbreviations (continued)

EC	Elite controller
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme linked immunosorbent spot
Env	Envelope
et al	et alii
ESCRT-I	Endosomal Sorting Complex Required for Transport-I protein
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gravity
GALT	Gut-associated lymphoid-tissue
gp	glycoprotein
HCMV	Human cytomegalovirus
HHV8	Human herpes virus-8
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSV	Herpes simplex virus
ICS	Intracellular cytokine staining
IFN-γ	Interferon-gamma
IL	Interleukin
IN	Integrase
IVDU	Intravenous drug users
kD	Kilodalton
KIR3DS1	Killer Immunoglobulin-like Receptor 3DS1
LTR	Long terminal repeat
M	Molar
MA	Matrix
mAb	Monoclonal antibody

Abbreviations (continued)

MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mg	Milligram
ml	Milliliter
mm^3	Cubic millimeter
MSM	Men who have sex with men
NC	Nucleocapsid
Nested-PCR	Nested-polymerase chain reaction
ng	Nanogram
NK cell	Natural Killer cell
nPA	Subject without protective-allele(s)
NTD	N-terminal domain
OD	Optical density
OIs	Opportunistic infections
OLP	Overlapping peptide
PA	Subject with protective-allele(s)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PB2	Polymerase basic 2
PCR	Polymerase chain reaction
PCR-SSOP	Polymerase chain reaction-sequence specific oligonucleotide primers
PCR-SSP	Polymerase chain reaction -sequence specific primers
pDC	Plasmacytoid dendritic cells
PE	*Phycoerythrin*
PE-Cy5, Cy7	*Phycoerythrin*-cyanine5, -cyanine7
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PHI	Primary infection phase

Abbreviations (continued)

PIC	Prointegration complex
PI[4, 5]P_2	Phosphoinositide phosphatidylinositol (4, 5) biphosphate
Pol-II	Polymerase II
PR	Protease
PTAP	Pro-Thr-Ala-Pro
PVDF	Polyvinylidene difluoride
pVL	Plasma HIV-1 load
RER	Rough endoplasmic reticulum
Rev	Regulatory of expression of viral protein
RNA	Ribonucleic acid
RP	Rapid progressor
Rpm	Round per minute
RRE	Rev-responsive element
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
R10	RFMI 1640 medium supplemented with 10% fetal bovine serum
SEB	Staphylococcus enterotoxin B
SFU	Spot forming unit
SH3	Src homology 3
SIV	Simean immunodeficiency virus
SP	Slow progressor
ssRNA	Single strand ribonucleic acid
SU	Surface
TAR	Tat-responsive stem-bulge loop
TBE	Tris borate ethylenediamine tetraacetic acid
TCR	T cell receptor
TLR	Toll-liked receptor
TM	Transmembrane

Abbreviations (continued)

TNF-α	Tumor necrosis factor-alpha
TP	Typical progressor
tRNA	Transfer ribonucleic acid
TP	Typical progressor
UNAIDS	United Nation programme on HIV/AIDS
µg	Microgram
µl	Microliter
VC	Viraemic controller
Vif	Virus Infectivity Factor
vol/vol	Volume by Volume
VZV	Varicella-zoster virus
WBC	White blood cell

CHAPTER I

INTRODUCTION

Human Immunodeficiency Virus-1 (HIV-1) is a pathogenic human lentivirus responsible for an Acquired Immunodeficiency Syndromes (AIDS) in human. Without proper treatment with anti-retroviral therapy (ART), its fatality rate is as high as 100% [1]. Though antiretroviral therapy (ART) has been world-widely demonstrated as an effective way to reduce morbidity, mortality and, more importantly disease spreading, its deleterious side-effects and cost also put a lifelong burden on its users, both healthily and economically [2, 3]. Another effective intervention for the world HIV-1 epidemic control is HIV-1 vaccine, either prophylactic or therapeutic. Decade of HIV-1 vaccine development has pressed us to reconsider what are the immune correlates of protection against HIV-1 infection [4]. Among HIV-1 infected individuals, there are a small, unique group of individuals who seem to control HIV-1 naturally (HIV controllers; plasma HIV-1 load less than 2,000 copies/ml without antiretroviral therapy). Study in this unique group of individuals might provide us a chance to investigate the role of host immune responses in natural HIV-1 protection.

Several immunological factors have been linked with HIV-1 control in which of HIV-1-specific-$CD8^+$ T cells convincingly are the most important component [5]. Impacts of HIV-1-specific-$CD8^+$ T-cells have been evidently demonstrated in a large number of studies, including: reduction of peak viraemia observed during primary infection, higher Simean Immunodeficiency Virus (SIV) load and rapid disease progression in $CD8^+$-depleted rhesus macaques, direct killing of HIV-1 infected $CD4^+$ T cells and suppression of HIV-1 replication [6-12]. Moreover, immune escape mutations are frequently observed in HLA class I (HLA-I) restricted epitopes [13-15]. Though the protective effects of HIV-1-specific-$CD8^+$ T cells seem solemnly solid, correlation between magnitude of $CD8^+$ T cell responses and plasma HIV-1 load have remained inconclusive

[16-18]. Advances in flow-cytometry technology over the past decade have allowed us to investigate several parameters, including both cellular phenotypes and functions at a single cell level. While there is still not definite about the HIV-1 control mediated phenotype, findings from several previous studies have demonstrated a strong association between HIV-1-specific-$CD8^+$ T cell functional quality and HIV-1 control [16, 17, 19, 20].

Another unequivocal evidence supporting the roles of HIV-1-specific-$CD8^+$ T cells is that some certain HLA-I alleles are frequently presented in HIV controllers [21, 22]. Evidently, there are 3 HLA-I alleles have been regarded as "protective alleles (PA)": HLA-B*27, -B*57 and -B*58 [21-25]. These associations are a result of the particular HIV-1 specific epitopes presented by them which mostly are immunogenic, functional constrained epitopes of highly conserved HIV-1 Gag capsid protein [25-29]. HIV-1 Gag-specific-$CD8^+$ T cell responses were the only response that inversely correlated with plasma HIV-1 load [30].

Therefore, I hypothesized that the natural HIV-1 controlling ability observed in HIV controllers is a result of high functional quality of $CD8^+$ T cell responses directly against highly conserved HIV-1 Gag protein. To test this speculation, the functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses of 3 groups of clinically distinct HIV-1 infected individuals; elite controllers (EC: with undetectable plasma HIV-1 load), viraemic controllers (VC: plasma HIV-1 load equal to/lower than 2,000 copies/ml) and typical progressors (TP: plasma HIV-1 load higher than 2,000 copies/ml) were assessed and compared in this study. In addition, the functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses were also compared between the group of HIV-1 infected individuals carrying the same protective HLA-I alleles: HLA-B*27, -B*57 and –B*58 and also mounting the same epitope specific responses but were with discordant plasma HIV-1 load.

To my best knowledge this is the first study investigating the protective effects of HIV-1 Gag p24-specific-$CD8^+$ T cell responses at a single epitope specific-level. Findings from this study will provide key information suggesting that even at a single epitope specific-level, the functional quality of $CD8^+$ T cells is still a critical factor impacting HIV-1 clinical outcomes.

CHAPTER II

OBJECTIVES

The objectives of this study are:

1. To analyze HIV-1 Gag p24 polymorphism of the recently circulating strain of HIV-1 in Thailand

2. To identify the immunodominant segments of HIV-1 Gag p24 targeted by T cells in viraemic controllers and typical progressors

3. To analyze magnitude and functional quality of HIV-1 Gag p24-specific-$CD8^+$T cell responses in viraemic controllers and typical progressors

4. To compare the function quality of HIV-1 Gag p24-specific-$CD8^+$T cell responses between "protective HLA-B alleles" matched viraemic controllers and typical progressors

CHAPTER III

LITERATURE REVIEWS

Human Immunodeficiency Virus 1 (HIV-1) is a pathogenic human lentivirus responsible for an Acquired Immunodeficiency Syndromes (AIDS) in human. Without proper treatment with anti-retroviral therapy (ART), its fatality is as high as 100% [1]. AIDS-related deaths are mostly due to an in ability to fight opportunistic infections and pathogen-related malignancies. In 2009, joint United Nation programme on HIV/AIDS estimated that a total number of 33.3 million individuals were infected with HIV-1 worldwide in which 2.6 million were newly infected [31]. In Thailand, there were approximately 530,000 HIV-1 infected individuals in which mostly were adult (older than 15 years old) male [31]. Annually, approximately 12,000 individuals were newly infected and 28,000 were dead from AIDS-related illness. HIV-1 prevalence in most-at-risk group was as followed; 38.7% in intravenous drug users (IVDU), 13.5% in men who have sex with men (MSM) and 2.8% in female sex workers.

Part I. Virology of HIV-1

1. HIV-1 Genome

HIV-1 is a member of Family *Retroviridae*, Genus *Lentivirus* with two single-stranded RNA (ssRNA), each was approximately 9.2 kilo base pairs long. This RNA genome is bounded at both ends with a non-coding long terminal repeat (LTR) region (5'LTR and 3'LTR). HIV-1 has 3 major genectic loci (*gag, pol* and *env*) which encoded core proteins, viral enzymes and envelope protein, respectively, similar with all other lentiviruses. In addition, HIV-1 possesses a group of loci encoding 7 accessory proteins; Tat, Rev, Vpr, Vpx, Vpu, Vif and Nef. Tet and Rev are required in HIV-1 replication process while others 5 are associated with HIV-1 full virulence [32]. Figure 1 shows genomic organization of HIV-1.

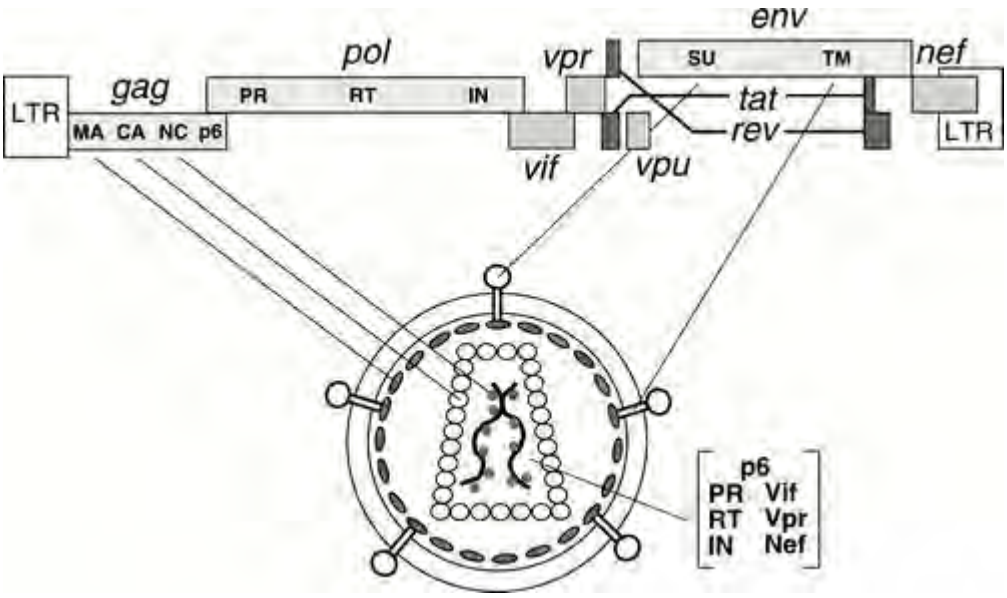


Figure 1. Genomic organization of HIV-1 and schematic representative of mature HIV-1 virion (adapted from [33])

2. HIV-1 structure

HIV-1, when viewed under an electron microscope, is an enveloped virion, approximately 100-120 micrometers in diameter with a typical lentiviral cone-shaped core, similar to other retroviruses [34]. Mature HIV-1 virions are composed of the following proteins. The envelope protein (Env) is composed of a membrane bilayer and a total of 72 trimeric surface glycoprotein spikes (Env gp120 and gp41 proteins) which protrude outwardly. Inside the envelope is lined with Gag p17 matrix proteins which provide the basic structure for viral structure. The cone-shaped core is formed by multimerization of Gag p24 capsid proteins. This core contains two identical ssRNAs of the HIV-1 genome which are associated with reverse transcriptase enzyme (Pol p66/p51 RT), integrase enzyme (Pol p31 IN) and Gag p7 nucleocapsid proteins. Accessory proteins, Vpr and Nef are also contained inside viral core (Figure 1) [32].

3. HIV-1 proteins

HIV-1 proteins are initially translated as polyprotein precursors and subsequently processed by both cellular and viral protease into mature proteins. Gag and Pol are translated as a Gag/Pol precursor ($Pr160^{Gag\text{-}Pol}$) which is auto-catalysed into Gag precursor ($Pr55^{Gag}$) and 3 viral enzymes; protease (PR, p10), integrase (IN, p32) and heterodimeric reverse transcriptase (RT, p51/66). During or after the budding of progeny virus, Gag precursor is further processed by viral protease (PR) into matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6. Env precursor (gp160) is cleaved by cellular protease into gp120 surface (SU) and gp41 transmembrane (TM). The remaining 7 accessory proteins are all translated in their mature form [32].

3.1. Envelope (Env, gp160)

Translation of Env mRNA is occurred in ribosomes at the rough endoplasmic reticulum (RER) at which newly translated gp160 are glycosylated at the same time. At ER, gp160 undergoes an oligomerization into trimer. Trimeric gp160 is subsequently transported to

the golgi complex and proteolytically cleaved with cellular protease into mature gp120 and gp41 [35, 36]. The gp120 and gp 41 are linked with each other by a non-covalent association forming a mature gp160 glycoprotein. This mature gp160 glycoprotein is then transported to cell surface waiting to be incorporated into new virion.

3.2. Gag (p55)

As with other retroviruses, Gag proteins are important for an HIV-1 assembly. Roles of Gag proteins in HIV-1 assembly include; a) formation of a structural framework of the virion, b) encapsidation of viral genomic RNA (gRNA), c) targeting of the new viral particle for export from the cell and d) acquiring of lipid bilayer and its associated Env glycoprotein [37].

3.2.1. Matrix (MA, p17)

Matrix domain (MA, P17) of $Pr55^{Gag}$ plays many crucial roles during HIV-1 life cycle. Its roles include; a) regulation of intracellular Gag localization, b) binding of Gag protein and plasma membrane and c) incorporation of Env glycoprotein to HIV-1 virions. Transport of $Pr55^{Gag}$ directly toward plasma membrane is directed by MA domain. The myristic acid moieties which are covalently attached to glycine residue and the cluster of basic residues at the N-terminal of MA are responsible for this plasma membrane localization of $Pr55^{Gag}$, by binding with inner leaflet of plasma membrane[38-40]. In addition, interactions between N-terminal myristic acid of MA and certain cellular molecules such as phosphoinositide phosphatidylinositol (4, 5) biphosphate ($PI[4,5]P_2$) and clathrin adaptor protein complex 3 (AP3) at plasma membrane are also important in directing HIV-1 assembly to occur at plasma membrane [41, 42]. Depletion of $PI[4,5]P_2$ or AP3 from plasma membrane results in an intracellular relocation of HIV-1 and enormously disrupts viral particle production [41]. After budding of new viral particle, cleavage at MA-CA junction by viral protease (PR) results in release of CA from MA and maturation of premature virion, leaving MA to act as an inner lining of virion membrane.

3.2.2. Capsid (CA, p24)

Capsid domain (CA, p24) of $Pr55^{Gag}$ has 2 major roles; promoting HIV-1 assembly and forming an outer layer of HIV-1 core in a mature viral particle. CA protein is composed of 2 domains; N-terminal domain (NTD, amino acids residues 1-146) and C-terminal domain (CTD, amino acids residues 148-231) which are linked with each other by a short flexible linker [43, 44]. The proper two-domain conformation of CA is very important for HIV-1 assembly. Mutations within CA that affected this organization result in HIV-1 assembly defect, improper maturation or impaired infectivity [45]. Cleavage of $Pr55^{Gag}$ by viral protease (PR) during budding of viral particle enables the multimerization of CA into a hexameric ring in which each CA is linked together by CTD [46]. This hexameric ring is a basic unit in the typical lentiviral-conical-shaped core seen in mature viral particle [47]. In addition, a cyclophilin A binding activity of cyclophilin A binding loop on NTD is very important for HIV-1 infectivity [48, 49]. Mutations in a cyclophilin A binding loop result in a decreased infectivity of HIV-1.

3.2.3. Nucleocapsid (NC, p7)

A principal function of nucleocapsid (NC, p7) is to encapsidate full-length, unspliced viral RNA into a newly formed virion in which both its zinc finger motif and clustered basic residues are the key players [50-52]. NC mediates viral genome encapsidation by interacting with an encapsidation element (ψ) on a 5' long terminal repeat (5'LTR) [53]. NC also plays a part in Gag multimerization by acting as a template for $Pr55^{Gag}$ alignment and packaging [54]. In addition, NC also possesses the nucleic acids chaperone activities [55, 56]. Firstly, NC catalyzes the destabilization of nucleic acid duplex which allows the reverse transcription process to occur [57]. And also, induction of nucleic acid aggregation by NC promotes transferred RNA (tRNA) primer binding and facilitates the annealing reaction. Moreover, NC zinc finger motif and surrounding basic residues are also important in the stimulation of DNA integration [58, 59].

3.2.3 P6

P6 plays a critical role in releasing of new virion from plasma membrane in which the highly conserved Pro-Thr-Ala-Pro (PTAP) motif at the N' terminal of P6, termed "late domain", is very important [60, 61]. P6 uses this late domain in binding with Tumour Susceptibility Gene 101 (Tsg101) protein, a component of ESCRT-I (Endosomal Sorting Complex Required for Transport-I) protein of the endosomal sorting machinery and usurps this cellular machinery to promote HIV-1 release from plasma membrane [62, 63]. In addition, P6 also directs an incorporation of Vpr and Vpx proteins into HIV-1 virion [64, 65].

3.3. Polymerase (Pol)

HIV-1 Pol encodes 3 HIV-1 enzymes; reverse transcriptase, integrase and protease (RT, IN and PR, respectively). These enzymatic products of Pol protein are the key player in several steps of HIV-1 life cycle.

3.3.1. Reverse transcriptase (RT, p51/p66)

One of the key features of all retroviruses is their ability to reverse-transcribe viral single-stranded RNA (ssRNA) into double-strand DNA (dsDNA) after viral entry into the cell [66, 67]. Viral enzyme, reverse transcriptase (RT, p51/p66) is responsible for this process. Mature HIV-1 RT holoenzyme is a heterodimer of 2 subunits; 66 and 51 kilodaltons (kD) in size (also termed, p66 and p51, respectively). Both domains are folded into multiple subdomains and arranged into the secondary structure suitable for an active reverse-transcription activity [68-71]. HIV-1 RT possesses 2 enzymatic activities; DNA polymerase and RNaseH activity [72-74]. DNA polymerase activity of RT is responsible for both an RNA template-dependent synthesis of minus-stranded DNA and a DNA template-dependent synthesis of plus-stranded DNA. RNaseH activity is used to degrade tRNA primer and viral genomic RNA in a RNA-DNA hybrid formed during synthesis of minus-strand DNA. Final products of HIV-1 reverse-transcription are a double-stranded viral DNA (dsDNA) ready to be integrated into host cell chromosome [32].

3.3.2. Integrase (IN, p32)

Another key feature of retroviruses is an ability to integrate their freshly reverse-transcribed dsDNA into host cell chromosome and serves as a template for viral RNA synthesis. IN is composed of 3 functionally and structurally distinct domains lining up from N' to C' terminal [75, 76]. N-terminal zinc binding domain (amino acids residues 1-55) which adopts a helix-turn-helix motif conformation, is responsible for the formation of higher structure of IN protein [77, 78]. Mutations in this domain result in disruption of IN multimerization and its integrase activity [78]. Central catalytic core domain (amino acids residues 50-212) has the similar structure with other polynucleotidyl transferase in which it is composed of a 5-stranded β-sheet flanked by helices [79]. This central domain of IN protein plays a critical role in the integrase activity of the enzyme [80, 81]. C-terminal domain adopts a 5-stranded β-barrel folding which bears a resemblance with Src homology 3 (SH3) domain [82, 83]. Its role is still elusive but with its ability to bind with nucleic acid in a non-sequence specific manner raises the suggestion that this domain might contribute to an association between IN and viral genome during reverse-transcription [84, 85].

3.3.3. Protease (PR, p10)

HIV-1 protease (PR, p10) plays an important role in turning an immature doughnut shaped virion into a mature virion with a conical-shaped core [86, 87]. HIV-1 PR is related to cellular aspartic protease such as pepsin and rennin [88]. Active PR is a dimer of 2 PR monomers which are connected to each other by 4 stranded, antiparallel β-sheets forming a substrate-binding cleft in the middle of this 2 monomers [89-91]. Enzyme active site is located in a middle of this substrate-binding cleft [91, 92]. Proteolytic activity of HIV-1 PR is of the same mechanism as other aspartic protease in which 2 apposed aspartic acid residues at the active site coordinates water molecule and hydrolyses peptide bond of targeted peptide [88].

3.4. Accessory proteins

Tat and Rev is acting as a regulator of HIV-1 transcription and protein expression, respectively, thus their presence are indispensable for HIV-1 replication cycle. The other 5 accessory proteins; Vif, Vpr, Vpx, Vpu and Nef, though they don't have any direct role in HIV-1 replication cycle, their presence guarantee a success in establishment of HIV-1 infection.

3.4.1. Tat

Tat is a 14 kD, 101 amino acids nuclear protein acting as a regulator of HIV-1 transcription. Tat active sites are located on a 58 amino acids at N'terminal which compose of transactivation domain (amino acids residues 1-48) and 5'TAR (Tat-responsive stem-bulge loop) binding domain (amino acid residues 49-57) [32]. In order to start HIV-1 transcription, a complex formed between viral gRNA, Tat and cellular co-factors is a prerequisite [93, 94]. Tat binds with TAR by the protein-nucleotide interaction between TAR binding domain and bulge structure of TAR. While host cofactors are brought into complex by interacting with Tat transactivation domain and subsequently brought into contact with loop structure of TAR [32]. This complex helps promoting the formation of RNA polymerase-II (RNA pol II) transcription complex.

3.4.2. Rev (Regulatory of Expression of Viral protein)

As its name, Rev (Regulatory of Expression of Viral protein) protein is a regulator of viral protein expression [32]. In a HIV-1 replication cycle, a large number of transcribed RNA, different in both length and splicing pattern, are created [95, 96]. For instance, a full-length unspliced gRNA which serves as mRNA for Gag-Pol precursor synthesis is produced in the same nucleus which a completely spliced Tat mRNA is created. Rev protein helps preserve all HIV-1 transcripts in their appropriate pattern by binding with an RRE (Rev-Responsive Element) which located at the junction between gp120 and gp41 of Env [97, 98]. Mutation or deletion of Rev results in loss of all long unspliced transcripts and disruption of HIV-

1 replication [99]. In addition, Rev also plays a part as a transporter of newly transcribed mRNA into cytoplasm [32].

3.4.3. Vif (Virus Infectivity Factor)

HIV-1 infectivity promoting role of Vif only occur in a producer cell. In an active viral producing cell, Vif proteins counter the action of APOBEC3G (Apolipoprotein B mRNA-editing Enzyme, Catalytic polypeptide like 3G) during viral assembly resulting in an increased viral particle production [32].

3.4.4. Vpr

Vpr is a 14 kD, 96 amino acids protein which is incorporated into viral particle. Vpr has multiple functions such as promoting transport of prointegration complex (PIC) into nucleus after viral entry, stimulation and enhancement of cellular transcription and expression, and arresting infected cell-cycle at stage G_2 [100]. In association with Vpx, Vpr helps establishing HIV-1 infection in resting cell [101].

3.4.5. Vpx

Generally, Vpx gene has a high degree of similarity with Vpr and also its function. Vpx is incorporated into newly formed virion at a very high level [102]. After viral entry, Vpx plays a part in nuclear targeting of PIC and its presence is crucial for an efficient infection of non-dividing cell [101].

3.4.6. Vpu

Vpu is an exclusively unique protein of HIV-1. It is an 81 amino acids long, multimeric integral membrane phosphoprotein and is not presented in viral particles [32]. Vpu has 2 main functions; enhancing virus release from HIV-1 infected cell and degradation of CD4 molecules. Vpu promotes viral release by forming a cation-selective ion channel in a CD4-independent manner [103, 104]. Vpu also directly binds with cytoplasmic tail of CD4 in ER and

liberates intracellular CD4-bound Env glycoprotein which resulted in increased level of Env at the plasma membrane [105, 106]. Thus, main function of Vpu is to promote HIV-1 production.

3.4.7. Nef

Nef protein is a 27 kD myristoylated, membrane-associated phosphoprotein. Nef is exclusively presented in primate lentiviruses only. Nef does not play any part in HIV-1 replication but it is known to have several functions which help increasing HIV-1 infectivity. Nef protein functions as a downregulator of many immunological surface molecules, such as CD4, major histocompatibility complex class I (MHC-I), major histocompatibility complex class II (MHC-II), CD3-T cell receptor (TCR) complex and costimulatory CD28 molecules [32]. Notably, virus isolated from HIV-1 infected individuals with a slow disease progression shows mutation in Nef.

4. Molecular biology of HIV-1 replication

HIV-1 replication cycle begins with the binding of gp120 molecule of virus particle to a CD4 molecule on the surface of target cell (detailed descriptions are in section 5). Subsequently, this CD4-bound gp120 also binds with HIV-1 coreceptor (either a C-X-C motif chemokines receptor, CXCR4 or C-C motif chemokines receptor, CCR5) which results in changing of gp120 conformation and activating gp41 molecule [107-109]. The gp41 molecule plays a role in the fusion of viral and cellular membrane in a pH-independent manner [110]. After viral entry, subviral particle is uncoated and started to reverse-transcribe its gRNA into a partially dsDNA. This dsDNA is subsequently transported into nucleus as a PIC which also contains subset of Gag and Pol proteins [111, 112]. Host machinery is responsible for the transport of PIC to nucleus. Once inside the nucleus, dsDNA is integrated into chromosomal DNA and served as a template for RNA polymerase II (Pol-II) directed RNA synthesis [113]. Newly transcribed mRNA is then transported into cytoplasm with the help from HIV-1 Rev proteins (described in section 3.4.2). Translation of Env protein is done in ribosomes at RER while of Gag and Pol proteins are at free

cytoplasmic ribosomes [32]. Premature Gag and Pol proteins, accompanied with newly synthesized viral gRNA are transported to plasma membrane, at which mature gp160 Env glycoproteins are densely habituated, and undergone a budding process to become an immature viral particles. During or immediately after budding of an immature viral particle, premature Gag and Pol protein are cleaved by viral protease, generating the cone-shaped core characteristic of mature HIV-1 virion (described in section 7).

5. HIV-1 binding and entry

HIV-1 Env glycoprotein (gp160) plays an important role in an interaction between viral particle and host cell. Firstly, gp120 sueface (SU) molecule of gp160 binds to a CD4 molecule expressed on the surface of susceptible target cells (both T helper lymphocyte and monocyte lineage) [114]. This binding induces a primary conformational change of gp 120 molecule enabling it to bind with coreceptor (CXCR4 or CCR5, depend on virus isolates) and forming a ternary CD4-gp120-coreceptor complex [32]. Subsequently, gp41 transmembrane (TM) molecule undergoes a secondary conformational change which exposes its hydrophobic region at N'terminal called fusion peptide [115]. This fusion protein is then inserted into cellular plasma membrane resulting in a tertially conformational change of gp41 and a fusion of viral and cellular lipid bilayer [110].

6. HIV-1 assembly

HIV-1 assembly takes place at lipid rafts, the cholesterol- and glycosphingolipid-enriched domains which located at the plasma membrane. As with other retroviruses, Gag proteins are important for an HIV-1 assembly [116]. Roles of Gag proteins in HIV-1 assembly including; a) formation of a structural framework of the virion, b) encapsidation of viral gRNA, c) targeting of the new viral particle for budding from the cell and d) acquiring of lipid bilayer and associated Env glycoprotein [37, 117]. After the translation of $Pr55^{Gag}$ in cytoplasmic ribosomes,

these polyprotein precursors are rapidly transported to cell membrane at the lipid rafts where the mature Env glycoproteins are densely populated.

7. HIV-1 maturation

As previously described in section 3.3.3, PR cleavage of Gag-Pol precursor liberates CA, NC and p6 leaving only MA binding with viral membrane. Subsequently, CA, NC and P6 undergoes a higher order formation and forms a conical-shaped core [118]. This process is termed “maturation” since disruption of this process both by Gag mutation or inhibition of PR activity result in severely decreased HIV-1 infectivity [119, 120].

Part II. Clinical course of HIV-1 infection

HIV-1 clinical course can be divided into three phases; acute or primary infection phase (PHI), asymptomatic or chronic phase (CHI) and late or AIDS (Acquired Immuno Deficiency Syndrome) phase [121]. The duration of each phase is highly variable among HIV-1 infected individuals. Standard clinical determinants for monitoring disease progression are plasma HIV-1 load (pVL) (measured as viral copies/ml of plasma) and $CD4^+$ T cell counts (measured as cells/mm^3 of whole blood) in which the level of both parameters are used as criteria defining each stage of disease. Figure 2 shows a schematic clinical course of HIV-1 infection.

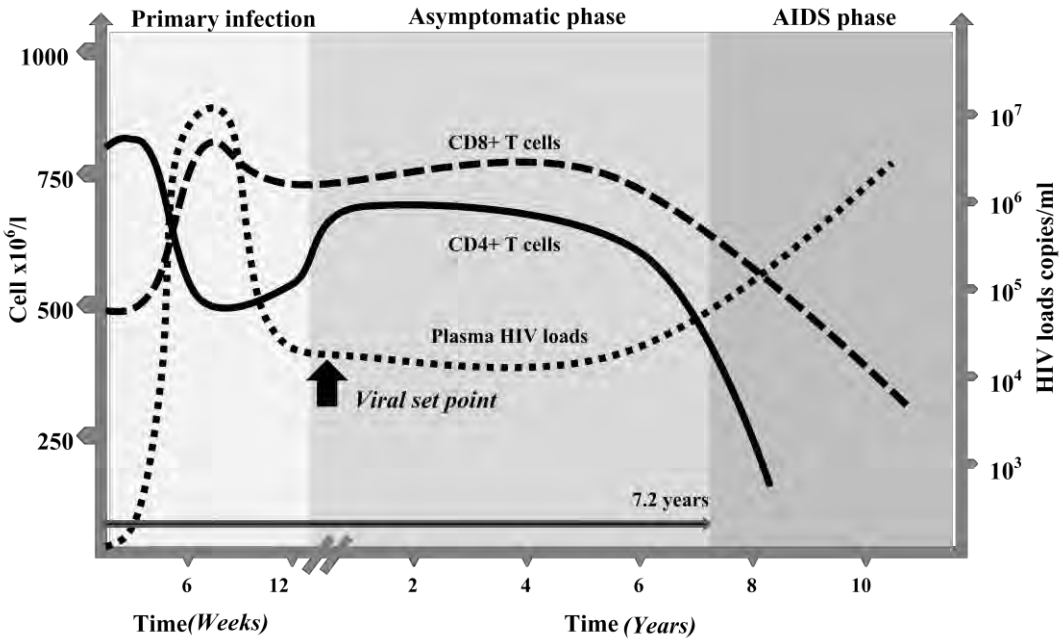


Figure 2. Schematic representative of HIV-1 infection clinical course in Thai population: $CD4^+$ T cell counts and plasma HIV-1 load are scaled on left and right-side Y-axis, respectively. Time is scaled on X-axis (weeks and years). Arrow-headed line at the lower part of the figure represents median time-to-AIDS after seroconversion in Thai population which is 7.2 years [122]. (adapted from [123])

1. Primary HIV-1 infection (PHI)

Hallmarks of primary HIV-1 infection (PHI) are a rise of pVL, dramatic depletion of $CD4^+$ T cells and an occurrence of relatively non-specific symptoms, termed as "acute retroviral syndrome" which usually begins at 2 weeks after HIV-1 infection [124]. Approximately, between 50% - 70% of HIV-1 infected individuals show these clinical manifestation of acute HIV-1 infection in the form of fever, maculopapular rash, diarrhoea and weight loss [125]. Typically, pVL increases continuously until reaching its peak, termed "peak viraemia" with the median between $10^6 – 10^7$ copies/ml, approximately [126, 127]. Massive $CD4^+$ T cell depletion can be observed both in peripheral blood and in the gut-associated lymphoid-tissue (GALT). These manifestations are clinically observable for 3-6 weeks until an appearance of HIV-1 specific immunological responses which are shown to have a substantial impact on HIV-1 clinical outcome [1]. After reaching its peak level, pVL begins to drop gradually which coincides with the increase in numbers of HIV-1 specific $CD8^+$ T cells. Then, pVL continuously decreases until it reaches a steady level, termed as "HIV-1 viral set-point" [126, 127]. Concurrently with this decrease in pVL, $CD4^+$ T cell also increase in numbers until reaching a steady level which is lower than its pre-infection level. This steady level of pVL and $CD4^+$ T cells represents a balance between an ability of virus to replicate under strong immunological pressures and host ability to replace dead $CD4^+$ T cells [1]. The level of pVL, together with the level of immune-reconstituted $CD4^+$ T cells at this viral set-point is highly variable and strongly associated with the speed of disease progression.

2. Chronic HIV-1 infection (CHI)

Chronic HIV-1 infection is the period of time ranging from reaching of the viral set-point to the start of clinically-defined AIDS status (as defined by $CD4^+$ T cell counts below 350 cells/mm^3 and/or occurrence of opportunistic infections) [128]. Median length of this phase in Thai population is 6.5 years with the median pVL at 12,923 copies/ml [129]. Normally, this phase of HIV-1 infection is clinically asymptomatic.

3. Acquired Immunodeficiency Syndrome phase (AIDS)

The drop in number of $CD4^+$ T cells (to the level below 350 cells/mm^3) usually accompanying with the uncontrollable rise in pVL are a symbolic character of AIDS phase [128]. As a

consequence, HIV-1 infected individuals become susceptible to many opportunistic infections (OIs), which normally are very limited in a healthy immunocompetent individual. A wide range of pathogens are responsible for these OIs, such as fungi: *Pneumocystis jiroveci* pneumonia, oral candidiasis (by *Candida albicans*); bacteria: crytococcal meningitis (by *Crytococcus neoformans*), *Mycobacterium avium* complex and *M. tuberculosis*; protozoa: toxoplasmosis (*Toxoplasma gondii*); and virus: cytomegalovirus (CMV), herpes simplex virus (HSV) and varicella-zoster virus (VZV). In addition, this stage of immunodeficiency is also associated with increased risk of some opportunistic virus-associated malignancy such as Burkitt's lymphoma (by Epstein-Barr virus (EBV)), cervical cancer (by human papilloma virus (HPV)) and polyclonal B-cell lymphoma (by human herpes virus type 8 (HHV8)) [1, 121]. Incidence of one or more of these OIs is also a definition of AIDS phase-independent of $CD4^+$ T cell counts.

4. Rate of disease progression

As describe earlier, both pVL and number of $CD4^+$ T cells at the steady viral set-point are associated with rate of disease progression (depicted in part II, section 2). Reaching viral set-point with low pVL and high $CD4^+$ T cell counts is associated with slow disease progression. On the other hand, high pVL and low $CD4^+$ T cells set-point are associated with fast disease progression. Disease progression rate can be used to categorize HIV-1 infected individuals into three groups: rapid, typical and slow progressors (RP, TP and SP, respectively). Study in a cohort of HIV-1 infected individuals in Thailand revealed that median time-to-AIDS for TP is 7.2 years after seroconversion while it is only lower than 3 years in RP and longer than 10 years in SP [129]. Identification of host and viral factors that attribute to, or at least are correlated with different rate of HIV-1 progression might provide the important information for the development of HIV-1 vaccine and prospective treatment strategies.

Part III. Host immune responses against HIV-1

Integration of HIV-1 proviral DNA into cellular genome results in a life-long persistent infection. Unlike acute-resolved viral infections, recovery form a persistent viral infection is not necessarily associated with viral eradication but rather from an immune-mediated containment of virus instead.

1. Innate immune response

Several components of innate immunity are active against HIV-1 infection. Firstly, plasmacytoid dendritic cells (pDC), though they are also infected, are activated by an interaction between toll-liked receptor 7 (TLR7) and HIV-1 RNA. Activated pDC secrete a wide range of cytokines in a large quantity including Interferon-alpha (IFN-α), Interleukin-12 (IL-12), Tumour Necrotic Factor-alpha (TNF-α) and IL-6. Natural Killer cells (NK cells) might also play some parts as observed by increased in their numbers during PHI. Moreover, an association observed between slow disease progression and certain NK cell receptor, Killer Immunoglobulin-like Receptor 3DS1 (KIR3DS1) and its ligand HLA-Bw4 provides another evidences supporting NK cell role in HIV-1 immunity [130].

2. Adaptive humoral immune response

HIV-1 specific antibodies can be detected within weeks after infection. However, these early-producing antibodies mostly are a non-neutralizing antibody [131]. This is partly due to the heavily glycosylated nature of HIV-1 gp120. In immunocompetent HIV-1infected individuals, only 10% of all HIV-1 specific antibodies are neutralizing [1]. Moreover, HIV-1 can rapidly evolve and escape from these neutralizing antibodies. Recent study have shown that even in the presence of robust, broad HIV-1 specific neutralizing antibody activity, infected individuals are still living with rather high pVL (10^2-10^4 copies/ml) [132]. These evidences suggest that HIV-1 specific humoral immune responses, though are robustly observed, their roles might not critical enough in HIV-1 containment.

3. Adaptive cellular immune response

3.1. $CD4^+$ T lymphocytes

Almost every HIV-1 infected individuals mounted strong $CD4^+$ T cell responses during PHI as measured by lymphocyte stimulation assay (LS) [1]. However, these responses are gradually lost over the years. For unknown reason, HIV-1 specific $CD4^+$ T cell responses are skewed toward Gag protein. This might be a result of highly immunogenic nature of Gag protein and high number of Gag proteins being produced in infected cells [5, 21, 133]. Normally, HIV-1 specific T cells responses are of lower magnitude than those of $CD8^+$ T cells, as measured by interferon-gamma (IFN-γ) production [134]. The major role of HIV-1-specific $CD4^+$ T cells is to mediate an effective $CD8^+$ T cell responses which are more active in controlling HIV-1 and others chronic viral infections [135, 136]. This assumption is supported by one study showing that addition of $CD4^+$ T cells isolated during PHI into the functionally-exhausted $CD8^+$ T cells culture help restore the long-lost proliferative function of these $CD8^+$ T cells [136].

3.2. $CD8^+$ T lymphocytes

Antigen-specific $CD8^+$ T lymphocytes ($CD8^+$ T cells) have been shown to play an important role in containment of many persistent viral infections, such as Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and also HIV-1 infection. During PHI, drop in pVL concurrently occurs with the appearance of HIV-1-specific-$CD8^+$ T cells, suggesting that these cells might possess an active antiviral property [9, 11, 137]. Studies in animal model of HIV-1 infection also support this suggestion. Depletion of $CD8^+$ T cells by administration of anti-CD8 specific antibody resulted in dramatically increased viral load in chronically SIV infected rhesus macaques [7]. And also, when $CD8^+$ T cells reappear, pVL returns into its normal level. Moreover, immune escape mutations are frequently observed in Human Leukocyte Antigen class I (HLA-I) restricted epitopes [13-15]. These studies indicate that $CD8^+$ T cells have an important role in determining viral set-point during PHI which in turn determining rate of disease

progression. Though the roles of HIV-1-specific-$CD8^+$ T cells are very obvious, it is still unclear which mechanisms or properties of these cells are correlated with effective HIV-1 control.

Canonically, antigen-specifi-$CD8^+$ T cells recognize their cognate antigens in a form of short, 8-12 amino acids long epitope which is presented on a Major Histocompatibility Complex class I (MHC-I) or Human Leukocyte Antigen (HLA) class I (in human) molecule, termed "peptide-MHC complex" (pMHC complex) on infected cells [138, 139]. After being activated by a specific binding with its cognate pMHC complex, $CD8^+$ T cells are capable to perform many effector functions including proliferation, secretion of cytokines and cytotoxic function [20, 140]. Proliferative capacity of virus-specific-$CD8^+$ T cells is very important for a long-term containment of persistent viral infection. In an HIV-1 infection, $CD8^+$ T cells from slow progressors (SP) showed an increased proliferative capacity comparing with $CD8^+$ T cells from typical progressors (TP) [141]. Finding from a recent study showing that full HIV-1 suppression by an ART treatment could not restore the proliferative capacity of $CD8^+$ T cells in TP, also supports that proliferating HIV-1-specific-$CD8^+$ T cells are vital in a long-termed HIV-1 control [142]. In addition to proliferation, upon specific antigen stimulation, $CD8^+$ T cells degranulate many pre-formed cytotoxic-granules (perforin and granzyme) and chemokines (Macrophage Inhibitory Protein-1 alpha and beta (MIP-1α, MIP-1β) and Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) and also undergo de novo synthesis of many cytokine including; Interleukin-2 (IL-2), Tumour Necrotic Factor-α (TNF-α) and Interferon-γ (IFN-γ) [143]. Perforin and granzyme both play an important role in $CD8^+$ T cell-mediated cytolytic killing of HIV-1 infected cells [141, 144, 145]. MIP-1α, MIP-1β and RANTES are C-C motif chemokines which bind to C-C receptor CCR5 [143]. Since CCR5 plays a crucial role in HIV-1 life cycle by acting as a coreceptor required during viral entry (described in part I, section 1.2.5), these natural CCR5 ligands have been shown to be in a strong correlation with non-cytolytic suppression of HIV-1 replication [146-148]. Each of those de novo-synthesized cytokine has its own unique role, acting both in a paracrine and autocrine fashions.

For instance, main function of TNF-α is to induce apoptosis cascade of both tumour cells and virus-infected cell by binding with its cognate receptor, TNF receptor I and II (TNF-RI, -RII) [149, 150]. In addition to its role as an apoptosis inducer, TNF-α is also acting as an immune response amplifier by inducing synthesis of IL-12, IL-18 and IFN-γ [143]. Despite its usage as a standard determinant of cell-mediated immune responses, IFN-γ does not have any direct effect on virus-infected cells [143]. Binding of IFN-γ to IFN-γ receptor (IFNGR) on the cell surface activates cellular antiviral state. As a result, cell undergoes many active activities which include, increasing level of TNF-R expression, promoting immunoproteasome, TAP transport protein functions and enhancement of MHC-I expression. By binding with its specific receptor (CD25), IL2 promotes proliferative response, an ability that has been shown to be preserved in HIV-1 SP and natural HIV-1 controllers [141, 151, 152]. Normally, $CD4^+$ T cells are the main source of IL-2 production *in vivo*. However, in a setting of massive $CD4^+$ T cells depletion of HIV-1 infection, an ability of $CD8^+$ T cells to synthesize IL-2 which in turn promoting their own proliferation might be of vital importance for a life-long protection against HIV-1 infection [5, 153, 154]. While there is still no definite conclusion about the characteristics of $CD8^+$ T cells that might mediate HIV-1 control, findings from several previous studies have demonstrated a strong association between functional quality of HIV-1 specific-$CD8^+$ T cells and HIV-1 control [16, 17, 19, 155, 156]. In general, the functional quality of T cells is defined by specific combination of functions that antigen-specific-T cells are able to perform in response against their specific targets [20] (details are described in part VI, section 2.3).

3.3. Antigen-specific-$CD8^+$ T cell functional assay

Cytotoxic assay is one of the most effective ways to determine the cytotoxic function of antigen-specific-$CD8^+$ T cells *in vitro*. This assay measures the amount of chromium released from chromium (^{51}Cr)-labeled target cells. However, this assay is not so practical since it requires special facility to work with radioactive-substance and can only investigate $CD8^+$ T cell responses against single epitope at a time. Development of a high-throughput ELIspot

(Enzyme-Linked Immuno Spot assay) provides us a chance to semi-quantitatively investigate T cell responses against a panel of antigenic peptides at the same time. This assay measures single cytokine production of $CD8^+$ T cells in response against antigenic peptides or optimal epitopes [157]. However, ELISpot assay only provides a quantitative data such as breadth (the number of peptides being responded) and magnitude (the number of responding cells against each peptide), both of which are highly varied among individuals and not strongly correlated with HIV-1 control [16, 18, 121]. Advances in multi-parametric flow-cytometry technology over the past decade have allowed us to simultaneously investigate several parameters, including both cellular phenotypes and functions at a single cell level [158, 159]. Moreover, recent studies have demonstrated an application of flow-cytometry in both cytotoxic assay and proliferative capacity determination, urging the important role of flow-cytometry-based assay as the universal cellular function analyzer.

3.4. Other host factors

In addition to the direct roles of both innate and adaptive immune responses, other host factors including which mostly are genetic factors. One of the most studied host genetic factors that are correlated with HIV-1 resistance is a 32-nucleotide deletion in C-C chemokine receptor CCR5 (CCR5Δ32). This mutation results in protein truncation and lost of CCR5 co-receptor function for HIV-1 entry. When compared with one another, individuals with natural CCR5 gene are more susceptible to HIV-1 infection than those with heterozygous CCR5Δ32, while those with homozygous CCR5Δ32 are the least susceptible. The frequency of this CCR5Δ32 allele has been reported to be at 10% approximately, in Caucasian population but nearly absent in Asian and African population. In Thai population, both hetero- and homozygous CCR5Δ32 alleles are not present or present at a very low frequency, if do exist [160, 161].

The other important host immunogenetic factors which have been reported to be correlated with HIV-1 clinical course is MHC-I [21, 162]. MHC-I which is also termed as HLA-I is a heterodimeric transmembrane protein, presented on the surface of all nucleated cells. HLA

class I molecules consist of a 44 kD heavy chain (alpha chain (α-chain)) which is non-covalently bound with the 12 kD beta-2-microglobulin light chain (β_2m (β-chain). The extracellular portion of the α-chain can be divided into three domains; α1, α2 and α3. The α1 and α2 domains together are forming a peptide-binding groove, a secondary structure of 8 anti-parallel β-pleated sheets floor and 2 separated α-helices. This peptide binding groove acts as a platform for the presentation of small endogenously processed self and non-self antigenic peptides. Typically, this peptide groove is able to accommodate peptides of about 8 to 11 amino acids in length. An amino acid at position 2 and at C' terminal of antigenic peptide is anchored into the floor of this groove, while the others bulge outwardly in a solvent-exposed conformation for T cell receptor (TCR) recognition. Some certain HLA-I alleles have been reported to be associated with slow HIV-1 progression, while several others with rapid progression, hence termed "protective HLA-I alleles" (PA) and "susceptible HLA-I alleles" (SA), respectively [21, 22]. There are 3 HLA-I alleles evidently regarded as "protective alleles": HLA-B27, -B57 and -B58 [21-25]. These certain HLA-I alleles are frequently presented in a unique group of HIV-1 infected individuals termed "viraemic controllers" (VC), who are able to naturally control HIV-1 replication (pVL lower than 2,000 copies/ml in the absence of ART). These associations are a result of the particular HIV-1 specific epitopes presented by them which mostly are immunogenic, functional constrained epitopes of highly conserved Gag capsid protein of HIV-1 [25-29]. HLA-B27-restricted epitope, KRWIILGLNK (KK10, gag 263-272) is highly conserved. This KK10 epitope is located within helix 7 of a mature HIV-1 capsid protein in which its proper structure is required for a multimerization of capsid protein [163]. Immune escape mutation of this epitope resulted in reduction of HIV replicative capacity [26, 27]. This high fitness cost is also observed in HLA-B57/58 restricted epitopes, such as KAFSPEVIPMF (KF11), TSTLQEQIGW (TW10) (Gag 162-172, 240-249, respectively) [28, 29]. Findings from these studies have suggested that an association observed between any certain HLA-I allele and HIV-1

protection is a result of an ability of that HLA-I allele to focus HIV-1-specific-$CD8^+$ T cell responses toward the vital, high fitness cost target.

Part IV. Rationale of this study

Therefore, we imply that natural HIV-1 control observed in HIV-1 controllers is the combined results of both higher functional quality of $CD8^+$ T cell responses and the functional constrained property of their targeting epitopes (Figure 3). HIV-1 Gag p24 protein is an ideal target for an effective immunological control of HIV-1 infection, mainly due to its highly immunogenic properties and highly conserved nature [21]. HIV-1 Gag p24 proteins from HIV-1 pre-integration complex (PIC) are abundant enough to be processed and presented before the integration of HIV-1 gRNA, hence HIV-1 infected cells can be detected and killed by Gag p24-specific-$CD8^+$ T cells, very early in the course of HIV-1 infection [164]. In addition, Gag p24 protein is the most immunogenic of all HIV-1 proteins which more than 20 times higher being produced than Pol [165, 166]. Moreover, compared to all other HIV-1 proteins, Gag p24 is the most conserved protein with only 10% of sequence variation among HIV-1 infected individuals [30]. In combination, all the properties of Gag p24 protein make it an ideal target for an effective T cells-based vaccine. In order to develop a successful vaccine, immune correlates of protection against that infection are inevitable. However, immune correlates of HIV-1 infection still remain inconclusive [4]. Findings from several studies showing that the high quality, not the quantity, of $CD8^+$ T cell responses is an immune-characteristic, preferentially observed in both HIV-1 slow progressors and HIV-1 controllers, have raised the importance of using the functional quality assay as an immune-correlate assessment [19, 133, 151, 156]. In this study, HIV-1 Gag p24-specific-$CD8^+$ T cell responses of three clinically different HIV-1 infected individuals; elite controllers (EC), viraemic controllers (VC) and typical progressors (TP) are being assessed to determine the association between the functional quality of these responses and different clinical outcomes observed between individuals. This assessment is also performed in a single epitope-specific manner, comparing between VC and TP carrying the same protective HLA-I alleles (those certain alleles which have been shown to be associated with HIV-1 control; HLA-B27 and -B57/B58 alleles). Findings from this study not only support the role of functional quality

assessment for defining HIV-1 immune correlates of protection but also provide an insight into the impacts of the single epitope-specific-$CD8^+$ T cell responses on HIV-1 clinical outcomes.

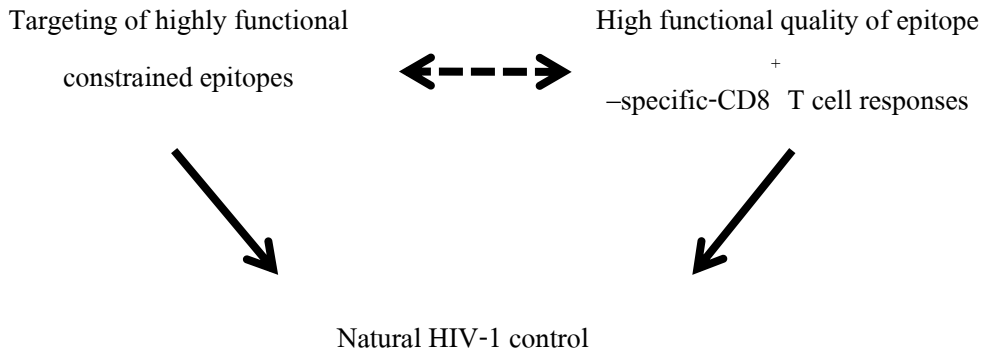


Figure 3. Schematic representative depicts the conceptual framework of an effective natural control of HIV-1 infection observed in HIV-1 controllers: Arrow-headed solid line represents a strongly evident association while double-headed dotted line depicts an inconclusive association.

CHAPTER IV

MATERIALS AND METHODS

Part I. Sample preparation

1. Study population

Total number of 45 chronic HIV-1-infected individuals, aged between 18-70 years old from King Chulalongkorn Memorial Hospital, Anonymous clinic of Thai Red Cross Society, and HIV Netherlands Australia Thailand Research Collaboration center were enrolled into this study. All donors were antiretroviral drug-naïve and had no opportunistic infections. This study was approved by an institutional review board (Human Research Ethics Committee, faculty of medicine, Chulalongkorn University) and signed consent was obtained from all the individuals before an enrolment. Each individual was categorized into three groups which were elite controllers (EC), viraemic controllers (VC) and typical progressors (TP), according to their plasma HIV load. The criteria for each group were shown in the table 1. Additional inclusion criterion for controller group is $CD4^+$ T cell counts (CD4 count) more than 450 cells/mm^3.

Table 1. HIV-1 infected individual grouping criteria

Groups	Plasma HIV load (copies/ml)
Elite Controllers (EC)	below detectable level (<40)
Viraemic Controllers (VC)	equalto/below 2,000
Typical Progressors (TP)	above 2,000

2. Peripheral blood mononuclear cells (PBMC) isolation and cryopreservation

Thirty milliliters of venous blood was collected in an ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood collection tubes (Beckton Dickinson, USA). For peripheral blood mononuclear cells (PBMC) isolation, standard density gradient centrifugation was conducted. All tubes were centrifuged at 3,000 rounds per minute (rpm) for 10 minutes at 25°C using centrifuge (Beckman Coulter, USA). Upper plasma fraction was collected and stored at -80°C for further usage. Lower cell fraction was first diluted with RPMI 1640 medium (Gibco, USA) and transferred to two 50 ml sterile polypropylene tubes (Becton Dickinson, USA) and diluted again with RPMI 1640 medium to a total volume of 35 ml each. Then, the diluted blood was transferred and overlaid upon 15 ml of Ficoll Hypaque (density gradient 1.077 g/l) (Amersham Bioscience, Sweden) in another two 50 ml sterile polypropylene tubes (Becton Dickinson, USA) and centrifuged at 1,500 rpm for 30 minutes at 25°C. Then, PBMC were harvested, transferred to new 50 ml sterile polypropylene tubes, washed twice with RPMI 1640 medium and centrifuged at 1,500 rpm for 5 minutes at 25°C centrifugation. And then, PBMC were resuspended in R10 medium (10% FBS and 90% RPMI 1640 medium). PBMC number and viability were determined using a standard trypan blue staining (Sigma-Aldrich, Germany) and a Neubauer haemocytometer (Boeco, Germany) as shown in part I, section 6. The rest of the PBMC apart from the amount required for an Interferon-γ ELISpot assay was cryopreserved using the described protocol in part I, section 3 for further usage.

3. PBMC cryopreservation

Firstly, PBMC were centrifuged at 1,500 rpm for 5 minutes at 25°C, resuspended with 500 µl of fetal bovine serum (FBS) (Bio Whittaker, USA) per $1x10^6$ PBMC and pre-cooled at 4°C for 15 minutes. And then, 500 µl of pre-cooled freezing media per $1x10^6$ PBMC consisted of 20% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and 80% FBS was drop-wisely added into PBMC suspension. PBMC suspension was transferred to a 2 ml sterile cryo-tube (Sarstedt,

Germany), and kept in a freezing container (Nalgene, USA) at -80°C for 24 hours and transferred to a liquid nitrogen tank (-196 °C).

4. PBMC thawing

Cryopreserved PBMC were thawed in a 37°C water bath until there was only a visible pea-sized ice-flake and then transferred to a 15 ml sterile polypropylene tube (Becton Dickinson, USA). RPMI 1640 medium (Gibco, USA) enriched with 10% volume/volume of FBS and 1% volume/volume of 2 mg/ml DNAse (Sigma-Aldrich, Germany) was used as thaw medium. PBMC were gently resuspended with 15 ml of thaw medium and centrifuged at 1500 rpm for 5 minutes at 25°C. After discarding the supernatant, PBMC pellet was resuspended with 15 ml of thaw medium and centrifuged at 1500 rpm for another 5 minutes at 25°C. The supernatant was then discarded and the PBMC pellet was gently resuspended with 1 ml of R10 medium with DNAse. Cells number and viability were determined using a standard trypan blue staining as shown in part I, section 6.

5. PBMC resting

Freshly isolated and cryopreserved PBMC subjected to functional quality determination were adjusted to a concentration of $1x10^6$ PBMC/ml in R10 with DNAse. Two milliliters of cell suspension were added into each well of a 24-well plate (Corning, USA) and incubated at 37°C in 5% CO_2 overnight.

6. Cell Counting

All PBMC needed to be counted were conducted as followed. Firstly, PBMC suspension was mixed with trypan blue at a ratio of 1: 20 (10 µl PBMC: 190 µl trypan blue) and loaded onto a Neubauer haemocytometer (Boeco, Germany). Only viable cells, which were without trypan blue stained, were counted in all four quadrants. Total number of viable cells was calculated using a standard formula:

Total PBMC number (cells/ml) = [(total PBMC count / 4) x 20 (dilution factor)]

x suspension volume (ml)

Part II. HLA class I typing

Genomic DNA was extracted from $1x10^6$ cryopreserved PBMC of each individual using Gentra® Puregene® kit (Qiagen, Germany) according to manufacturer's instruction. Briefly, cryopreserved PBMC were thawed according to method provided in part I, section 4 with R10 medium (without DNAse). PBMC pellet was resuspended with 200 µl of R10 medium without DNAse in a 15 ml sterile polypropylene tube (Becton Dickinson, USA). Three milliliters of cell lysis solution which was pre-supplemented with 15 µl of RNAse were added into cell suspension. Tube was inverted for 25 times and incubated at 37°C in 5% CO_2 for 15 minutes. Tube was put on ice for another 3 minutes and 1 ml of protein precipitation solution was added subsequently into cell suspension. Tube was mixed for 20 seconds and centrifuged at 2000 g at 25°C for 5 minutes. Supernatant was aspirated into a new 15 ml sterile polypropylene tube which contained 3 ml of absolute isopropanol (Merck, Germany). Tube was inverted for 50 times and centrifuged at 2000 g at 25°C for 3 minutes. After discarding the supernatant, 3 ml of 70% ethanol (Merck, Germany) were added into the tube and centrifuged at 2000 g at 25°C for another 3 minutes. Supernatant was discarded and the tube was kept at room temperature in a horizontal position for 10 minutes to air-dry the pellet. Two hundred and fifty microliters of DNA hydration solution were subsequently added into tube. Tube was mixed for 5 minutes and incubated in a 65°C water bath for an hour. After this incubation period, tube was kept overnight on a shaker-incubator at 37°C. DNA concentration was quantified by determining its absorbance at 260 nanometers (A_{260}). DNA was also checked for purity by determining an A_{260}/A_{280} ratio. Both DNA concentration and purification were determined on Nanodrop1000 using ND-1000 software version 3.7.1 (Thermo Scientific, USA). DNA was adjusted with DNAse-RNAse-free distilled water (Invitrogen, USA) to a concentration of 20 µg/ml. All 3 specific HLA class I alleles (HLA-A, -B and -C) typing were performed by Proimmune Ltd. (United Kingdom) using both PCR-sequence specific oligonucleotides (PCR-SSOP) for low resolution and PCR-sequence specific primers (PCR-SSP) for high resolution.

Part III. Complete blood count (CBC), $CD4^+$ T cell counts, $CD8^+$ T cell counts and plasma HIV-1 load quantification

1. Complete blood count (CBC)

Complete blood counts were performed on an automated hematology analyzer (Sysmex xs-1000i) (Sysmex, USA). This assay was carried out at the Immunological unit, King Chulalongkorn Memorial Hospital**,** Bangkok, Thailand.

2. $CD4^+$ T cell and $CD8^+$ T cell counts

$CD4^+$ and $CD8^+$ T cell counts were performed on flow cytometer (Beckman Coulter, USA) with the following specific fluorochrome-conjugated antibodies; FITC conjugated anti human CD45 antibody, PC5 conjugated anti human CD3 antibody and RD1 conjugated anti human CD4/CD8 antibody (Cyto-stat® Trichrome™) (Beckman Coulter, USA). This assay was carried out at the Immunological unit, King Chulalongkorn Memorial Hospital**,** Bangkok, Thailand.

3. Plasma HIV load quantification

Plasma HIV load quantifications were performed by using an *in vitro* nucleic acid amplification based COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 test (Roche, USA) on an automated COBAS® Taqman® 48 analyzer (Roche, USA). This test had the range of HIV-1 RNA detection between 40-10,000,000 copies/ml. All the quantifications were carried out at the Virological Unit, King Chulalongkorn Memorial Hospital**,** Bangkok, Thailand.

Part IV. HIV-1 *gag* p24 gene sequencing

1. HIV-1 *gag* p24 specific primer design

HIV-1 *gag* p24 gene specific primers were designed based on Thailand HIV-1 CRF_01AE wild type CM240 sequence with the accession number U5477 available from: http://www.ncbi.nlm.nih.gov. There were 3 pairs of primer for an outer polymerase chain reaction (PCR) amplification and another pair for inner amplification. Final PCR product was 932 nucleotides long and covered from position 640 to 1571 of full-length HIV sequence, CM240. All primer sequences were shown in table 2.

Table 2. HIV-1 *gag* p24 gene amplification primers

Primer names	Forward primers (5'-3')	Reverse primers (5'-3')
Outer1	GAGGTGCACACAGCAAGAGGCG	CCCCCTATCATTTTTGGTTTCC
Outer2	AAATCTCTAGCAGTGGCGCCCGAACAG	TAACCCTGCGGGATGTGGTATTCC
Outer3	GCGRCTGGTGAGTACGCC	RGGAAGGCCAGATYTTCC
Inner	GACACCAAGGARGCTTTRGA	GGGGCCCTGCAATTTCTGGC

2. Amplification of HIV-1 *gag* p24 gene

Viral RNA was extracted from fresh or cryopreserved plasma using High Pure® Viral RNA kit (Roche, USA) according to manufacturer's instruction. In brief, 200 µl of plasma were mixed with 400 µl of binding buffer and 4 µl of poly-A tail in a 1.5 ml DNAse-RNAse-free microcentrifuge tube (Axygen, USA). Tube was mixed and incubated at room temperature for 20 minutes. Plasma mixture was aspirated into a spin column and centrifuged at 11,000 rpm for 30 seconds. Five hundred microliters of inhibitor removal buffer were added into column and centrifuged at 11,000 rpm for 1 minute. For washing, 450 µl of wash buffer were added into column and centrifuged at 11,000 rpm for 1 minute twice. Column was subsequently centrifuged at 14,000 rpm for 1 minute to remove all remaining wash buffer. Filter-tube was put in a new 1.5 ml DNAse-RNAse-free microcentrifuge tube. To elute RNA, 30 µl of elution buffer were added into column and centrifuged at 11,000 rpm for 1 minute. Extracted RNA was either used immediately or aliquot at 10 µl into 200 µl DNAse-RNAse-free microcentrifuge tubes (Axygen, USA) and kept at -80 °C until use. All the polymerase chain reactions (PCR) were performed in a thermocycler (Eppendorf, Germany). HIV-1 *gag* p24 gene was amplified in a nested PCR reaction. Firstly, RNA was reverse transcribed and amplified using a one-step reverse transcription-polymerase chain reaction kit (RT-PCR) (Qiagen, Germany) in 3 separated RT-PCR reactions. These 3 outer RT-PCR reactions used different sets of primer which were shown in table2. All reactions were performed in 50 µl reaction mixtures which consisted of 10 µl of 5X Qiagen OneStep RT-PCR buffer, 2.0 µl of 10 µM dNTP (Deoxyribonucleotide triphosphate), 3 µl of Qiagen OneStep RT-PCR enzyme mix, 0.6 µM each outer primer-pair and 3 µl of RNA template. All 3 RT-PCR reactions with different primer-pair were performed simultaneously with the following condition; reverse transcription at 50ºC for 30 minutes, DNA polymerase activation at 95ºC for 15 minutes, 35 cycles of DNA amplification at 94ºC for 30 seconds, 54ºC for 30 seconds and 72ºC for 2.25 minutes and 68 ºC for 20 minutes for final extension. PCR products from these 3 RT-PCR reactions were mixed together and used as a template for an inner

amplification reaction later. Inner PCR reaction was also performed in a 50 µl reaction mixtures with the following reagents; 5 µl of 10X Titanium® Taq buffer, 1.0 µl of 10 µM dNTP (Fermentas, Canada), 1 µl of Hot Start Titanium® Taq DNA Polymerase (ClonTech, USA), 0.6 µl of inner primer-pair (Table 2) and 2 µl of outer-reaction PCR products mixture. The condition for inner DNA amplification was as followed by DNA polymerase activation at 94 ºC for 1 minute, 35 cycles of DNA amplification at 94ºC for 1 minute, 58ºC for 1 minute and 68ºC for 1 minute and a final extension at 68ºC for another 10 minutes. HIV-1 *gag* p24 PCR products from this nested-PCR reaction were checked for a positive band by gel electrophoresis using 1% of agarose gel (Research Organics, USA) (Figure 4). All PCR products were purified by using a QIAquick® PCR purification kit (Qiagen, Germany) according to manufacturer's instruction. In brief, PCR product was mixed with 250 µl of PBI buffer in a 1.5 ml DNAse-RNAse-free microcentrifuge tube. Mixture was aspirated into a spin column and centrifuged at 13,000 rpm for 1 minute. After discarding the flow-through suspension, 750 µl of PE buffer were added into mixture and centrifuged at 13,000 rpm for 1 minute. After discarding the flow-through, column was centrifuged at 14,000 rpm for 1 minute to remove all the leftover. Spin column was put in a new 1.5 ml DNAse-RNAse-free microcentrifuge tube. To elute purified PCR product, 50 µl of EB buffer were added into column, incubated at room temperature for 1 minute and centrifuged at 13,000 rpm for 1 minute. PCR products were sent to AIT biotech (Singapore) for nucleotide sequencing with the same inner primer-pair. All sequences were analyzed using Bioedit Sequence Alignment Editor version 7.0.9.0.

Figure 4. HIV-1 Gag p24 PCR amplification products

Representative picture of gel electrophoresis of amplified HIV-1 Gag p24 gene from donors' plasma HIV-RNA is shown. A-F are code-names for each individual sample. Bp is 100 base-pair marker.

Part V. HIV-1 Gag p24 overlapping peptides

1. HIV-1 Gag p24 overlapping peptide design

Ten frozen plasma samples were randomly selected from Virological unit of King Chulalongkorn Memorial Hospital, Bangkok. These samples were from chronically HIV-1 infected individuals who came to routinely check up their plasma HIV load between January-June 2008. All frozen plasma samples were extracted for viral RNA which were amplified and sequenced for HIV-1 *gag* p24 gene as described in part II, section 2. These nucleotide sequences were translated into amino acids using the Expert **P**rotein **A**nalysis **Sy**stem proteomics server (ExPASy) which were subsequently aligned and determined for their consensus sequence using Bioedit Sequence Alignment Editor version 7.0.9.0. This amino acid sequences alignment and consensus sequence were shown in figure 5. Then, the consensus amino acid sequence was used as a template for a HIV-1 Gag p24 overlapping peptide design on PeptGen tool available from: http://www.hiv.lanl.gov. Each peptide was 20 amino acids long and overlapped by 10 amino acids with each other. There were 23 overlapping peptides spanning whole HIV-1 Gag p24 protein as depicted in figure 6. And, 7 additional HLA-B27, -B57 and -B58 restricted epitopes were also defined according to their position located in database available from: http://www.hiv.lanl.gov/ content/immunology. All peptides were synthesized by Mimotopes (Australia). All the overlapping peptides and epitopes were listed in a table 3.

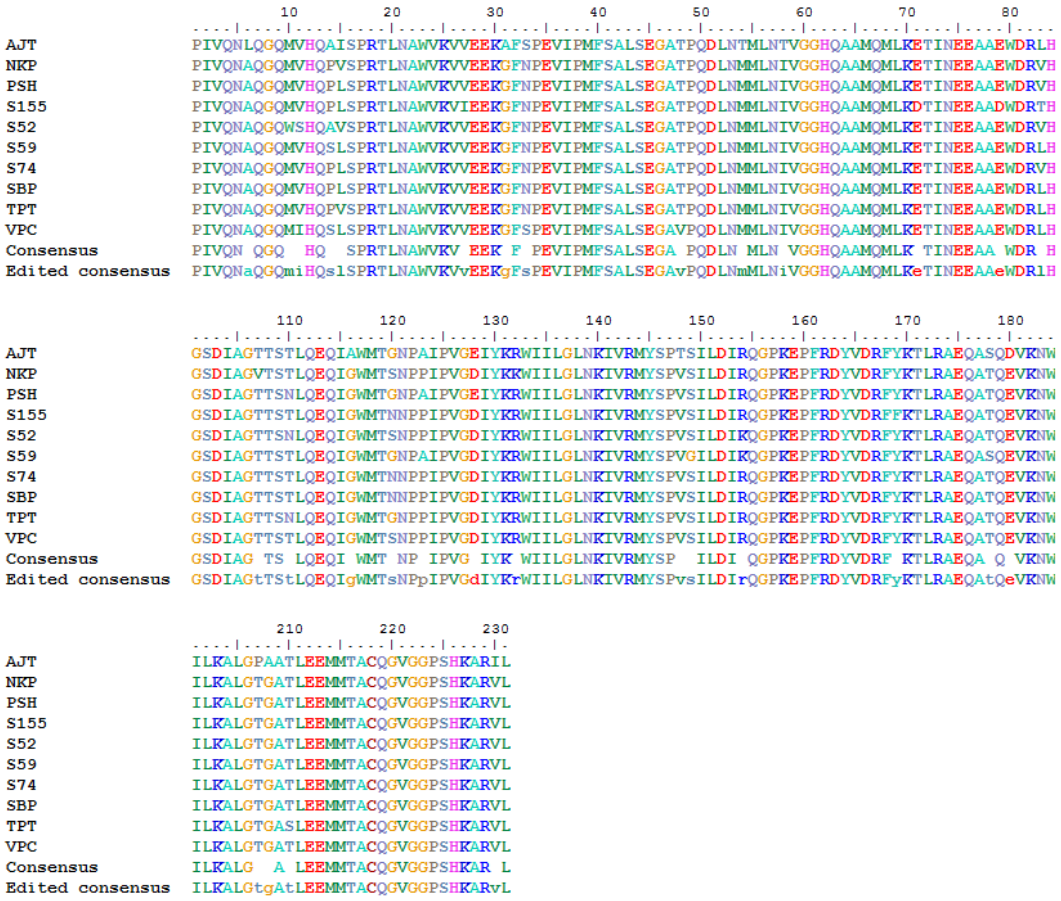


Figure 5. HIV-1 Gag p24 amino acid sequences alignment and consensus sequence. Amino acid sequences derived from PCR products of 10 randomly selected chronic HIV-1 infected donors (AJT, NKP, PSH, S155, S52, S59, S74, SBP, TPT and VPC). Consensus sequence was generated with a gap to identify polymorphic residues between 10 sequences. Edited consensus sequence was a consensus sequence in which the gaps were filled with the most frequent amino acid residue of all 10 sequences. Sequences alignment and consensus sequence were analyzed and edited on Bioedit Sequence Alignment Editor version 7.0.9.0.

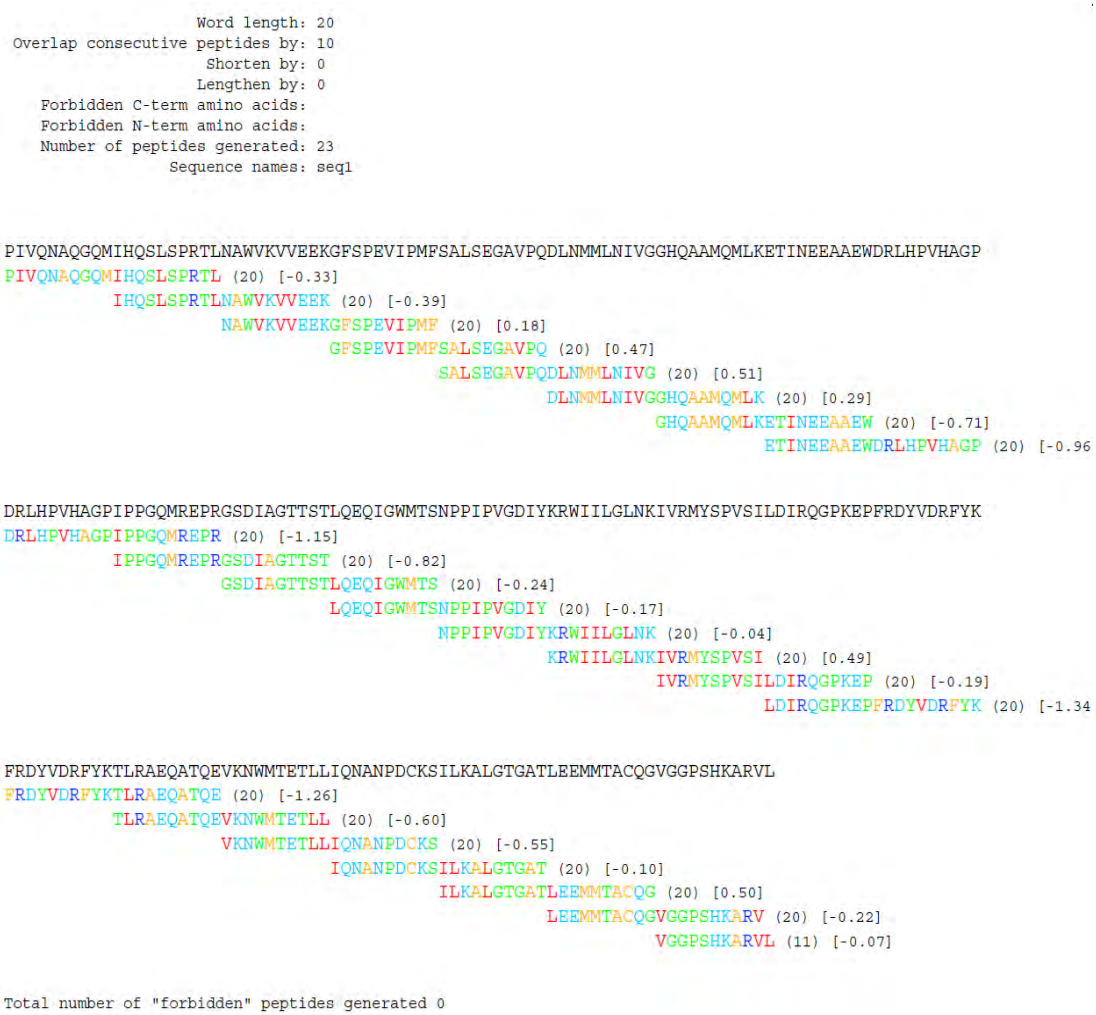


Figure 6. Design of HIV-1 Gag p24 overlapping peptides. A total number of 23 peptides were generated by using PeptGen tool available from: http://www.hiv.lanl.gov. Each peptide was 20 amino acids long and overlapped by 10 amino acids with each other.

Table 3. HIV-1 Gag p24 overlapping peptides and HLA-B*27, -*57 and -*58 restricted epitopes

Peptide names	Amino acid sequences
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
KK10	KRWIILGLNK
LW9	LSPRTLNAW
KF11	KGFNPEVIPMF
EW10	ETINEEAAEW
QW9	QATQEVKNW
GM9	GTGATLEEM
TW10	TSTLQEQIGW

2. Peptide preparation

All lyophilized peptide powders were prepared according to manufacturer's recommendation available from: http://www.mimotopes.com. Briefly, a lyophilized peptide powder was first dissolved with 20 µl of absolute DMSO (Sigma-Aldrich, Germany). Ten microliters of peptide solution were then pipette into a 2 ml cryo-tube (Sarstedt, Germany) and diluted with 490 µl of sterile PBS (Sigma-Aldrich, Germany). The concentration of peptide in sterile PBS was quantified using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA) according to the manufacturer's instructions. Briefly, albumin (bovine serum albumin, BSA) standards were prepared to cover 8 concentrations, ranging from 25-2,000 µg/ml. Working reactions (WR) were prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50: 1, Reagent A: B) at which the required volume was calculated according to the formula:

Total volume WR required = (# standards + # unknowns) × (# replicates) × (volume of WR per sample)

There are 30 peptides and 9 standards (8 standards + 1 blank), so the required total WR volume was 7,800 µl (7800 µl of reagent A and 156 µl of reagent B). Subsequently, 10 µl each of standard and peptide were mixed with 200 µl of WR in a 1.5 ml DNAse-RNAse-free microcentrifuge tube. All tubes were incubated in 37°C water bath for 30 minutes and cooled at room temperature. All samples were aspirated into a cuvette and measured for the absorbance at 562 nm (A_{562}) using SmartSpec™ 3000 spectrophotometer (Bio-Rad, USA). Standard curve was prepared by plotting the average blank corrected A_{562} measurement for each standard versus its concentration in µg/ml. Protein concentration of each peptide was determined by using the prepared standard curve. All peptides were adjusted to a concentration of 1 mg/ml (stock concentration) or 200 µg/ml (working concentration) using sterile PBS. Stocked peptides were kept at -80°C to prevent protein degradation. Fifty microliters of working peptides were aliquot into 1.5 ml DNAse-RNAse-free microcentrifuge tube and kept at 4°C for daily usage.

Part VI. Determination of HIV-1 Gag p24-specific-CD8$^+$ T cell responses

1. Interferon-γ (IFN-γ) Enzyme Linked Immunosorbent Spot (ELISpot) assay

In order to determine an area on HIV-1 Gag p24 protein that was targeted by HIV-1 infected individuals' T cells, IFN-γ ELISpot assay was performed. Polyvinylidene difluoride plate (PVDF) (Millipore, USA) was coated with 15 µg/ml anti-human interferon-gamma monoclonal antibody 1-D1K (Mabtech, Sweden) in 100 µl of sterile PBS (Sigma-Aldrich, Germany) at 37°C in 5% CO_2 for 3 hours. The plate was washed 6 times with sterile PBS and blocked with R10 medium at room temperature, dark for an hour. After tapped the blocked media out, 100 µl of PBMC suspension which was pre-adjusted to a concentration of $2.5x10^6$ PBMC/ml with R10, was added into each well of the plate. Five microliters of 200 µg/ml overlapping peptides were added into each well in duplicate. Negative control well contained only $2.5x10^6$ PBMC/ml in 100 µl R10 medium. Phytohemagglutinin-A (PHA-A) (Sigma-Aldrich, Germany) at a final concentration of 10 µg/ml was used as a positive control. Recombinant pp65 protein (65 kilo dalton lower matrix phosphoprotein) of Human Cytomegalovirus (HCMV) (Miltenyi Biotech, Germany) was also used as other viral specific responses control at 2 µl per $2.5x10^5$ PBMC. The ELISpot plate was incubated at 37°C in 5% CO_2 for 15 hours. The following day, plate was washed 6 times by PBS with 0.05% Tween-20 (Sigma-Aldrich, Germany) then washed another 1 time with PBS. Fifty microliters of 1 mg/ml anti-human interferon-gamma monoclonal antibody 7-B6-1-Biotin (Mabtech, Sweden) were added to each well, the plate was incubated at 37°C in 5% CO_2 for 3 hours and washed as above. Fifty microliters of 1 mg/ml streptavidin-conjugated alkaline phosphatase (Mabtech, Sweden) were added to each well and then incubated for another 1 hour at 37°C in 5% CO_2. The plate was washed as above and developed colour by adding alkaline phosphatase conjugate substrate kit (Biorad, USA). The plate was kept at room temperature, dark for 5 minutes, and then washed 10 times with tap water and allowed to dry at room temperature overnight. An automated ELISpot reader (Karl-Zeiss, USA) was used to count

the number of the spot and calculated as spot forming units (SFU) per 10^6 PBMC. Positive well was one with three times spot above negative well and was above 75 SFU/10^6 PBMC.

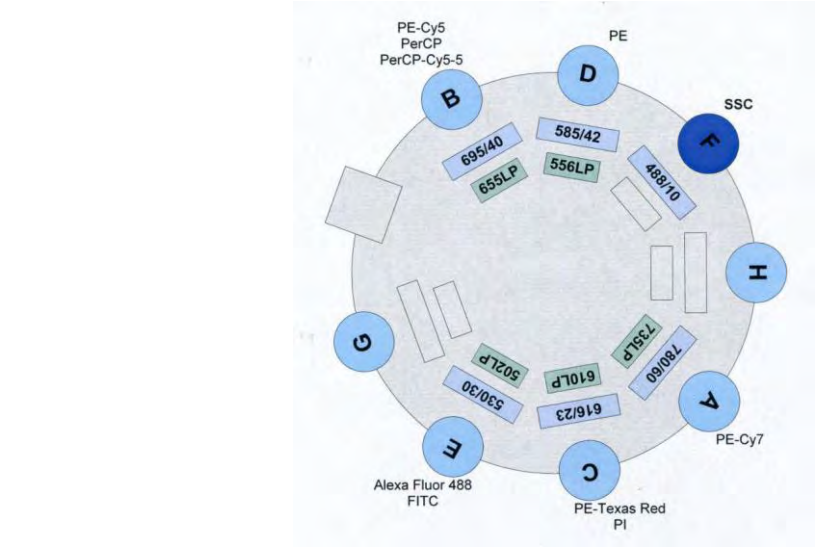
2. Functional quality determination by Intracellular Cytokine Staining (ICS) assay

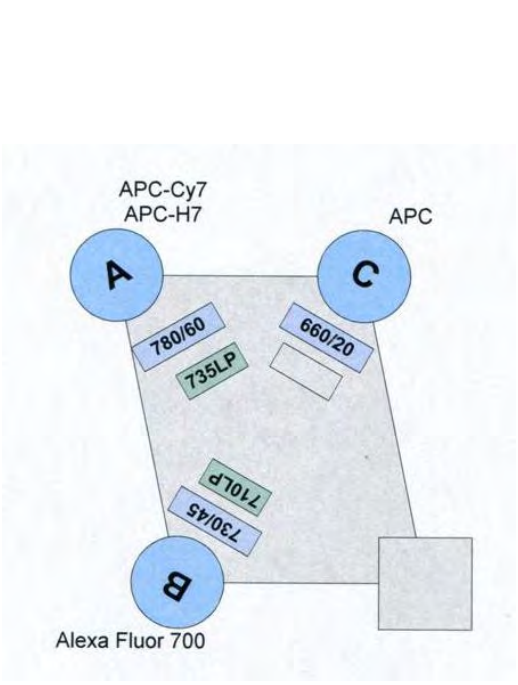
2.1. Flow-cytometer configuration

Multiple-parameter flow-cytometry analysis in this study was performed on a flow-cytometer (FACS Aria II) (Becton Dickinson, USA). Flow-cytometer configuration was shown in figure 7.

2.2. Antibody selection and titration

Both extracellular and intracellular staining antibodies used in this study were selected according to flow cytometer configuration. The antibodies used in the functional quality determination of HIV-1 Gag p24 specific $CD8^+$ T cell responses were shown in table 4. All antibodies were first titrated to define the optimal concentration which was the concentration that provided the maximal separation between positive and negative population (Figure 8). The titration series of all antibodies were started from a reference concentration recommended by manufacturer and continued with 6 to 8 two-folded serial dilution. The antibody titration steps were performed under the same condition as the assay condition part VI, section 2.3 using fresh PBMC from HIV-1 seronegative healthy donors. Staphylococcus enterotoxin B (SEB) (Sigma-Aldrich, Germany) was used as a stimulator instead of peptide for the titration step.





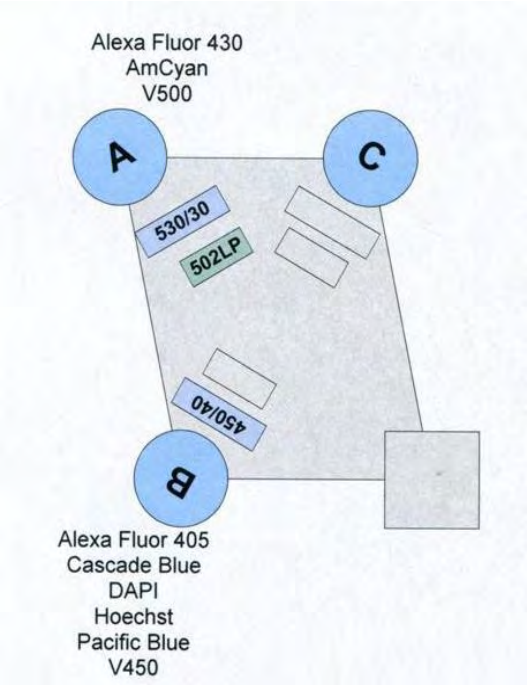


Figure 7. Configuration of FACS Aria II system used in this study. Figure showed an octagon® and trigon® configuration in which fluorescent signal that was emitted from conjugated antibodies was selected/reflected by dichroic filters (indicated with its specific wavelength) and detected by their compatible detectors. Upper octagon was for blue laser (488 nm), lower left was for red laser (633 nm) and lower right was for violet laser (405 nm) excited signals. Fluorochromes indicated next to detectors were the fluorochromes that compatible with their given detector.

Table 4. Monoclonal antibodies used for functional quality determination of HIV-1 Gag p24-specific-CD8$^+$ T cell responses

Markers	Fluorochromes	Companies	Volumes (µl)
CD3	APC-H7	Beckton Dickinson, USA	2.5
CD8	Pacific blue	Biolegend, USA	1.25
CD107a	PE-Cy5	Biolegend, USA	1.25
IL-2	FITC	Biolegend, USA	20
TNF-α	APC	Biolegend, USA	5
IFN-γ	PE-Cy7	Biolegend, USA	5
MIP1-β	PE	Beckton Dickinson, USA	0.5

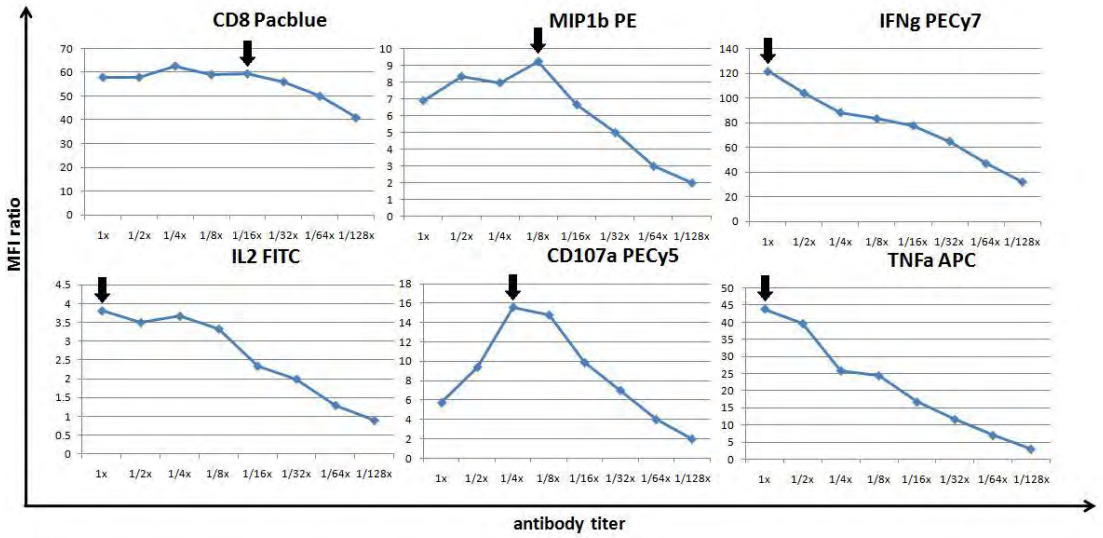


Figure 8. Titration curve of each antibody. Titration curve at which the MFI ratio (positive population MFI/negative population MFI) on y-axis was plotted against antibody titers on x-axis. Black arrow indicated peak MFI ratio of each antibody.

2.3 Functional quality determination of HIV-1 Gag p24-specific-CD8$^+$ T cell responses

Overnight-rested PBMC were collected from 24-well plate and centrifuged at 1500 rpm for 5 minutes at 25°C. After discarding the supernatant, cell pellet was resuspended with R10 medium and counted as described in section 2.1.5. Overnight-rested PBMC were then adjusted to a concentration of 10^7 PBMC/ml with R10 medium and aliquoted at 100 µl into 5 ml polystyrene round-bottom tubes (Becton Dickinson, USA) for each HIV-1 Gag p24 peptide-specific responses including 3 additional control tubes. These controls were composed of 1 positive control (SEB), 1 negative control (10% DMSO) and 1 irrelevant peptide control (MERIKELRDL, polymerase protein 2 of Influenza A virus). Co-stimulatory mixture was prepared as shown in table5 and aliquoted at 100 µl into each PBMC containing tube. PBMC were then stimulated with the IFN-γ ELISpot responding peptide or irrelevant peptide at a final concentration of 10 µg/ml. Final concentration of SEB at 0.5 µg/ml and DMSO at 0.1% was added into a positive and negative control tube, respectively. All tubes were incubated at 37°C in 5% CO_2 for 6 hours. Then, PBMC were centrifuged at 860 g for 4 minutes at 25°C and flicked to decant the supernatant. PBMC were washed with 500 µl of ICS wash solution (sterile PBS supplemented with 1% vol/vol FBS and 0.02% vol/vol sodium azide), and centrifuged again at 863 g for 4 minutes at 25°C. Supernatant was decanted, 100 µl of surface staining antibodies mixture (Table 6) were added into each tube and incubated at 37°C in 5% CO_2 for 20 minutes. PBMC were washed with 500 µl of ICS wash solution, centrifuged at 860 g for 4 minutes at 25°C, flicked to decant the supernatant and then repeated with another round of these washing steps. PBMC were then permeabilized with 250 µl of Cytofix-Cytoperm reagent (Becton Dickinson, USA) and incubated at 4°C for 20 minutes. PBMC were washed with 1 ml of 1X Perm/Wash solution (Becton Dickinson, USA), centrifuged at 860 g for 4 minutes at 25°C, flicked to decant the supernatant and then repeated with another round of these washing steps. PBMC were then stained with 100 µl of intracellular staining antibodies mixture (Table 7) and incubated at 37°C in 5% CO_2 for 30 minutes. PBMC were washed with 1 ml of 1X Perm/Wash

solution, centrifuged at 860 g for 4 minutes at 25°C, flicked to decant the supernatant and then repeated with another round of these washing steps. PBMC were resuspended with 200 µl of 1% Paraformaldehyde (PFA) (Sigma-Aldrich, Germany) and kept at 4ºC until analysis. HIV-1 Gag p24-specific-$CD8^+$ T cell responses were analyzed on a flow-cytometer (Becton Dickinson, USA) using BD FACSDiVa software version 6.1.3 (Becton Dickinson, USA). Each sample was acquired until 10^6 events were achieved.

Table 5. Co-stimulator mixture

Reagents	Volume (1x)
Anti-human CD28 (Beckton Dickinson, USA)	0.2 µl
Anti-human CD49d (Beckton Dickinson, USA)	0.2 µl
Brefeldin A (10 mg/ml) (Sigma-Aldrich, Germany)	0.2 µl
CD107a-conjugated anti-human PE-Cy5 monoclonal antibody (Biolegend, USA)	1.25 µl
R10 medium	98.15 µl

Table 6. Surface staining antibodies mixture

Reagents	Volumes (1x)
APC-H7-conjugated anti-human CD3 monoclonal antibody (Beckton Dickinson, USA)	2.5 µl
Pacific blue-conjugated anti-human CD8 monoclonal antibody (Biolegend, USA)	1.25 µl
R10 medium	96.25 µl

Table 7. Intracellular staining antibodies mixture

Reagents	Volume (1x)
FITC-conjugated anti-human IL-2 monoclonal antibody (Biolegend, USA)	20 µl
APC-conjugated anti-human TNF-α monoclonal antibody (Biolegend, USA)	5 µl
PE-Cy7-conjugated anti-human IFN-γ monoclonal antibody (Biolegend, USA)	5 µl
PE-conjugated anti-human MIP1-β monoclonal antibody (Beckton Dickinson, USA)	0.5 µl
1X Perm/Wash solution (Beckton Dickinson, USA)	69.5 µl

3. Functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell analysis

To identify HIV-1 Gag p24-specific-$CD8^+$ T cell population, PBMC were gated on the lymphocyte subset (side scatter area (SSC-A) on y-axis versus forward scatter area (FSC-A) on x-axis), followed by gating on CD3 positive population (CD3-APC-H7 area (CD3-APC-H7-A) on y-axis versus forward scatter area (FSC-A) on x-axis) and subsequently gated on double CD3-CD8 positive population (CD3-APC-H7 area (CD3-APC-H7-A) on y-axis versus CD8 Pacific blue area (CD8-Pacific blue-A) on x-axis) (Figure 9). To identify each function performed by responding HIV-1 Gag p24-specific-$CD8^+$ T cells, $CD3^+CD8^+$ cells with each function were gated versus FSC-A (FSC-A on y-axis versus IL-2 FITC-A or TNF-α APC-A or IFN-γ PE-Cy7-A or CD107a PE-Cy5-A or MIP-1β PE-A on x-axis versus) (Figure 10). Positive population cutoff of all 5 functions was set according to the background level observed from negative control tube of each individual. To determine the percentage of all 32 functionally distinct population of HIV-1 Gag p24-specific-$CD8^+$ T cells, Boolean gating algorithm was applied by using FCOM subset analysis module on WinList software version 6.0 (Verity Software House, USA). FCOM equation was as followed:

FCOM (G8, G9, G10, G11, G12) + 0.5)*1024/32+FRND(2); in which

FCOM = name of the algorithm

G8-12 = each gated population of interest in which;

G8 = $CD3^+CD8^+$ IL-2^+

G9 = $CD3^+CD8^+$ TNF-α^+

G10 = $CD3^+CD8^+$ IFN-γ^+

G11 = $CD3^+CD8^+$ $CD107a^+$

G12 = $CD3^+CD8^+$MIP-1β^+

0.5 = Value indicated that the first population was offset from the axis (it doesn't start at 0)

1024 = Value indicated that how many channels the populations should be spread. Value at 1024 was chosen according to a resolution of 1024 channels, as suggested by software programmer.

/32 = This value indicated the number of possible combination resulted from Boolean gating algorithm. In this case, there were 5 gating population so total number of possible combination = $2^5 = 32$.

FRND(2) = This value was a random number added to the FCOM peak position to create a Gaussian distribution. Having a Gaussian distribution allows us to more easily see the population and apply color. As the number of gating populations was increased, this number needed to be reduced to prevent overlap. The value of 2 was chosen according to software programmer suggestion.

All data from peptide stimulated PBMC were subsequently corrected with data from a negative control and an irrelevant peptide control to obtain true positive population of responding HIV-1 Gag p24-specific-$CD8^+$ T cells. By using $CD8^+$ T cell counts obtained from a complete blood count (CBC) data, an absolute number of each subset of all 32 functionally distinct populations was calculated using the following equation:

Absolute number of responding $CD8^+$ T cells = Percentage of responding $CD8^+$ T cells

x $CD8^+$ T cell counts

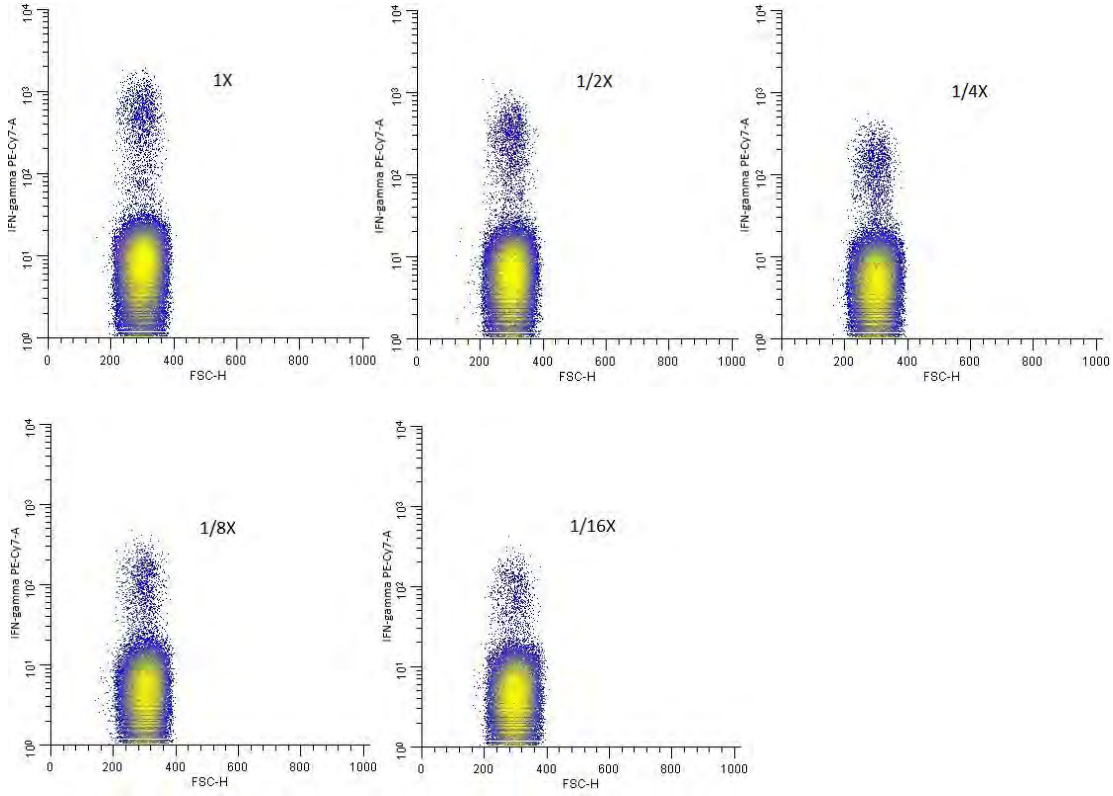


Figure 9. Comparison of dot plots from the same antibody with different titer. Each plot of IFN-γ PE-Cy7 intensity (y-axis) versus forward scatter (x-axis) was compared for separation between positive populations and negative populations. Antibody titer of each plot was indicated on the top of the plot. Background level was similar in every antibody titers. The maximum separation was observed at antibody titer of 1X.

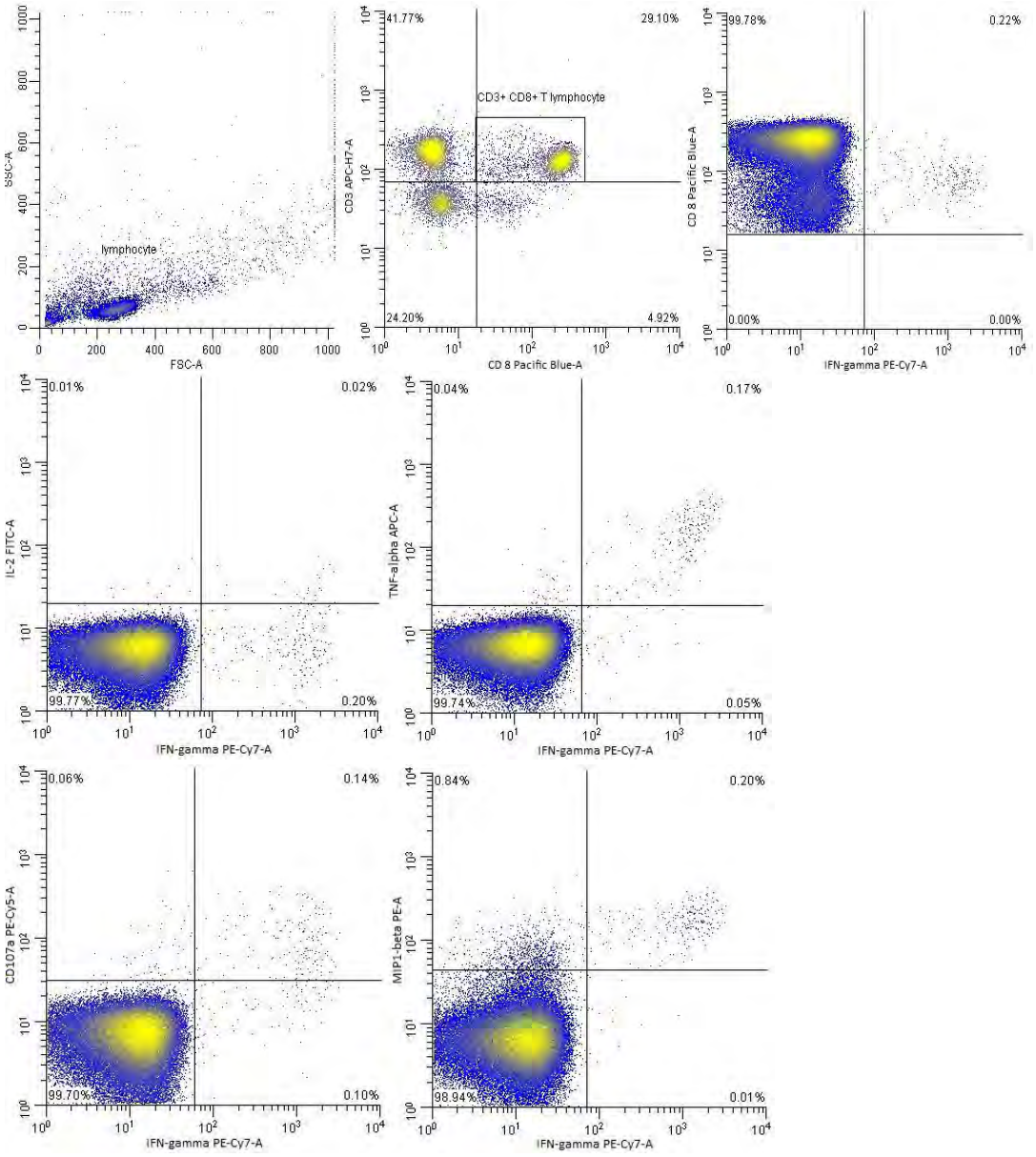


Figure 10. Gating strategy for identification of high functional quality responding CD8$^+$ T cells. Plots shown were a representative data from HLA-B58 restricted QW9 specific CD8$^+$ T cell response from subject HN30, a viraemic controller. Upper column showed a gating scheme for CD3$^+$CD8$^+$ T cell population. The upper rightmost plot and 4 lower plots showed a gating scheme for CD3$^+$CD8$^+$ T cells responded with IFN-γ, IL-2, TNF-α, CD107a and MIP-1β, respectively.

Part VII. Statistical analysis

All statistical analyses were performed using Prism version 5 (Graphpad Software). Two-tailed, Mann-Whitney U test was used to compare between 2 groups and Spearman R test was used to determine correlation and p value < 0.05 was considered statistic significance.

CHAPTER V

RESULTS

Part I. Demographic data of the study population

From 45 chronic HIV-1 infected individuals enrolled in this study, there were 3 elite controllers (EC), 10 viraemic controllers (VC) and 32 typical progressors (TP). Their demographic data was summarized in table 8. There were 21 female and 24 male individuals in this group at age between 18-66 years old and a median of 30 years old. Their HIV-1 duration was from 6 months to 13 years with a median of 3 years. Their $CD4^+$ T cell count (CD4 counts) ranged from 126 to 1,319 cells/mm^3 with a median of 493 cells/mm^3. Their $CD8^+$ T cell count (CD8 count) ranged from 382 to 2,232 cells/mm^3 with a median of 942 cells/mm^3. Their plasma HIV-1 load (pVL) ranged from <40 to 1,132,883 copies/ml with a median of 9,165 copies/ml.

Part II. Human Leukocyte Antigen class I (HLA-I) of the study population

Specific Human Leukocyte Antigen class I (HLA-I) alleles of each individual enrolled in this study were shown in table 9. The 4 most frequent HLA-A alleles were HLA-A*11 (60%), followed by A*24 (42.5%), A*02 (25%) and A*33 (20%) (Figure 11A). For HLA-B alleles, HLA-B*15 (35%) was the most frequent allele which followed by B*40 (30%), B*27 (20%) and B*58 (20%) (Figure 12A). HLA-C*07 (45%) was the most frequent HLA-C alleles, followed by C*03 (35%), C*08 (27.5%) and C*01 (22.5%) (Figure 13A). Interestingly, when the frequency of HLA-I alleles were determined with a high typing-resolution (4 digits designation), there were some changes in allelic ranking. For HLA-A alleles, the two most frequent alleles were still the same; HLA-A*1101 (52.5%) and A*2402 (25%). Instead of A*02 allele, HLA-A*3303 (17.5%) became the third frequent allele (Figure 11B). This was partly due to many specific HLA protein (4 digits designation) observed in HLA-A*02 types; HLA-A*0201 (5%), A*0203 (7.5%), A*0205 (7.5%) and A*0207 (5%) (Figure 12B). HLA-B*1502 (22.5%) still remained the most frequent HLA-B alleles. The second most frequent alleles were changed to HLA-B*5801 (20%), followed by B*4601 (17.5%) and B*4001 (15%) (Figure 13B). HLA-B*27 consisted of 3 specific alleles; HLA-B*2704 (12.5%), B*2705 (2.5%) and B*2706 (5%). Similarly, HLA-B*40 also consisted of 3 specific alleles; HLA-B*4001 (15%), B*4002 (7.5%) and B*4006 (7.5%). For HLA-C alleles, the most frequent alleles were still HLA-C*0702 (40%), followed by HLA-C*0801 (27.5%), C*0102 (22.5%) and C*0302 (17.5%) (Figure 14B). This change in ranking of HLA-C allele frequency was also due to the presence of multiple specific HLA proteins (4 digits) (Figure 14B) in a single HLA type (2 digits) (Figure 14A).

Table 8. Demographic data of the study population

	ID	Sex	Age	Time since seroconversion (years)	CD4 counts (cells/mm^3)	CD8 counts (cells/mm^3)	pVL (copies/ml)
EC	HN5	F	28	7	834	942	<40
	HN10	F	38	5	670	507	<40
	HN31	M	52	10	470	745	<40
VC	HN1	M	18	1	878	na†	1,999
	HN2	M	26	3	1,169	1,814	1,175
	HN9	M	43	9	639	na†	936
	HN12	M	27	1	495	791	1,549
	HN15	M	30	3	578	1,388	742
	HN20	F	24	3	493	1,104	1,034
	PRT	M	19	1	551	633	1,123
	HN24	F	28	9	699	810	151
	HN26	M	51	na†	735	780	1,318
	HN30	M	34	3	1,319	2,232	165
TP	JSM	F	36	2	265	1,348	6,569
	NOT	M	27	4	195	624	16,925
	VKJ	F	29	3	381	1,313	154,253
	SUL	M	45	4	493	896	5,385
	RSR	F	42	4	187	974	503,411
	KRR	F	37	6	126	743	88,597
	SYY	F	49	12	368	1,104	9,540
	HN21	M	44	3	881	1,222	299,077
	PNN	F	33	10	319	2,008	5,728
	HN16	M	21	1	561	1,148	12,231
	HN22	M	26	1.5	754	785	4,612
	CHL	F	66	13	448	na†	3,993

	ID	Sex	Age	Time since seroconversion (years)	CD4 counts (cells/mm^3)	CD8 counts (cells/mm^3)	pVL (copies/ml)
TP	NKM	M	37	12	173	na†	112,042
	TBT	M	21	2	185	na†	4,973
	PPK	F	40	4	219	na†	43,061
	UKY	F	33	8	249	na†	309,949
	HN8	F	36	1	659	na†	230,942
	HN17	F	28	1	325	382	10,961
	HN18	F	30	1	385	823	51,900
	HN19	M	26	2	519	1,338	17,313
	HNN5	M	29	2	375	1,259	27,487
	HNN7	M	23	1	525	1,003	30,145
	HN3	F	24	1	452	521	3,307
	HN7	M	23	3	540	582	12,501
	HN11	F	58	5	450	865	3,094
	HN14	F	28	1	551	694	32,744
	HN23	M	35	1	427	1,029	3,312
	HNN4	F	34	5	187	550	1,132,883
	HN25	F	27	0.5	622	1,172	5,689
	HN27	M	24	0.5	855	1,489	8,790
	HN28	M			490	979	58,523
	HN29	M			428	663	31,329

na† Not available

Table 9. HLA class-I alleles of all the enrolled HIV-1 infected individuals

	ID	HLA-A alleles		HLA-B alleles		HLA-C alleles	
	HN5	2402	1101	1502	4001	0303	0801
EC	HN10	2402	1101	5401	4001	0102	0304
	HN31	3201	6802	3502	5301	0401	0401
	HN1	2402	1101	1301	2705	0202	0403
	HN2	0302	1101	0801	2706	0304	0702
	HN9	1101	3101	3501	4001	0303	0702
	HN12	0101	1101	5701	1801	0602	0701
VC	HN15	0206	1102	2704	4006	0801	1202
	HN20	1101	1101	1502	4601	0102	0801
	PRT	1101	1102	5201	2704	0702	1202
	HN24	0101	0206	5701	4002	0304	0602
	HN26	0203	1101	4601	4601	0102	0102
	HN30	0101	3101	5101	5701	0501	0602
	JSM	1101	2410	1802	2704	na†	na†
	NOT	0206	1102	2704	3701	0602	1202
	VKJ	1101	3303	1301	5801	na†	na†
	SUL	1101	3303	1502	5801	0302	0801
	RSR	3101	3303	5102	5801	0302	1502
	KRR	2402	3303	3802	5801	0302	0702
TP	SYY	3303	3303	5801	5801	0302	0302
	HN21	2601	1102	3901	2704	0702	1202
	PNN	2402	3303	2706	5801	0302	0702
	HN16	1101	3303	5801	4001	0302	0702
	HN22	0101	1101	5201	5701	0602	0702
	CHL	0201	0207	5201	5603	0102	1202
	NKM	2402	2402	1501	4002	0401	0702
	TBT	0203	1101	4001	4001	0304	0702

	ID	HLA-A alleles		HLA-B alleles		HLA-C alleles	
	PPK	2407	2407	3505	4601	na†	na†
	UKY	1101	1101	1301	4601	0102	0406
	HN8	0201	1101	1502	4601	0102	0801
	HN17	0203	0207	1525	4002	0304	0702
	HN18	0207	2407	1502	4601	0102	0801
	HN19	0207	0207	4001	4601	0102	0702
	HNN5	2402	2407	1502	3901	0403	0801
TP	HNN7	2402	1101	5401	4006	0102	0702
	HN3	2407	1103	1502	3701	0602	0801
	HN7	2407	3401	1502	1535	0702	0801
	HN11	3401	3001	1302	1535	0602	0702
	HN14	2402	1101	1502	1502	0801	0801
	HN23	2402	6801	1505	1513	0801	1602
	HNN4	2410	3301	5801	1802	0302	0704
	HN25	1101	1101	3802	4006	0702	1502
	HN27	0203	3301	3802	3503	1203	0702
	HN28	0201	1101	1301	5502	0302	1203
	HN29	na†	na†	4601	4601	0102	0102

na† Not available

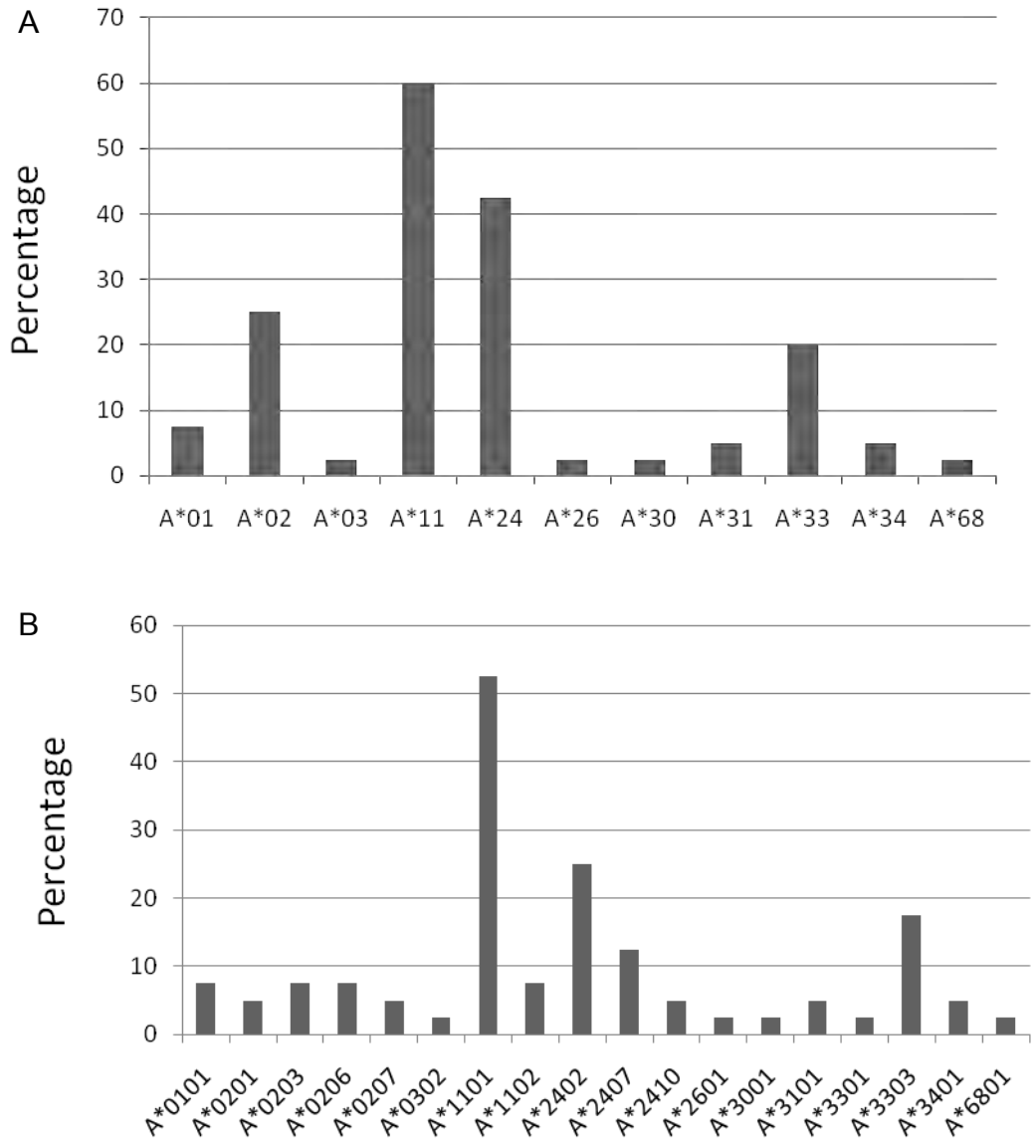


Figure 11. Proportion of each HLA-A allele in study population; A. Low resolution typing (2 digits) and B. High resolution typing (4 digits)

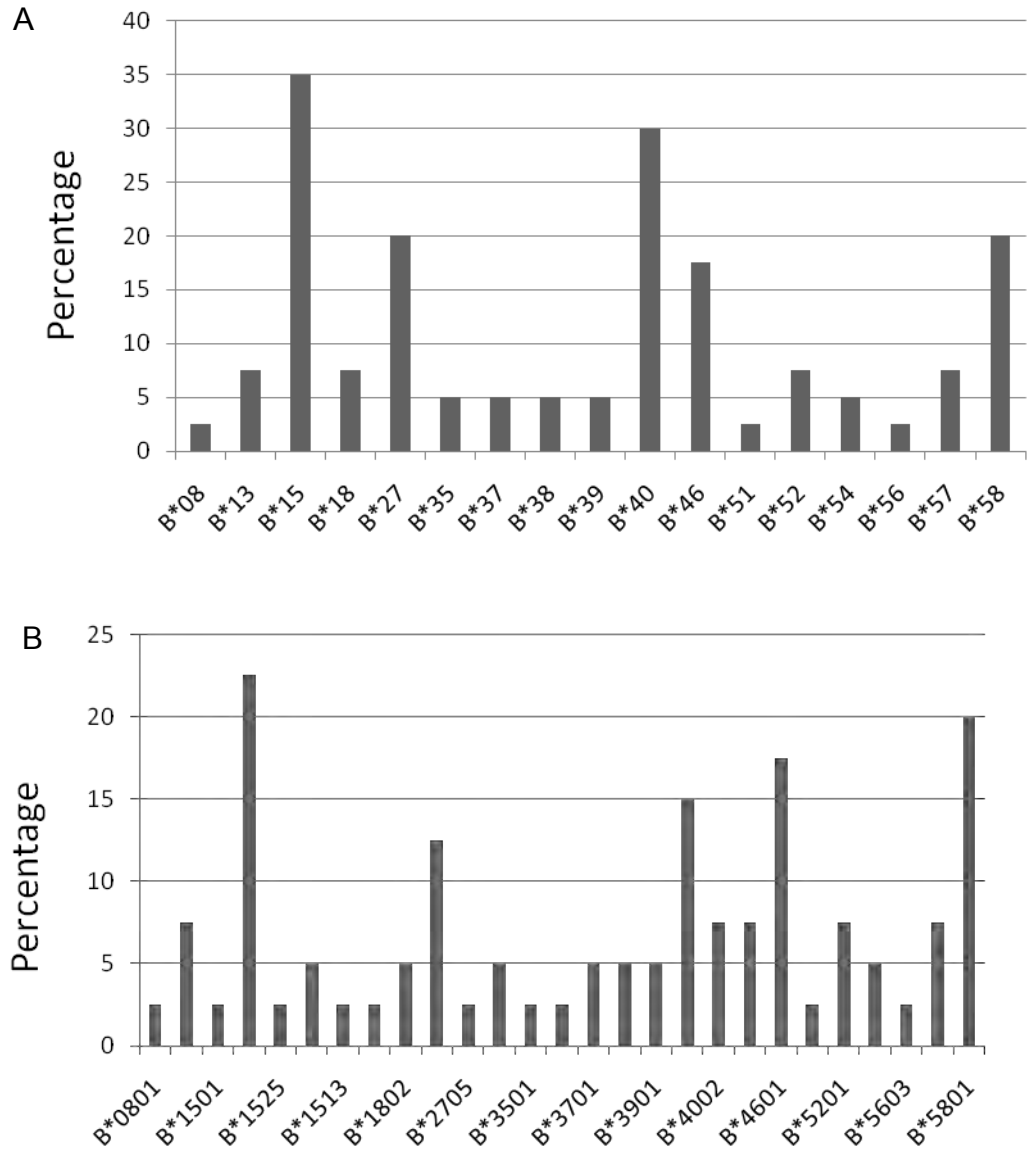


Figure 12. Proportion of each HLA-B allele in study population; A. Low resolution typing (2 digits) and B. High resolution typing (4 digits)

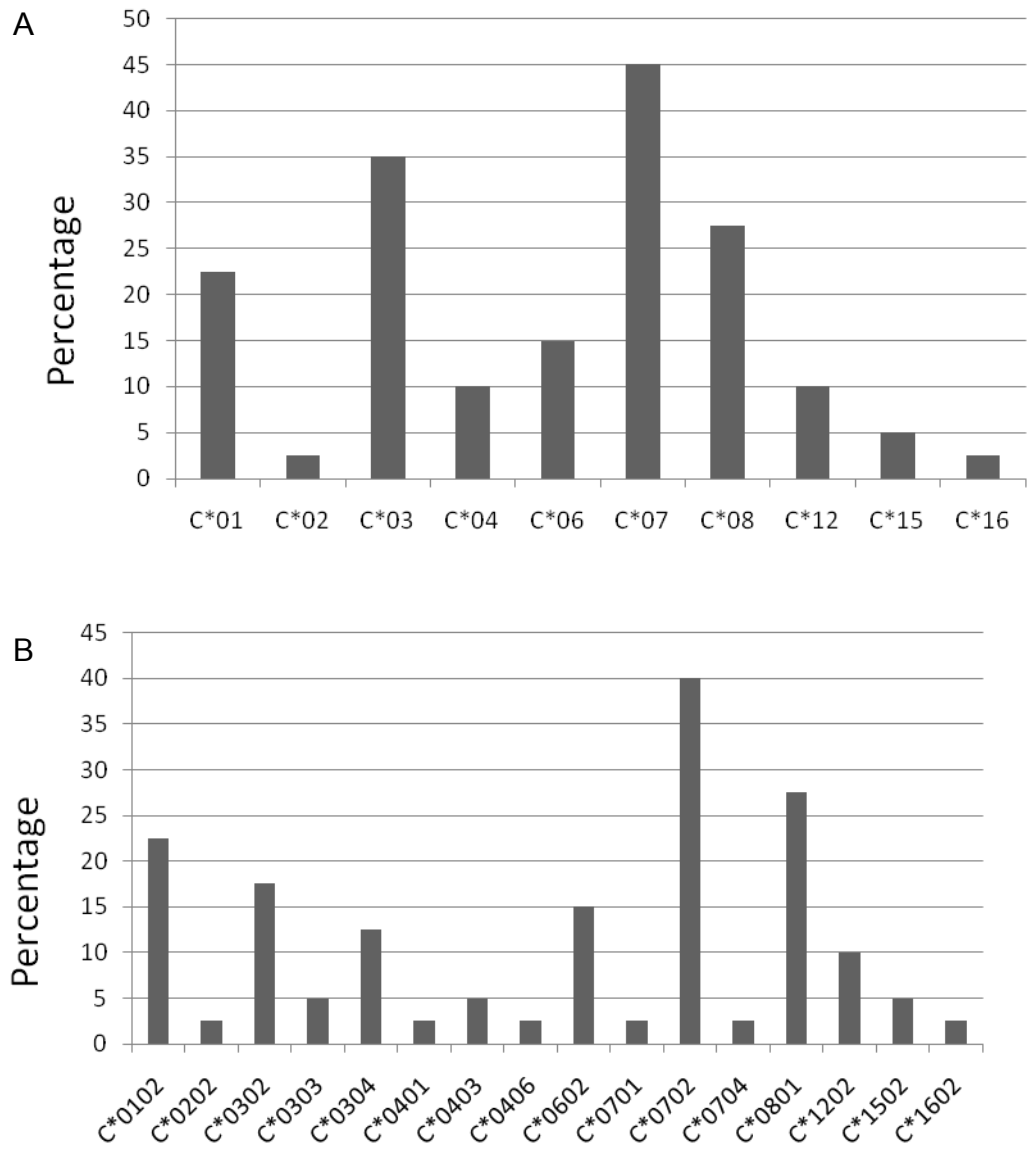


Figure 13. Proportion of each HLA-C allele in study population; A. Low resolution typing (2 digits) and B. High resolution typing (4 digits)

Part III. Comparison of demographic data between HIV-1 infected individual groups

1. HIV-1 infected individuals grouped by plasma HIV-1 load

Demographic data was compared between groups to determine if there were any characteristics that might be associated with natural HIV-1 control. Age, sex, time after seroconversion and $CD8^+$ T cell count were not different between 3 groups (Table 10). Both EC and VC had better HIV-1 clinical outcome than TP as reflected by their higher $CD4^+$ T cell counts (Table 14). However, statistically significance was only observed when $CD4^+$ T cell counts were compared between VC and TP ($p = 0.0006$, figure 14A). Lacking of statistic significance observed when EC and TP were compared, might due to limited number of EC (only 3 individuals, figure 14A). Since subjects were grouped according to their plasma HIV-1 load (pVL), it was obvious that highest pVL level was observed in TP while VC and EC were with significantly lower level (Figure 14B).

Table 10. Demographic data of three clinically distinct HIV-1 infected individual groups which were categorized according to their plasma HIV-1 load (pVL)

Groups	Age (years)	Sex (M:F)	Years after seroconversion	$CD8^+$ T cell counts (cells/mm^3)	$CD4^+$ T cell counts (cells/mm^3)	Plasma HIV-1 load (copies/ml)
Elite controllers (EC)	38 (28-52)	1:2	7 (5-10)	745 (507-942)	670 (470-834)	<40
Viraemic controllers (VC)	27.5 (18-51)	8:2	3 (1-9)	957 (633-2,232)	669 (493-1,319)	1,079 (151-1,900)
Typical progressors (TP)	33 (21-66)	15:17	2.5 (0.5-13)	976.5 (382-2,008)	427.5 (126-881)	17,119 (3,094-1,132,883)

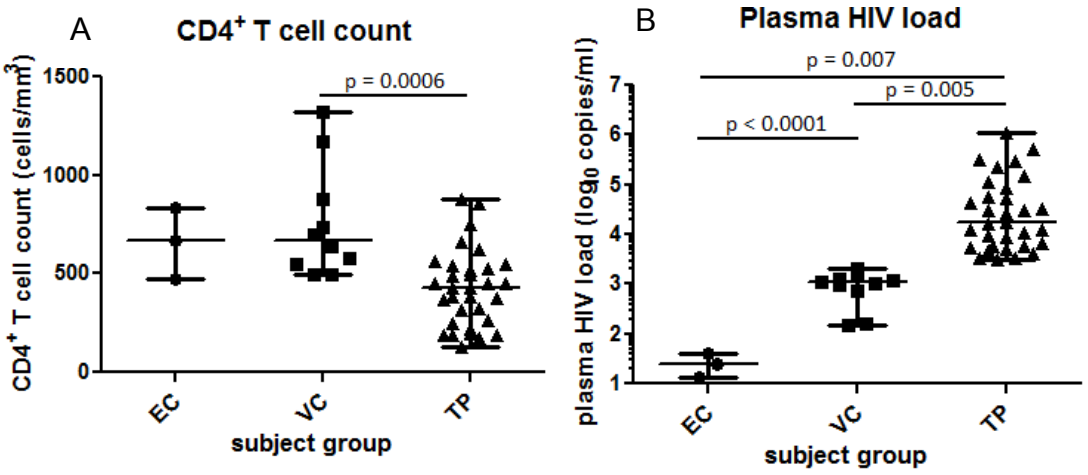


Figure 14. Comparison of CD4$^+$ T cell counts; A. and plasma HIV-1 load and B. between elite controllers (EC), virsemic controllers (VC) and typical progressors (TP)

2. HIV-1 infected individuals grouped by the presence of protective HLA-I alleles

HLA-I alleles are also an important factor in HIV-1-specific immune responses. Three HLA-I alleles; HLA-B*27, -B*57 and –B*58 have been evidently demonstrated to be in a strong association with HIV-1 control. Hence, these HLA-I alleles were termed "protective HLA-I alleles". To determine the impacts of these protective HLA-I alleles in this study, HIV-1 infected individuals were categorized according to their HLA-I alleles into 2 groups; subject with protective-allele(s) (PA) and subject without protective-allele(s) (nPA). PA were HIV-1 infected individuals positive for HLA-B*27, -B*57 and –B*58 while nPA were subjects who were positive for others HLA-I alleles (Table 9). There were 8 HLA-B*27 positive individuals and 11 HLA-B*57/58 positive individuals in this study population. Surprisingly, both CD4 T^+ cell counts and plasma HIV-1 load were not different between PA and nPA (Table 11). This comparable clinical outcome was independent of years after seroconversion and all other demographic data (Table 11). The only difference that reached statistic significance was $CD8^+$ T cell counts in which higher in PA than nPA ($p < 0.0433$, figure 15).

Table 11. Demographic data of HIV-1 infected individuals categorized by the presence of protective HLA-I alleles

Groups	Age (years)	Sex (M:F)	Years after seroconversion	$CD8^+$ T cell counts ($cells/mm^3$)	$CD4^+$ T cell counts ($cells/mm^3$)	Plasma HIV-1 load (copies/ml)
Subjects with protective-allele(s) (PA)	30 (18-49)	10:9	3 (1-12)	1,104 (550-2,232)	493 (126-1,319)	5,728 (151-1,132,883)
Subjects without protective-allele(s) (nPA)	29.5 (19-66)	12:10	1 (0.5-13)	865 (382-1,489)	451 (173-855)	14,137 (936-309,949)

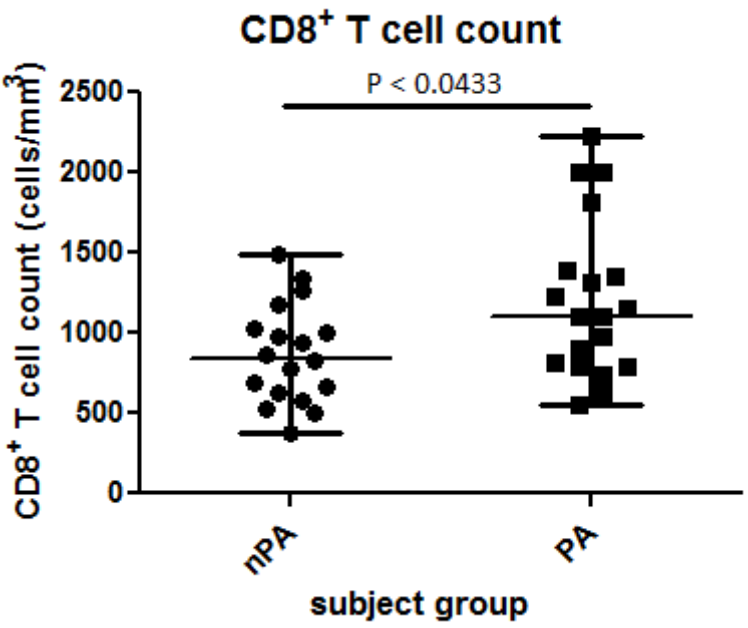


Figure 15. Comparison of $CD8^+$ T cell counts between subject with protective-allele(s) (PA) and subject without protective-allele(s) (nPA).

Part IV. HIV-1 Gag p24 specific primers design

Since the endemic HIV-1 genotype of Thailand is the recombinant form between clade A and clade E, termed Circulating Recombinant Form_01AE (CRF_01AE), overlapping peptide design based on Thailand own recently-circulating virus is more appropriate, in order to properly determine HIV-1 Gag p24-specific-T cell responses in Thai population. HIV-1 Gag p24 specific primers were designed as described in materials and methods part 4, section 1. All primer sequences were shown in table 2. The binding location of each primer-pair was shown in figure 16 which were also summarized in table 12.

Part V. HIV-1 Gag p24 amplification

Details of the polymerase chain reaction (PCR) amplification of HIV-1 Gag p24 were thoroughly described in materials and methods part 4, section 2. According to the inner primer-pair binding location, final amplified products were 932 nucleotides in length, ranging from position 640-1571 of CM240 sequence (Figure 1, figure 16 and table 12).

Part VI. HIV-1 Gag p24 overlapping peptides design

PCR products from 10 randomly-selected HIV-1 infected individuals were determined for their nucleotide sequences, translated into amino acid sequences and aligned to obtain their consensus sequence. This consensus sequence was used as a template for designing a panel of recently circulating HIV-1 Thailand strain-based Gag p24 overlapping peptides. Detailed descriptions of this process were summarized in materials and methods part 5, section 1. Peptide sequences were summarized in table 3.

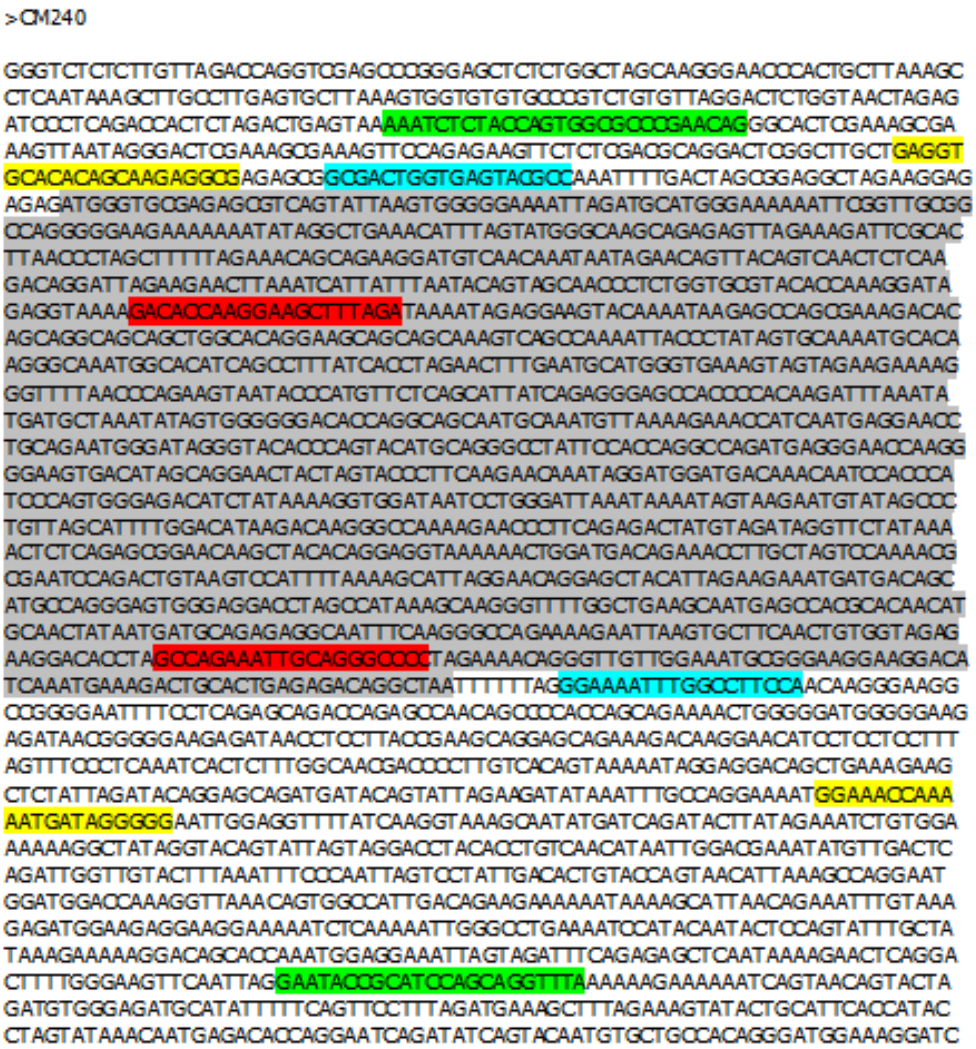


Figure 16. Scheme represents specific binding location of each pairs of HIV-1 Gag p24 primers. The background sequence showed nucleotide position 1-2590 of CM240 (accession no. U5477). The grey highlighted sequence was whole *gag* ranging from position 355-1866 with a total length of 1512 nucleotides. The green highlighted sequences were the binding site of outer2 primer-pair, forward and reverse, respectively. The yellow highlighted sequences were the binding site of outer1 primer-pair, forward and reverse, respectively. The cyan highlighted sequences were the binding site of outer3 primer-pair, forward and reverse, respectively. The red highlighted sequences were the binding site of inner primer-pair, forward and reverse, respectively.

Table 12. Summary of primer binding position

Forward primers	Binding position (CM240)	Reverse Primers	Binding Positon (CM240)
Outer1	276-297	Outer1	1951-1972
Outer2	169-195	Outer2	2401-2423
Outer3	304-321	Outer3	1652-1669
Inner	640-659	Inner	1552-1571

Part VII. Determination of HIV-1 Gag p24 specific T cell responses by an interferon-γ ELISpot assay

Interferon-γ (IFN-γ) Enzyme-Linked Immuno Spot (ELISpot) assays were used to determine the effects of T cell responses specifically against HIV-1 Gag p24 proteins on the HIV-1 clinical outcomes. Of all 45 individuals enrolled in this study, four were non-responders. Interestingly, all 3 EC (HN5, 10, 31) were non-responders. Another non-responder was a typical progressor (HN7). All these 4 non-responders were excluded from further investigations.

1. Comparison of HIV-1 Gag p24 specific interferon-γ ELISpot responses between typical progressors and viraemic controllers

Overall, both TP and VC mediated the same breadth of HIV-1 Gag p24-specific-T cell responses (Table 13). Although VC seemed to mount higher magnitude of HIV-1 Gag p24-specific-T cell responses than TP, this difference was not statistically significant (Table 13).

Table 13. Comparison of HIV-1 Gag p24 specific interferon-γ ELISpot responses between typical progressors and viraemic controllers

	Typical progressors (TP)	Viraemic controllers (VC)
Breadth (OLP)	3 (1-6)	3 (1-9)
Cumulative magnitude (SFU/10^6 PBMC)	1755 (322-7913)	2748 (644-16098)
Median magnitude (SFU/10^6 PBMC)	542 (98.75-2490)	684.5 (205-1855)

2. Comparison of HIV-1 Gag p24 specific interferon-γ ELISpot responses between typical progressors and viraemic controllers carrying the same protective HLA-I alleles

Both breadth and magnitude of T cell responses are under the influence of HLA-I restriction. Impacts of HLA-I alleles are likely due to the frequency of HLA-I alleles which, in turn, having an influence on the immunodominance of epitopes in a certain population. In order to exclude this impacts from HLA-I restriction, both HIV-1 Gag p24 overlapping peptides-specific- and protective HLA-I allele-restricted-epitope-specific-T cell responses of VC and TP were analyzed in a protective allele-matched manner.

In a group of HLA-B*27 positive individuals, significantly lower pVL was observed in VC (Table 14, figure 17A). Though $CD4^+$ T cell counts were also higher in VC, its difference did not reach statistically significant (Figure 17B). All the other demographic data were not different between HLA-B*27 positive TP and VC. However, HIV-1 Gag p24-specific IFN-γ responses, in term of breadth, median magnitude or cumulative magnitude were not different between HLA-B*27 positive TP and VC, though broader breadth and higher cumulative magnitude were obviously observed in VC (Table 14, figure 17B and 17C). Similarly, in an HLA-B*27-restricted epitope, KRWIILGLNK (KK10)-specific-T cell response, the magnitude of IFN-γ response against this epitope was not different between TP and VC (Table 14).

In a group of HLA-B*57/58 positive, VC had significantly better HIV-1 clinical outcomes than their TP counterparts, as reflected by their higher $CD4^+$ T cell counts and lower pVL (Table 14, figure 18A and 18B). However, better HIV-1 clinical outcomes observed in these HLA-B*57/58 positive VC were not a result of HIV-1 Gag p24-specific-T cell responses, since they mediated the same breadth, cumulative magnitude and median magnitude of IFN-γ specific responses as HLA-B*57/58 positive TP (Table 14). These similarities were also observed when responses against 6 previously reported HLA-B*57/58-restricted epitopes of HIV-1 Gag p24 protein were determined (Table 14).

Table 14. Comparison of HIV-1 Gag p24 specific interferon-γ ELISpot responses between typical progressors and viraemic controllers carrying the same protective HLA-I alleles

	HLA-B*27 group		HLA-B*57/58 group	
	TP (4)	**VC (4)**	**TP (9)**	**VC (3)**
Age (years)	34.5 (27-44)	25 (19-30)	34 (21-49)	28 (27-34)
HIV-1 duration (years)	3.5 (2-10)	3 (2-3)	4 (1.5-12)	2 (1-3)
$CD4^+$ T cell counts ($cells/mm^3$)	292 (195-881)	728 (493-1,169)	368 (126-658)	699 (495-1,319)*
$CD8^+$ T cell counts ($cells/mm^3$)	1285 (624-2,008)	1388 (1,104-1,814)	974 (550-2,008)	810 (791-2,232)
Plasma HIV-1 load (copies/ml)	11,747 (5,728-299,077)	1,104.5 (742-1,900)*	12,231 (3,026-1,132,883)	165 (151-1,549)**
p24 breadth of responses (no of OLPs)	2 (2-4)	7.5 (2-9)	2 (1-6)	4 (1-8)
p24 cumulative magnitude of responses ($SFU/10^6$ PBMC)	1,779 (719.5-1,804)	10,241 (1,024-16,098)	1,496 (542-7,913)	2,440 (644-3,056)
p24 median magnitude of responses ($SFU/10^6$ PBMC)	889.3 (167.3-902)	769 (512-1,149)	542 (98.75-1,445)	644 (205-725)
Breadth of specific HLA-I allele restricted epitopes responses (no of EPs) †	na††	na††	2 (0-4)	3 (2-4)
Median magnitude of specific HLA-I allele restricted epitopes responses ($SFU/10^6$ PBMC)	1,109 (0-2,547.5)	2,112 (0-5,834)	987 (0-3,435)	586 (483-751)
Cumulative magnitude of specific HLA-I allele restricted epitopes responses ($SFU/10^6$ PBMC)	na††	na††	1,860 (0-4058)	2,338 (966-3,678)

*p value < 0.05, ** p value < 0.01,

† HLA-B27-restricted epitope: KRWIILGLNK, KK10;

†HLA-B57/58-restricted epitopes: LSPRTLNAW, LW9; KGFNPEVIPMF, KF11; ETINEEAEW, EW10; QATQEVKNW, QW9; GTGATLEEM, GM9 and TSTLQEQIGW, TW10

†† not analyzed, only 1 HLA-B27 restricted epitope tested in this study

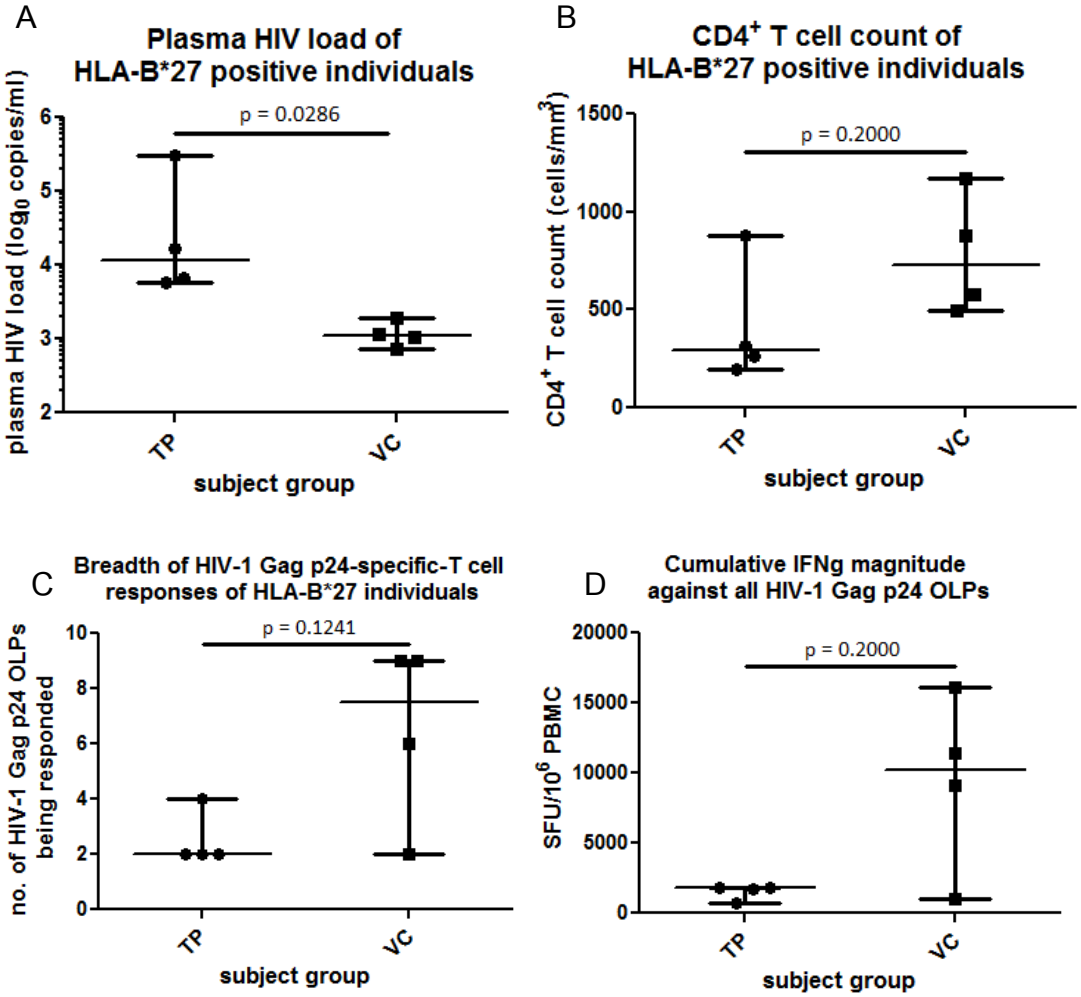


Figure 17. Comparison of plasma HIV-1 load (A), $CD4^+$ T cell counts (B), breadth (C), and cumulative magnitude of HIV-1 Gag p24-specific-T cell responses (D) between HLA-B*27 positive typical progressors (TP) and viraemic controllers (VC).

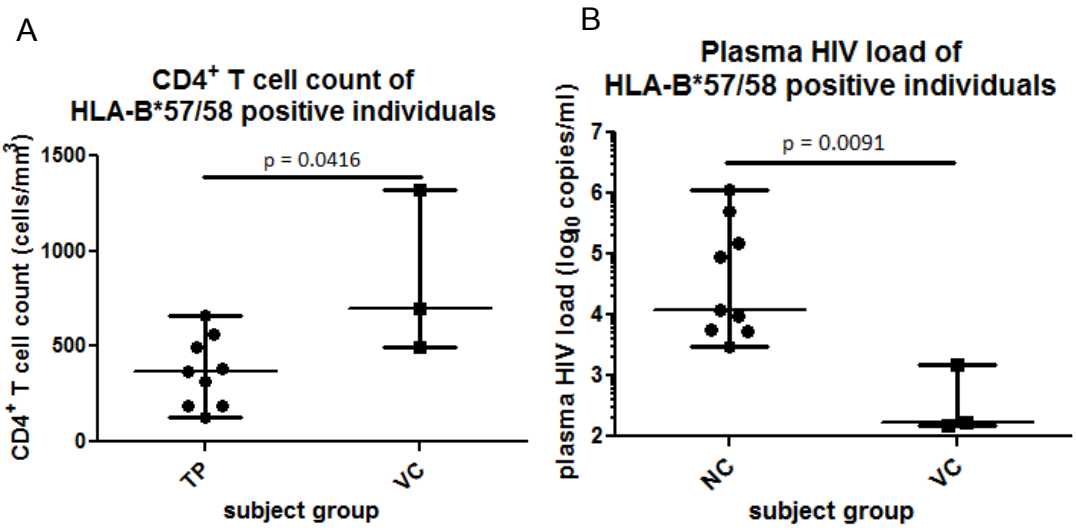


Figure 18. Comparison of CD4$^+$ T cell counts (A), plasma HIV-1 load (B), between HLA-B*57/58 positive typical progressors (TP) and viraemic controllers (VC)

Part VIII. Determination of HIV-1 Gag p24-specific- $CD8^+$ T cell responses functional quality by an intracellular cytokines staining (ICS) assay

1. Antibodies titration

All antibodies used in a flow-cytometry analysis were titrated to select the optimal antibody titer. Their optimal titer was determined by both median fluorescence intensity (MFI) ratio and position of negative population. MFI ratio is a proportion between MFI of positive population and MFI of negative population which stand for signal-to-background ratio (described in part VI, section 2.2) [159]. MFI ratio of each antibody titer was then plotted against its titer, setting up an antibody titration curve (Figure 8). In addition, the position of negative population was also taken into account to determine the background level (Figure 7). Consequently, optimal antibody titer was the titer at which minimal background was seen and provided peak MFI ratio. Optimal titer and volume for each antibody was summarized in table 15.

2. Optimization of functional quality determination protocol

After obtaining the optimal titer of each antibody, their compatibility was assessed by performing an intracellular cytokines staining (ICS) assay using the protocol described in [159]. Firstly, the functional quality of $CD8^+$ T cells was determined in a non-specific responses manner, using T cell inducing super-antigen, Staphylococcus enterotoxin B (SEB), as a stimulator. PBMC from 2 HIV-1 sero-negative healthy volunteers were used instead of the precious, limited in quantity, specimen collected from HIV-1 infected individuals.

Next, an ICS protocol was tried in a more specific, $CD8^+$ T cell response against human cytomegalovirus (HCMV) derived epitope stimulation. PBMC from another 2 HIV-1 sero-negative healthy individuals, in which their specific T cell responses against HCMV derived peptides have been identified in our laboratory.

Table 15. Summary of fluorochrome-conjugated antibodies titer and volume used in functional determination assay

Antibodies	Recommended volumes (1X)	Optimal titers	Optimal volumes
APC-H7-conjugated anti-human CD3 monoclonal antibody (Beckton Dickinson)	5 µl	1/2X	2.5 µl
Pacific blue-conjugated anti-human CD8 monoclonal antibody (Biolegend)	20 µl	1/16X	1.25 µl
PE-Cy5-conjugated anti-human CD107a monoclonal antibody (Beckton Dickinson)	5 µl	1/4X	1.25 µl
FITC-conjugated anti-human IL-2 monoclonal antibody (Biolegend)	20 µl	1X	20 µl
APC-conjugated anti-human TNF-α monoclonal antibody (Biolegend)	5 µl	1X	5 µl
PE-Cy7-conjugated anti-human IFN-γ monoclonal antibody (Biolegend)	5 µl	1X	5 µl
PE-conjugated anti-human MIP1-β monoclonal antibody (Beckton Dickinson)	5 µl	1/8X	0.5 µl

3. Freshly isolated PBMC showed an enhanced sensitivity of the functional quality determination assay

In this study, as described in part VI, section 2.3, instead of frozen specimen, functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses was determined in a freshly isolated PBMC sample. Though, labor intensive in its protocol, overnight-rested freshly isolated PBMC showed an enhanced sensitivity of cytokines detection as compared to frozen samples (Figure 15).

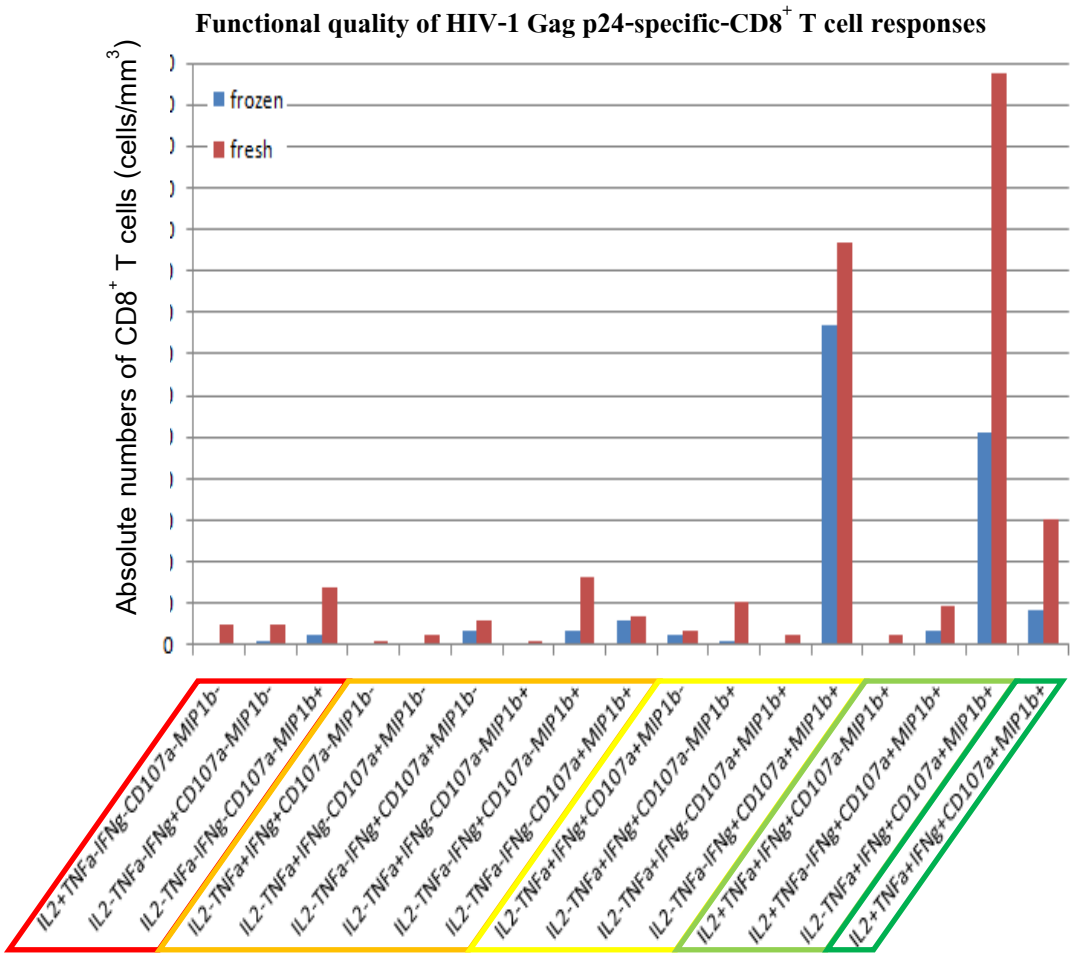


Figure 19. Enhanced sensitivity of the functional quality determination assay when freshly isolated PBMC were used: representative graph compared median absolute number of each functionally distinct subpopulation of $CD8^+$ T cells specific to HIV-1 Gag p24 overlapping peptide (OLP11) between frozen (blue column) and freshly isolated PBMCs (red column). Only positive subpopulations were shown.

4. **Comparison of the functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses between typical progressors and viraemic controllers**

Findings from the IFN-γ ELISpot assay had suggested that, at both the whole HIV-1 Gag p24 protein specific level and single epitope specific level, analysis of a single cytokine production might not accurate enough to illustrate the protective effect of HIV-1 Gag p24-specific-CD8$^+$ T cell responses. Hence, HIV-1 Gag p24-specific-CD8$^+$ T cell responses were next investigated by a newly optimized ICS assay to determine if there were any differences in the functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cells between TP and VC.

Due to the limited availability of specimen, total numbers of 20 HIV-1 infected individuals were included in this part of study. All the individuals whom were included in functional quality assay were listed in table 20. Their demographic data and HLA-I alleles were shown in table 8 and 9, respectively.

Overall, significantly larger number of HIV-1 Gag p24-specific-CD8$^+$ T cells with high functional quality (defined as responding with 4 or 5 functions simultaneously) were observed in VC as compared to NC (Figure 16). Next, absolute number of each subpopulation of all the 31 possible functionally-distinct HIV-1 Gag p24-specific-CD8$^+$ T cells were compared between TP and VC (Figure 16). In general, HIV-1 Gag p24-specific-CD8$^+$ T cells of VC showed a trend toward high functional quality responses (shifted to the right) while those of TP were with limited functional quality. Though many subpopulations were different between VC and TP, only 3 were of statistical significance; full 5 functions, IL-2$^+$TNF-α$^+$IFN-γ$^+$CD107a$^+$MIP1-β$^+$, 4 functions TNF-α$^+$IFN-γ$^+$CD107a$^+$MIP1-β$^+$ and 3 functions TNF-α$^+$ CD107a$^+$MIP1-β$^+$. The median of these CD8$^+$ T cell subpopulations in VC and TP were 47 vs. 0 cell/mm^3 ($p = 0.01$), 352 vs. 62 cell/mm^3 ($p = 0.0038$) and 91 vs. 9 cell/mm^3 ($p = 0.01$), respectively. In TP group, HIV-1 Gag p24-specific-CD8$^+$ T cell responses were dominated by single MIP1-β producing cells (Figure 17).

Table 16. List of HIV-1 infected individuals which were included for the determination of their HIV-1 Gag p24-specific-CD8$^+$ T cell functional quality

Typical Progressors (TP)		Viraemic Controllers (VC)	
ID	**Protective HLA-I alleles**†	**ID**	**Protective HLA-I alleles**
HN3	None††	**HN2**	HLA-B*2706
HN11	None	**HN12**	HLA-B*5701
HN18	None	**HN15**	HLA-B*2704
HN19	None	**HN20**	HLA-B*2704
HN21	HLA-B*2704	**HN24**	HLA-B*5701
HNN4	HLA-B*5801	**HN26**	HLA-B*4601
JSM	HLA-B*2704	**HN30**	HLA-B*5701
NOT	HLA-B*2704	**PRT**	None
PNN	HLA-B*2706/B*5801		
RSR	HLA-B*5801		
SUL	HLA-B*5801		
VKJ	HLA-B*5801		

† indicated that this individual was positive for one or more of the 3 protective HLA-I alleles, which included HLA-B*27, -B*57 and -B*58, †† indicated that this individual was negative for all of the 3 protective HLA-I alleles.

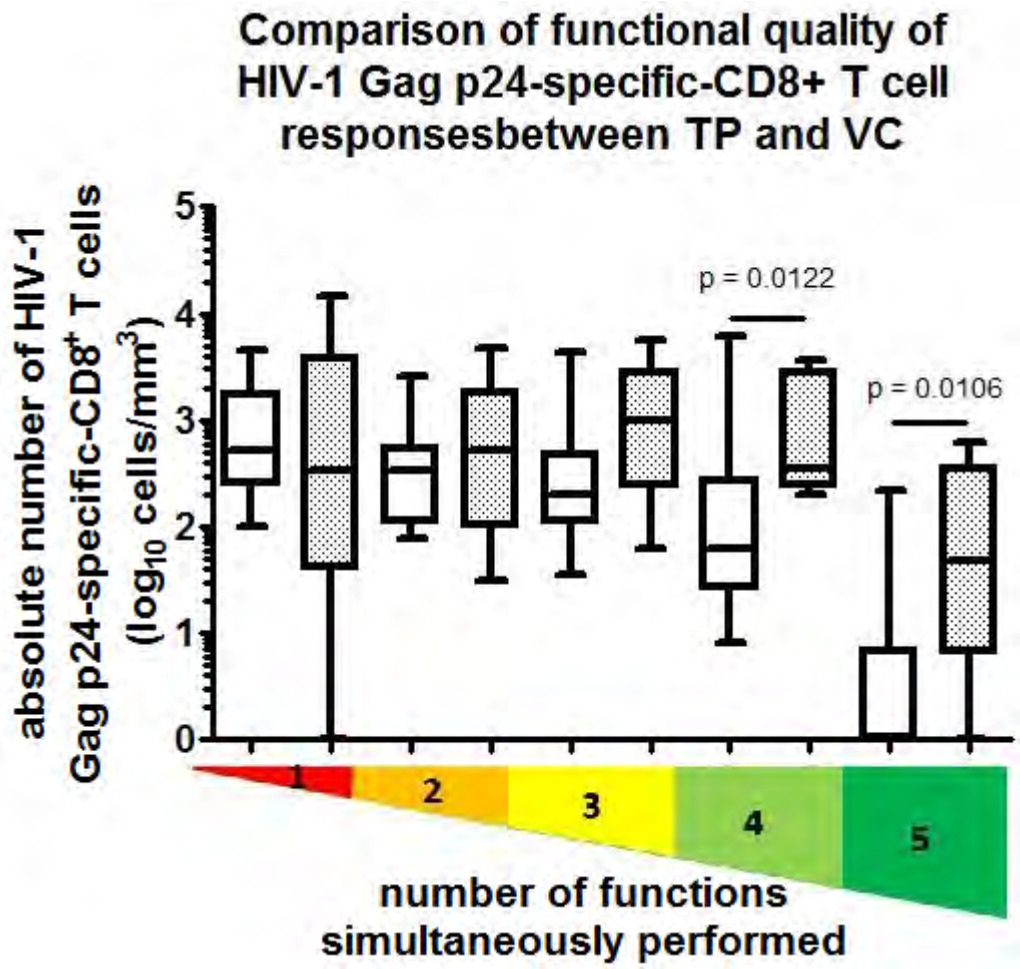


Figure 20. Comparison of functional quality of HIV-1 Gag p24-specifc-CD8$^+$ T cell responses between typical progressors and viraemic controllers. Cumulative absolute number of p24-specific CD8$^+$ T cells which simultaneously responded with 1, 2, 3, 4 or 5 functions (y-axis) were compared between groups; typical progressors, TP (clear column) and viraemic controllers, VC (dotted column). Color spectrum on x-axis represented numbers of functions; red =1 function, orange = 2 functions, yellow = 3 functions, light green = 4 functions and green = 5 functions.

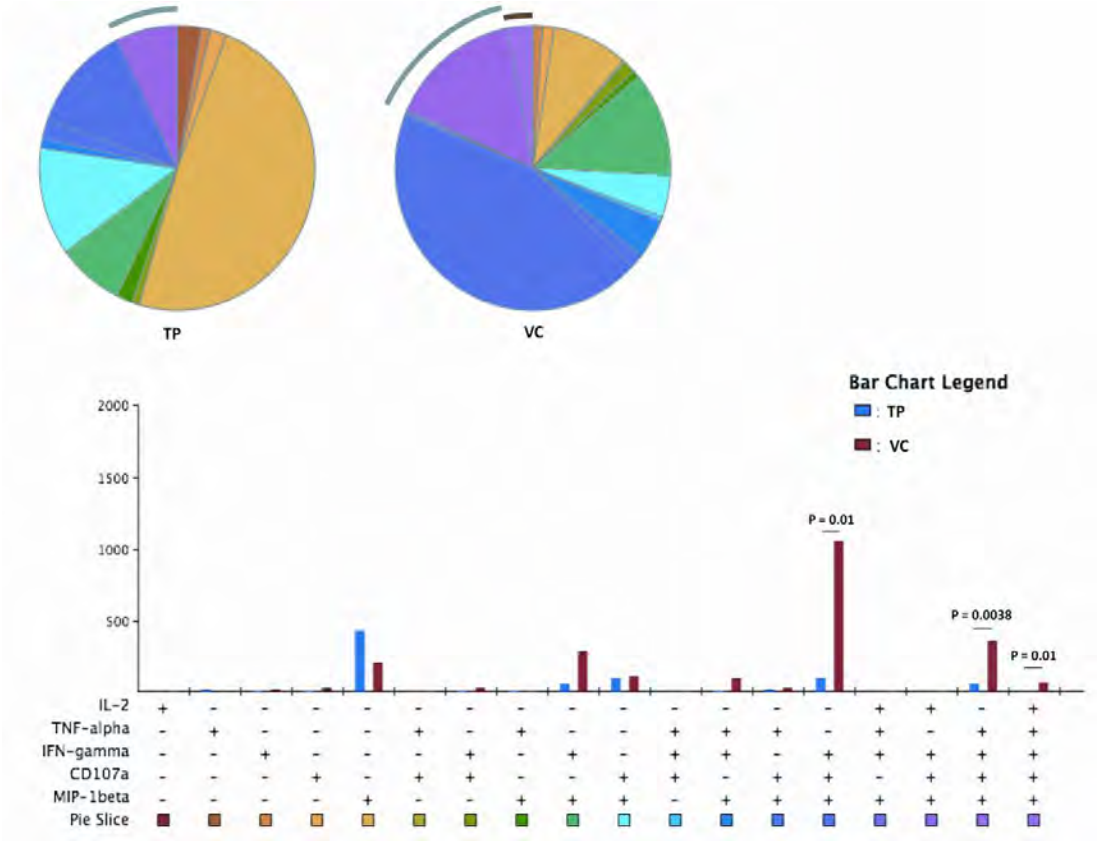


Figure 21. Comparison of HIV-1 Gag p24-specific-CD8$^+$ T cell functional quality between typical progressors (TP) and viraemic controllers (VC). Upper pie chart showed a schematic diagram of the contribution of each functionally distinct subpopulation. Each subpopulation was represented by individual color (also shown at the lowest part of the lower bar chart). Each pie slice area represented the proportion of that subpopulation in total responding HIV-1 Gag p24-specific-CD8$^+$ T cell population. Black arc that curved over the pie chart represented the full 5 functions responding subpopulation while the grey arc represented the simultaneous 4 functions responding subpopulations. **Lower bar chart** showed a median absolute number of each functionally distinct subpopulation of HIV-1 Gag p24-specific-CD8$^+$ T cells. Blue bar represented typical progressors while crimson bar represented viraemic controllers. Any subpopulations that were significantly different between TP and VC were indicated by linked-bar and p-value over the two comparative pairs. Only the positive subpopulations were shown.

5. Comparison of the functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses between HLA-B*27 positive typical progressors and viraemic controllers

Next, the functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses were compared between the group of HLA-B*27 positive TP and VC. There were enough PBMC sample for all 4 HLA-B*27 TP while only 3 out of 4 HLA-B*27 VC were available (Table 20). Due to the exclusion of one HLA-B*27 VC (HN1), demographic data of these 7 HLA-B*27 positive TP and VC was newly analyzed and shown in table 21. In general, there were some minor changes in median values of all the demographic data as compared with those shown in table 18. Major changes were observed in 2 parameters, median CD4 $^+$ T cell counts and plasma HIV-1 load (Table 21). Median CD4 $^+$ T cell counts of VC were decreased from 728 to 578 cells/mm^3, which was also not different from TP. Though the exclusion of subject HN1 from the HLA-B*27 positive VC group resulted in decrease of median plasma HIV-1 load from 1105 to 1034 copies/ml, this also lowered the numbers of VC population which also decreased statistical power. This resulted in a non-significant difference between TP and VC plasma HIV-1 load, though their difference was more than 1 log (11,747 vs. 1,034 copies/ml, p = 0.0571, figure 18).

When the absolute numbers of CD8$^+$ T cells specifically responding against whole HIV-1 Gag p24 protein were analyzed, HLA-B*27 positive VC possessed significantly larger number of high functional quality CD8$^+$ T cells than TP (Figure 19). Though larger numbers of simultaneously 3 and 4 functions responding CD8$^+$ T cells were also obviously observable, statistic significance was only observed when the full 5 functions responding CD8$^+$ T cells were compared between TP and VC (p = 0.0498, figure 19). Actually, full 5 functions (IL-2$^+$TNF-α^+IFN-γ^+CD107a$^+$MIP1-β^+) responding HIV-1 Gag p24-specific-CD8$^+$ T cells were absent in TP.

Of all 31 functionally distinct subpopulations of HIV-1 Gag p24-specific-$CD8^+$ T cells, HLA-B*27 positive VC had higher numbers of several subpopulations than those of TP (Figure 20). Similarly, only the full 5 functions responding $CD8^+$ T cells were significantly different ($p = 0.0498$, figure 20).

Table 17. Demographic data of HLA-B*27 positive individuals in the functional quality determination assay

Demographic data	TP (4)	VC (3)
Age (years)	34.5 (27-44)	26 (24-30)
Years after seroconversion	3.5 (2-10)	3 (3)
$CD4^+$ T cell count (cells/mm^3)	292 (195-881)	578 (493-1169)
$CD8^+$ T cell count (cells/mm^3)	1285 (624-2008)	1388 (1104-1814)
Plasma HIV-1 load (copies/ml)	11747 (5728-299077)	1034 (742-1175)†

† Difference in pVL was significant when all 4 HLA-B*27 positive TP were compared with 4 HLA-B*27 positive VC (11747 vs. 1104.5, $p = 0.0286$, figure 17A)

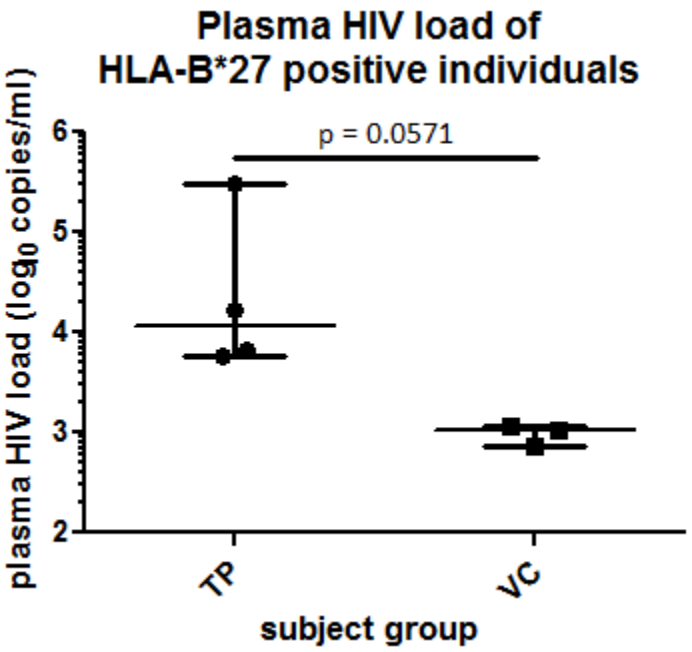


Figure 22. Comparison of plasma HIV-1 load between HLA-B*27 positive typical progressors (TP) and viraemic controllers (VC) whom were included in the functional quality determination assay.

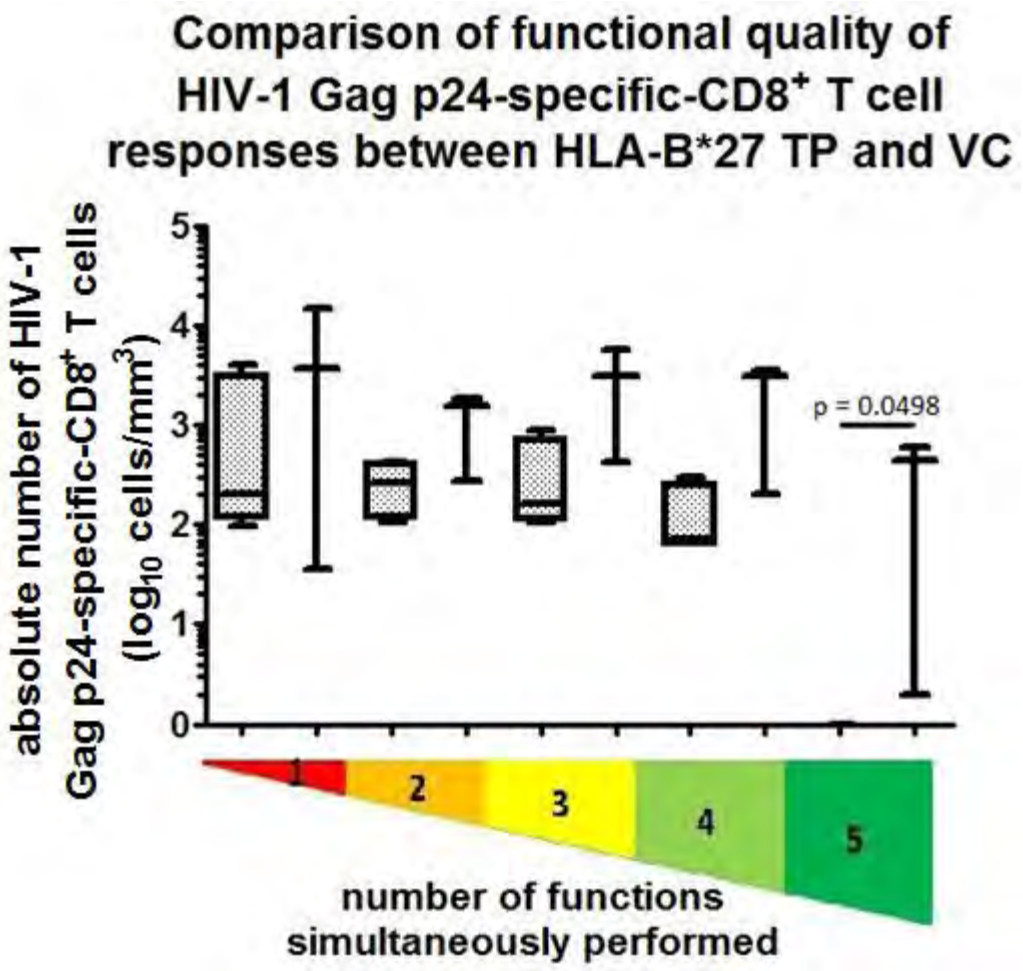


Figure 23. Comparison of the functional quality of HIV-1 Gag p24-specifc-CD8$^+$ T cell responses between HLA-B*27 positive typical progressors and viraemic controllers. Cumulative absolute number of p24-specific-CD8$^+$ T cells which simultaneously responded with 1, 2, 3, 4 or 5 functions (y-axis) were compared between HLA-B*27 positive individuals; typical progressors, TP (dotted column) and viraemic controllers, VC (clear cloumn). Color spectrum on x-axis represented numbers of functions; red =1 function, orange = 2 functions, yellow = 3 functions, light green = 4 functions and green = 5 functions.

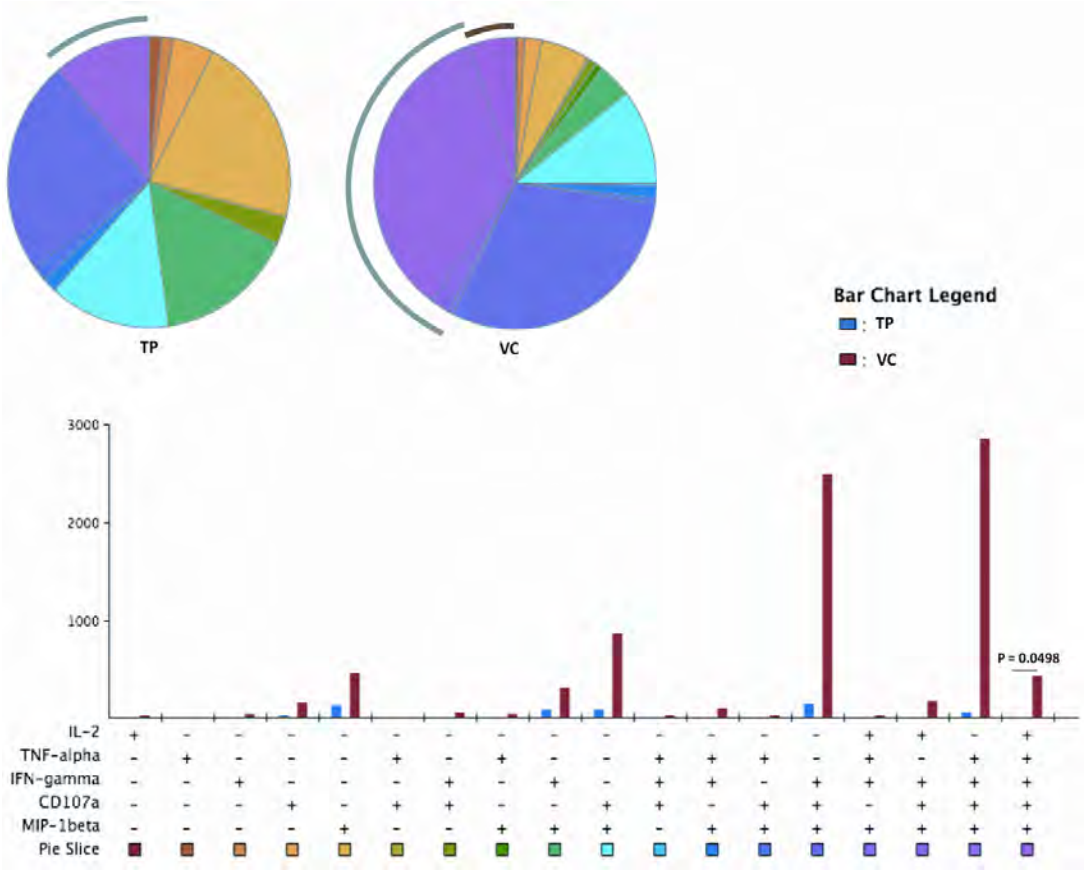


Figure 24. Comparison of HIV-1 Gag p24-specific-CD8$^+$ T cell functional quality between HLA-B*27 positive typical progressors (TP) and viraemic controller (VC). Upper pie chart showed a schematic diagram of the contribution of each functionally distinct subpopulation. Each subpopulation was represented by individual color (also shown at the lowest part of the lower bar chart). Each pie slice area represented the proportion of that subpopulation in total responding HIV-1 Gag p24-specific-CD8$^+$ T cell population. Black arc that curved over the pie chart represented the full 5 functions responding subpopulation while the grey arc represented the simultaneous 4 functions responding subpopulations. **Lower bar chart** showed a median absolute number of each functionally distinct subpopulation of HIV-1 Gag p24-specific-CD8$^+$ T cells. Blue bar represented typical progressors while crimson bar represented viraemic controllers. Any subpopulations that were significantly different between HLA-B*27 positive TP and VC were indicated by linked-bar and p-value over the two comparative pairs. Only the positive subpopulations were shown**.**

6. Comparison of the functional quality of HLA-B*27 restricted KK10-specific-CD8$^+$ T cell responses between HLA-B*27 positive typical progressors and viraemic controllers

Next, the functional quality of CD8$^+$ T cell responses against HLA-B*27 restricted eptiope, KRWIILGLNK (KK10) was determined and compared between these HLA-B*27 positive TP and NC. HLA-B*27 positive VC had significantly larger number of full 5 functions responding KK10-specific-CD8$^+$ T cells than HLA-B*27 TP ($p = 0.0436$, figure 21). HLA-B*27 positive TP also mediated a relatively high functional quality of response against KK10 epitope as reflected by the similar numbers of CD8$^+$ T cells responded with 4 functions observed between HLA-B*27 positive TP and VC (Figure 21). Similar to the finding observed in the whole HIV-1 Gag p24 protein-specific-responses, CD8$^+$ T cells responded with full 5 functions were absent in HLA-B*27 positive TP.

When each functionally distinct subpopulation of HLA-B*27 restricted KK10-specific-CD8$^+$ T cell population was taken into account, absence of full 5 functions responding CD8$^+$ T cells in TP was obviously observed (Figure 22). At this single immunodominant epitope level, TP did mediated some degree of high functional quality of responses as reflected by the 25% contribution of 4 functions responding CD8$^+$ T cells (TNF-α^+IFN-γ^+CD107a$^+$MIP1-β^+) (Figure 22). However, full 5 functions CD8$^+$ T cells were remained absent in TP in which a paucity of IL-2 production seemed to be the main reason. At this single epitope level, VC also showed some degree of enhanced CD8$^+$ T cell functional quality as obviously observed by the increased proportion of full 5 functions responding CD8+ T cells in total HLA-B*27 KK10-specific-CD8$^+$ T cell population (Figure 22).

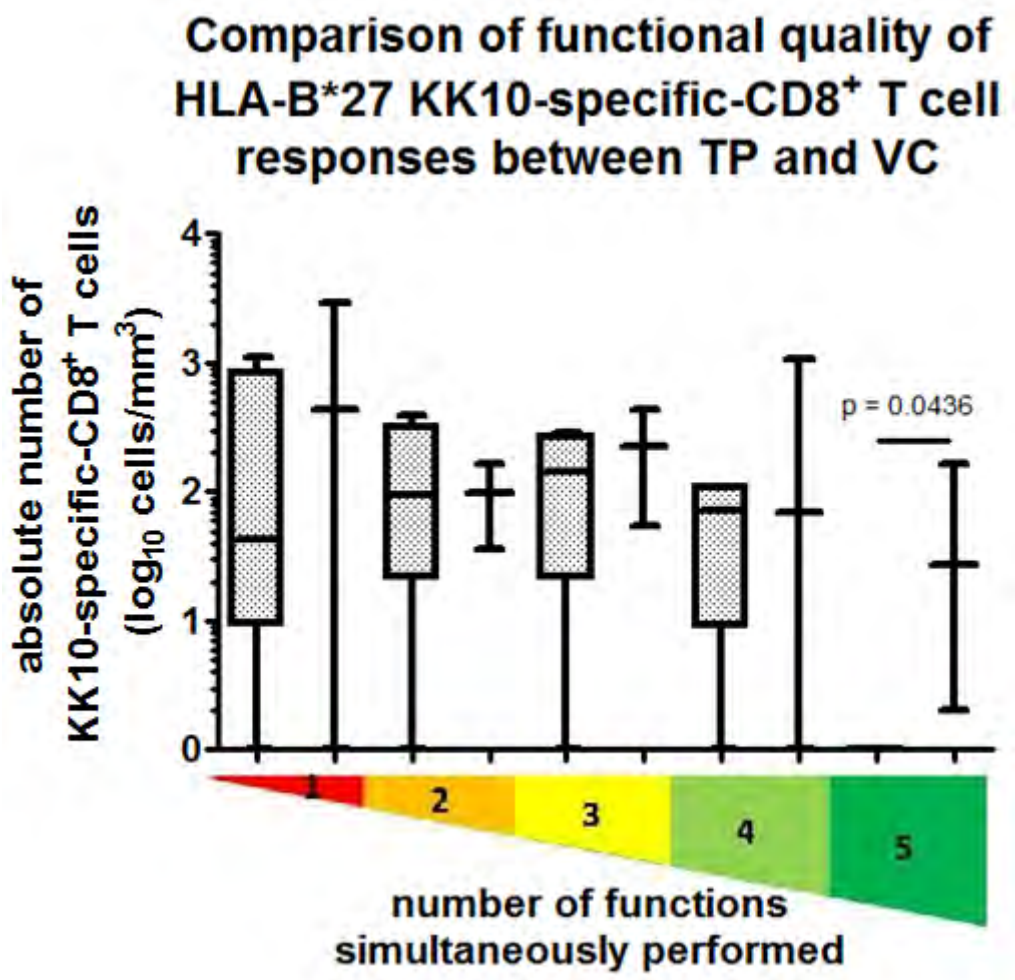


Figure 25. Comparison of the functional quality of HLA-B*27 KK10-specifc-CD8$^+$ T cell responses between HLA-B*27 positive typical progressors and viraemic controllers. Absolute number of HLA-B*27 KK10-specific CD8$^+$ T cells which simultaneously responded with 1, 2, 3, 4 or 5 functions (y-axis) were compared between HLA-B*27 positive individuals; typical progressors, TP (dotted column) and viraemic controllers, VC (clear cloumn). Color spectrum on x-axis represented numbers of functions; red =1 function, orange = 2 functions, yellow = 3 functions, light green = 4 functions and green = 5 functions.

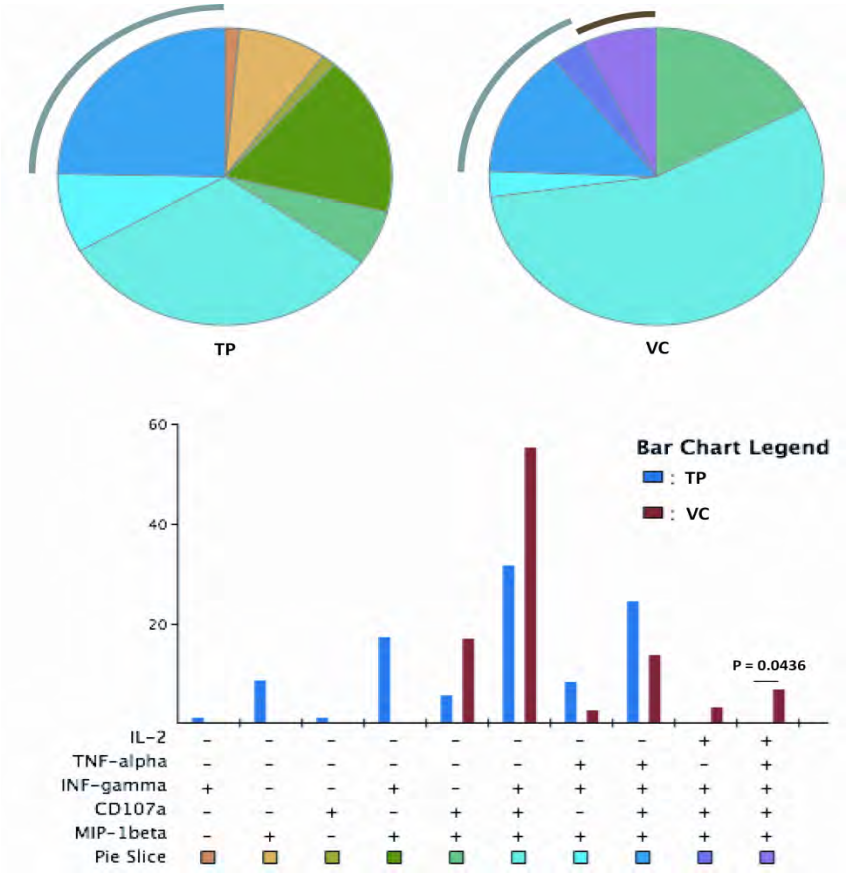


Figure 26. Comparison of HLA-B*27 restricted KK10-specific-CD8$^+$ T cell functional quality between HLA-B*27 positive typical progressors (TP) and viraemic controller (VC). **Upper pie chart** showed a schematic diagram of the contribution of each functionally distinct subpopulation. Each subpopulation was represented by individual color (also shown at the lowest part of the lower bar chart). Each pie slice area represented the proportion of that subpopulation in total responding HIV-1 Gag p24-specific-CD8$^+$ T cell population. Black arc that curved over the pie chart represented the full 5 functions responding subpopulation while the grey arc represented the simultaneous 4 functions responding subpopulations. **Lower bar chart** showed a median absolute number of each functionally distinct subpopulation of HLA-B*27 restricted KK10-specific-CD8$^+$ T cell. Blue bar represented typical progressors while crimson bar represented viraemic controllers. Any subpopulations that were significantly different between HLA-B*27 positive TP and VC were indicated by linked-bar and p-value over the two comparative pair. Only the positive subpopulations were shown.

7. Comparison of the functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses between HLA-B*57/58 positive typical progressors and viraemic controllers

The functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses were next compared between the group of HLA-B*57/58 positive TP and VC. Again, due to limited amount of samples, only 8 HLA-B*57/58 positive individuals were included in this functional quality determination assay, in which 5 and 3 individuals were typical progressors and viraemic controllers, respectively (Table 23). Demographic data of these 8 HLA-B*57/58 positive TP and VC was shown in table 22. As compared with table 18, median age, HIV-1 duration and CD8$^+$ T cell counts remained the same while slightly changes were observed in both CD4$^+$ T cell counts and plasma HIV- load (Table 22). Median CD4+ T cell counts of TP were lower from 368 to 319 cells/mm^3. Decreased level of TP's CD4$^+$ T cell counts resulted in a more significant CD4$^+$ T cell counts difference between HLA-B*57/58 positive TP and VC (from p value = 0.0416 to p-value = 0.0358, figure 18A vs. figure 23A). Though the median plasma HIV-1 load of TP was largely increased, from 11,747 to 154,253 copies/ml, degree of significance was lower when plasma HIV-1 load were compared between HLA-B*57/58 positive TP and VC (from p value = 0.0091 to p-value = 0.0357, Figure 14B vs. figure 23B). This decreased degree of significance might partly reflected the lost of statistical power due to the smaller population size (from 9 TP to 5 TP).

Significantly larger numbers of full 5 functions responding HIV-1 Gag p24-specific-CD8$^+$ T cells were observed in HLA-B*57/58 positive VC than their TP counterparts (p = 0.0262, figure 24). Unlike HLA-B*27 positive TP, there was a single HLA-B*57/58 positive TP who was capable of mediating the full 5 functions responses, though at a very limited degree (only 8 cells/mm^3).

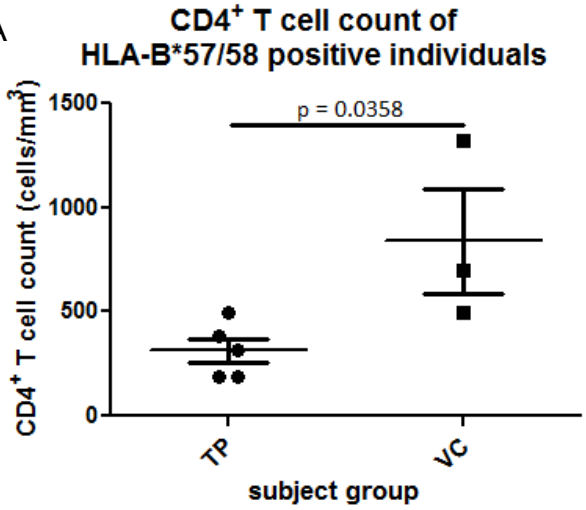
As shown in figure 25, approximately half of the HIV-1 Gag p24-specific-$CD8^+$ T responses mediated by HLA-B*57/58 positive viraemic controllers was of high functional quality (responded with 4 or 5 functions simultaneously) while only 8% was observed in TP. This was the highest contribution of high functional quality HIV-1 Gag p24-specific-$CD8^+$ T cells ever observed in this study. However, statistical significant was only observed when comparing the absolute number of full 5 functions responding HIV-1 Gag p24-specific-$CD8^+$ T cells between HLA-B*57/58 positive TP and VC (Figure 25).

Table 18. Demographic data of HLA-B*57/58 positive individuals in the functional quality determination assay

Demographic data	TP (5)	VC (3)
Age (years)	34 (29-47)	28 (27-34)
Years after seroconversion	4 (3-10)	3 (1-9)
$CD4^+$ T cell counts (cells/mm^3)	319 (187-493)	699 (495-1,319)*
$CD8^+$ T cell counts (cells/mm^3)	974 (550-2,008)	810 (791-2,232)
Plasma HIV-1 load (copies/ml)	154,253 (5385-1,132,883)	165 (151-1,549)**

* Significantly different at p = 0.0358, ** Significantly different at p = 0.0357

A



B

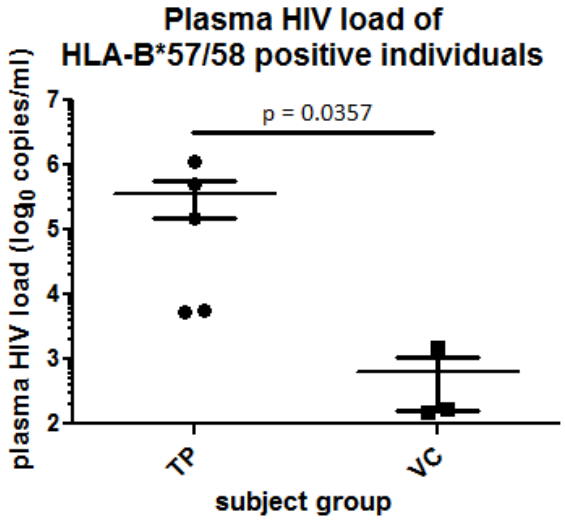


Figure 27. Comparison of $CD4^+$ T cell counts (A) and plasma HIV-1 load (B) between HLA-B*57/58 positive TP and VC whom were included in the functional quality determination assay

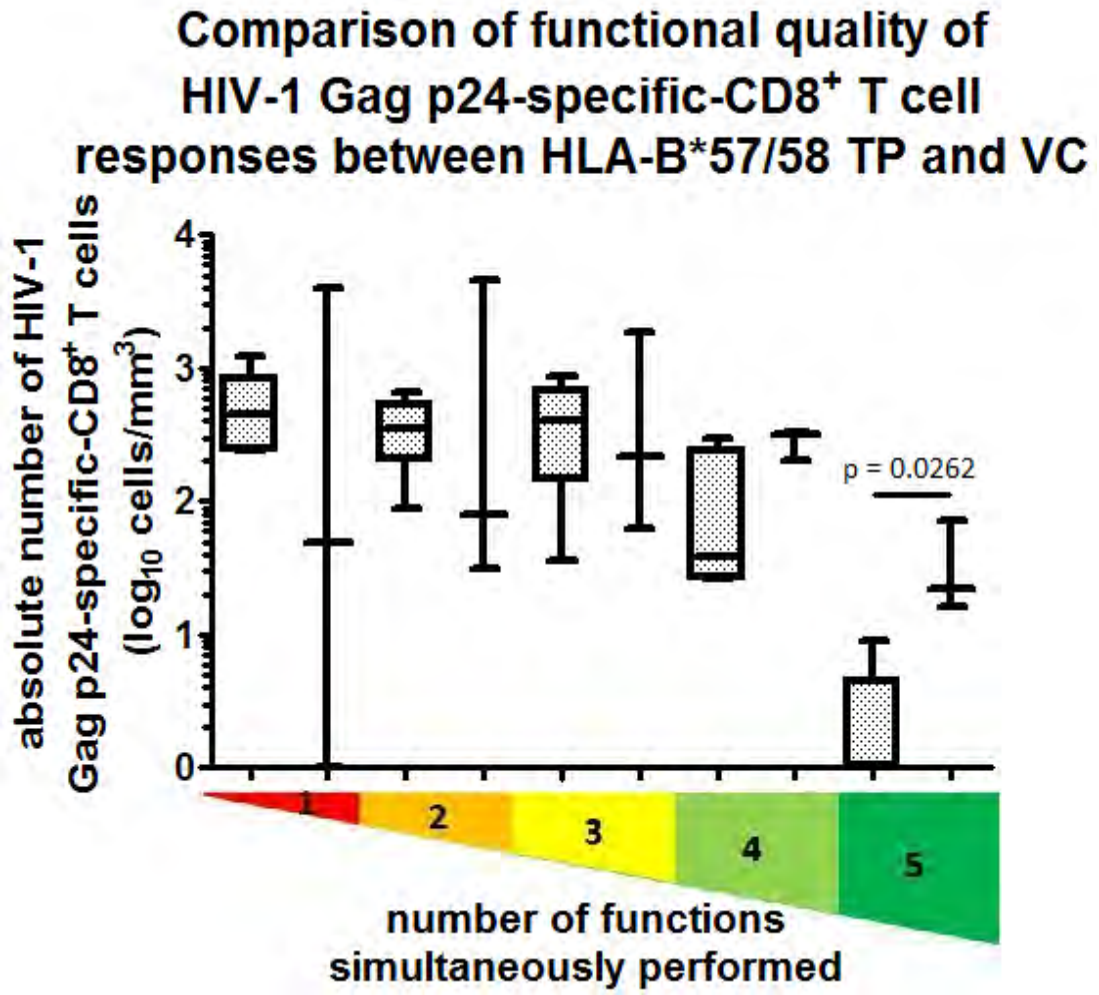


Figure 28. Comparison of the functional quality of HIV-1 Gag p24-specifc-CD8$^+$ T cell responses between HLA-B*57/58 positive typical progressors and viraemic controllers. Absolute number of HLA-B*27 KK10-specific CD8$^+$ T cells which simultaneously responded with 1, 2, 3, 4 or 5 functions (y-axis) were compared between HLA-B*27 positive individuals; typical progressors, TP (dotted column) and viraemic controllers, VC (clear cloumn). Color spectrum on x-axis represented numbers of functions; red =1 function, orange = 2 functions, yellow = 3 functions, light green = 4 functions and green = 5 functions.

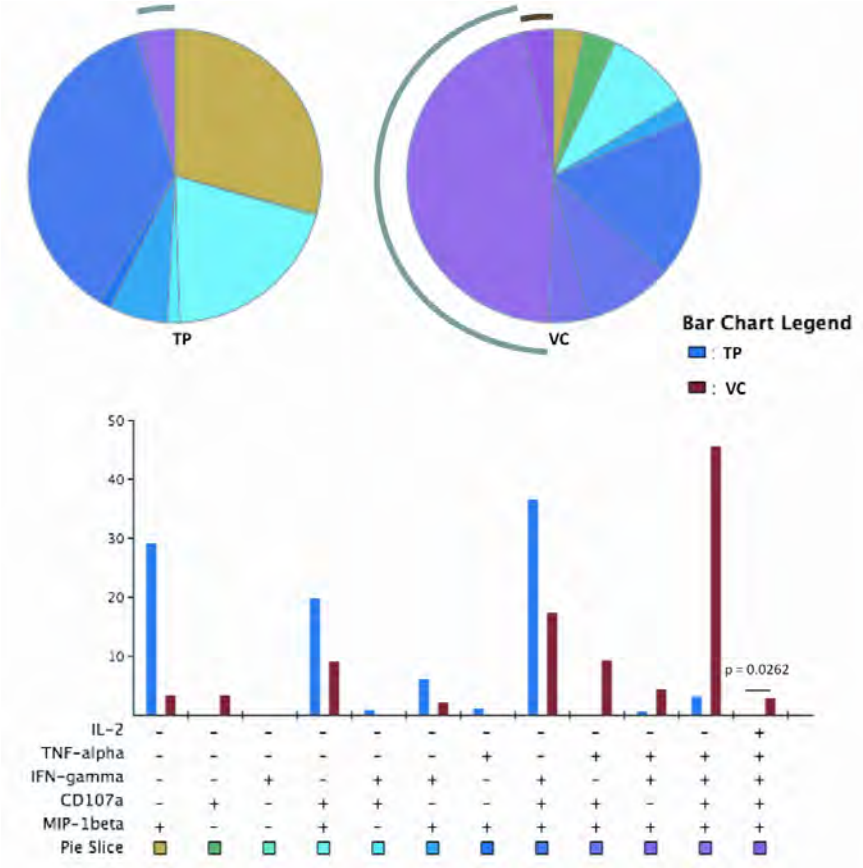


Figure 29. Comparison of HIV-1 Gag p24-specific-CD8$^+$ T cell functional quality between HLA-B*57/58 positive typical progressors (TP) and viraemic controller (VC). Upper pie chart showed a schematic diagram of the contribution of each functionally distinct subpopulation. Each subpopulation was represented by individual color (also shown at the lowest part of the lower bar chart). Each pie slice area represented the proportion of that subpopulation in total responding HIV-1 Gag p24-specific-CD8$^+$ T cell population. Black arc that curved over the pie chart represented the full 5 functions responding subpopulation while the grey arc represented the simultaneous 4 functions responding subpopulations. **Lower bar chart** showed a median absolute number of each functionally distinct subpopulation of HIV-1 Gag p24-specific-CD8$^+$ T cells. Blue bar represented typical progressors while crimson bar represented viraemic controllers. Any subpopulations that were significantly different between HLA-B*57/58 positive TP and VC were indicated by linked-bar and p-value over the two comparative pair. Only the positive subpopulations were shown.

8. Comparison of the functional quality of HLA-B*57/58 restricted epitopes-specific-CD8$^+$ T cell responses between HLA-B*57/58 positive typical progressors and viraemic controllers

There were 6 HLA-B*57/58 restricted epitopes being investigated in this study. However, none of the HLA-B*57/58 positive individuals showed positive ELISpot responses against all 6 epitopes. This resulted in an inadequate number of events for the statistical comparison of CD8$^+$ Tcell responses in a single epitope specific manner. As a consequence, cumulative responses against all 6 epitopes were used for an assessment of the functional quality of these 6 HLA-B*57/58 restricted epitopes specific CD8$^+$ T cell responses.

Larger numbers of full 5 functions responding epitopes-specific-CD8$^+$ T cells were observed in HLA-B*57/58 positive VC than their TP counterparts, however this difference was not statistically significant ($p = 0.0262$, figure 26). Similar to whole HIV-1 Gag p24-sepcific responses, the same HLA-B*57/58 positive TP who possessed full 5 functions responding CD8$^+$ T cells was also capable of mediating the full 5 functions responses

As shown in figure 27, at an epitope specific level, HLA-B*57/58 also possessed high functional quality CD8$^+$ T cells (responded with 4 or 5 functions simultaneously). Though numbers of these high functional quality cells were lower than those of VC, their difference did not reach statistical significance.

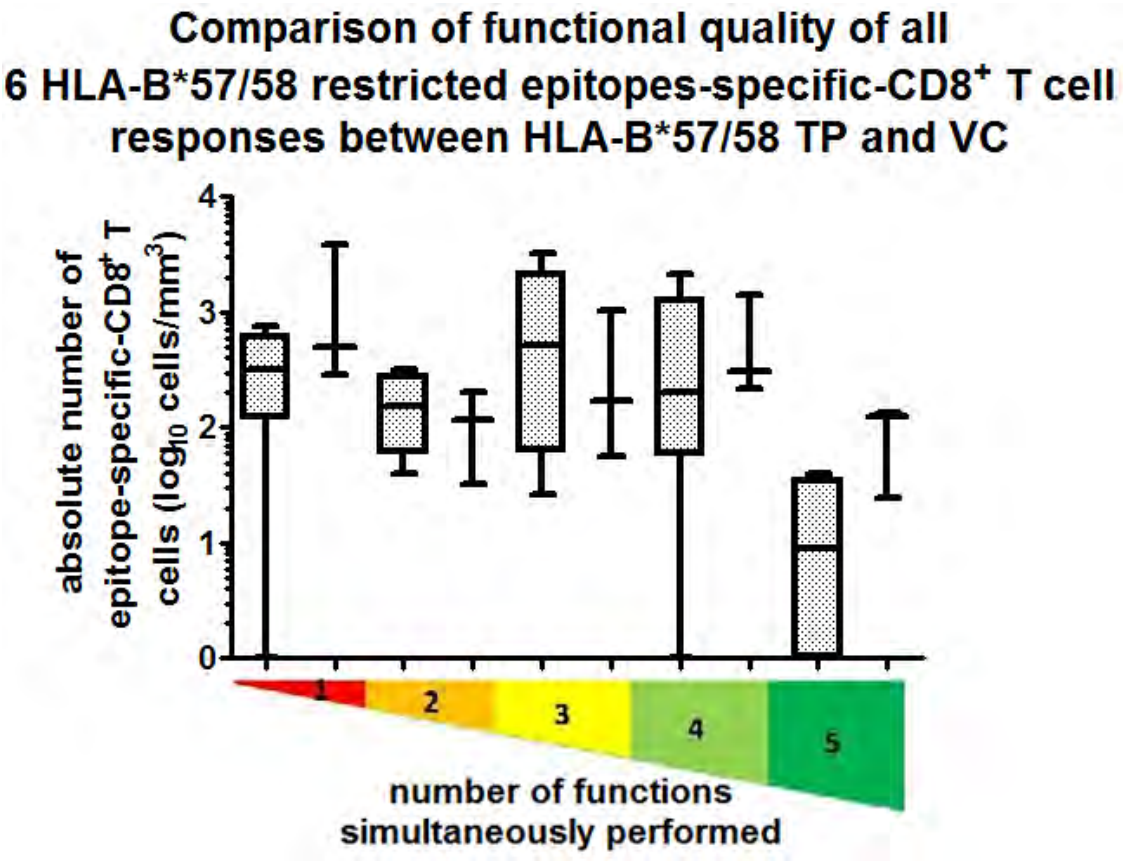


Figure 30. Comparison of the functional quality of all 6 HLA-B*57/58 restricted epitopes-specific-CD8$^+$ T cell responses between HLA-B*57/58 positive typical progressors and viraemic controllers. Absolute number of HLA-B*27 KK10-specific CD8$^+$ T cells which simultaneously responded with 1, 2, 3, 4 or 5 functions (y-axis) were compared between HLA-B*27 positive individuals; typical progressors, TP (dotted column) and viraemic controllers, VC (clear column). Color spectrum on x-axis represented numbers of functions; red =1 function, orange = 2 functions, yellow = 3 functions, light green = 4 functions and green = 5 functions.

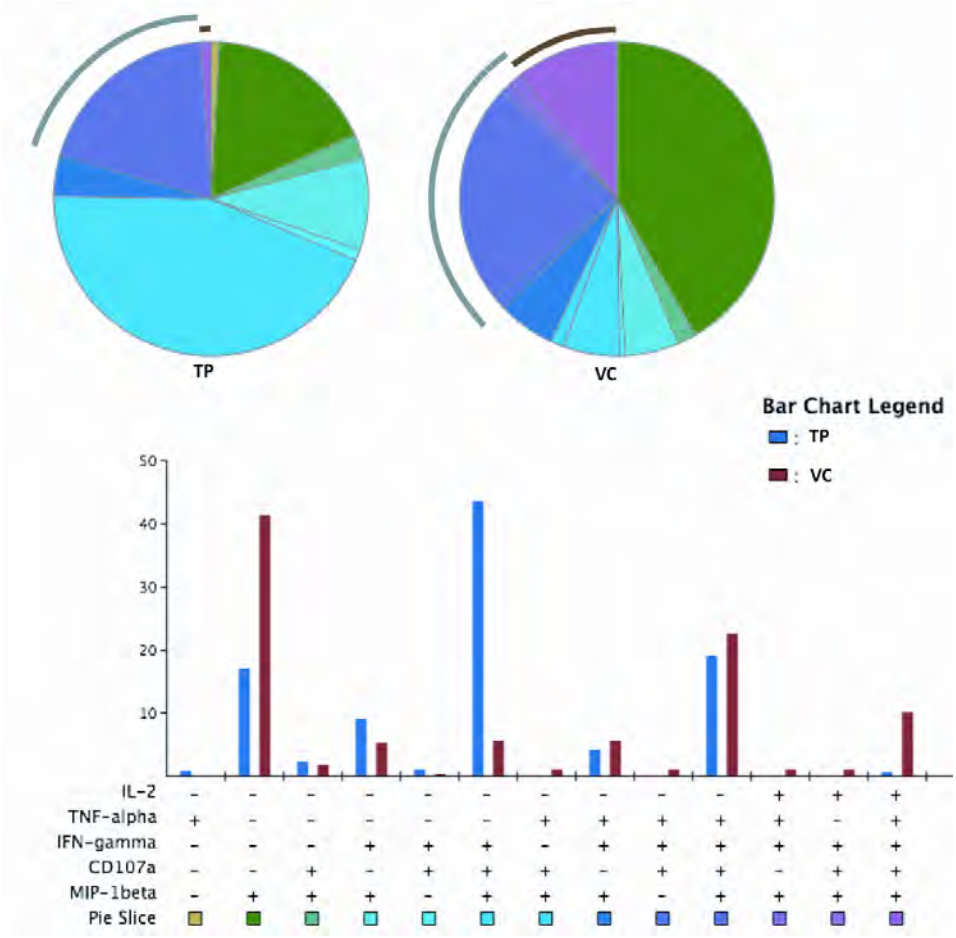


Figure 31. Comparison of the functional quality of CD8$^+$ T cells specifically responded against 6 HLA-B*57/58 restricted epitopes between HLA-B*57/58 positive typical progressors (TP) and viraemic controller (VC). Upper pie chart showed a schematic diagram of the contribution of each functionally distinct subpopulation. Each subpopulation was represented by individual color (also shown at the lowest part of the lower bar chart). Each pie slice area represented the proportion of that subpopulation in total responding HIV-1 Gag p24-specific-CD8$^+$ T cell population. Black arc that curved over the pie chart represented the full 5 functions responding subpopulation while the grey arc represented the simultaneous 4 functions responding subpopulations. **Lower bar chart** showed a median absolute number of each functionally distinct subpopulation of the cumulative number of all 6 HLA-B*57/58 restricted epitopes-specific-CD8$^+$ T cells. Blue bar represented typical progressors while crimson bar represented viraemic controllers. Only the positive subpopulations were shown.

Part IX. Relationship between high functional quality HIV-1 Gag p24-specific-CD8$^+$ T cells and HIV-1 clinical outcomes

Results from our previous experiments had demonstrated an association between high functional quality CD8$^+$ T cell responses and good HIV-1 clinical outcome in both HIV-1 Gag p24 protein specific level and in a single epitope level. Next, the relationship between functional quality of HIV-1 Gag p24-specific-CD8$^+$ T cell responses and readouts of HIV-1 clinical outcome (CD4$^+$ T cell counts and plasma HIV-1 load) were determined. Significantly lower level of pVL was observed in HIV-1 infected donors possessing HIV-1 Gag p24-specific-CD8$^+$ T cells with full 5 functions compared to those who did not (Figure 28A). In addition, these donors with full 5 functions CD8$^+$ T cells had significantly higher level of CD4$^+$ T cell counts than donors lacking these full functional responses (Figure 32B). Furthermore, an absolute number of CD8$^+$ T cells with full 5 functions and 4 functions were strongly in negative correlation with pVL ($r = -0.6984$, $p = 0.0006$ and $r = -0.5729$ $p = 0.0083$, respectively) (Figure 28C and 28E) and in positive correlation with CD4$^+$ T cell counts ($r = 0.5648$, $p = 0.0095$ and $r = 0.4567$, $p = 0.0429$, respectively) (Figure 28D and 28F). These findings suggested that good clinical outcome observed in VC was strongly associated with absolute number of HIV-1 Gag p24-specific-CD8$^+$ T cells with high functional quality.

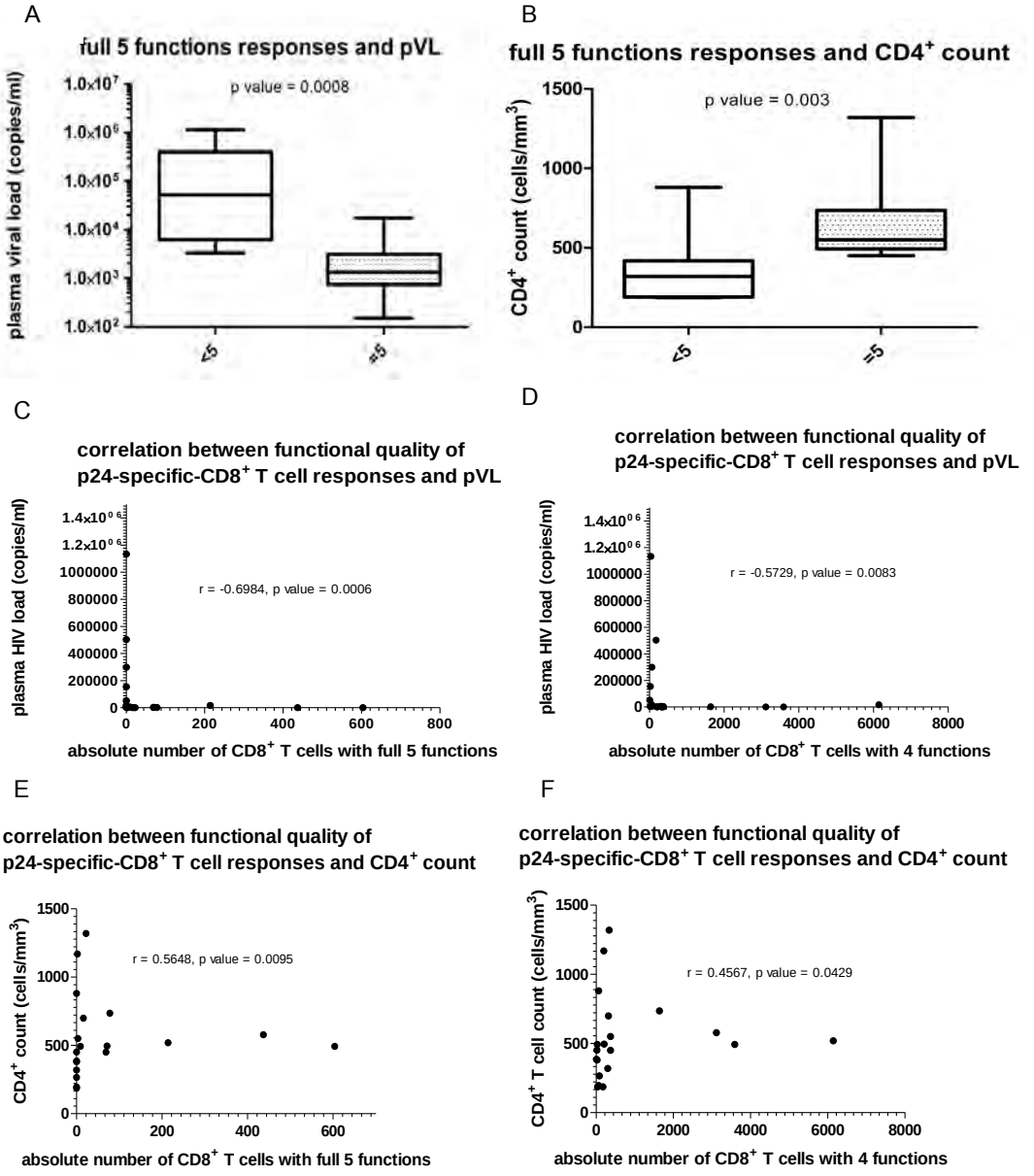


Figure 32. Relationship between HIV-1 Gag p24-specific-CD8$^+$ T cell responded with full 5 functions and HIV-1 control. Both plasma HIV-1 load (**A**) and CD4$^+$ T cell counts (**B**) were compared between donors who possessed HIV-1 Gag p24-specific-CD8$^+$ T cells responded with full 5 functions (=5) and those who did not (<5) Mann-Whitney U test. Plasma HIV-1 load (**C**) and CD4$^+$ T cell counts (**D**) were plotted as a function of absolute number of HIV-1 Gag p24-specific-CD8$^+$ T cell responded with full 5 functions (cells/mm^3). Plasma HIV-1 load (**E**) and CD4$^+$ T cell counts (**F**) was plotted as a function of absolute number of HIV-1 Gag p24-specific-CD8$^+$ T cell responded with full 4 functions (cells/mm^3). Each dot represented each individual. Spearman R test.

CHAPTER VI

DISCUSSION

These studies examined the functional quality of the immunodominant antigen-specific $CD8^+$ T cell responses, which have been shown to play an important role in HIV-1 control [19, 20, 156, 167]. Roles of host immune response in HIV-1 control are better illustrated in a unique group of HIV-1 infected individuals called "HIV controllers" who are able to maintain low plasma HIV-1 load without anti-retroviral treatment for years. In this study, we examined the protective effect of HIV-1 Gag p24-specific-$CD8^+$ T cell responses in control of HIV-1. Here, we demonstrate that mere presence of some certain HLA class-I alleles (HLA-I), have long been defined as "protective alleles" (HLA-B*27, -B*57 and –B*58) does not guarantee this HIV-1 controller status. Our finding demonstrate that an ability to control HIV-1 is rather strongly associated with exact number of HIV-1 Gag p24-specific-$CD8^+$ T cells responded with high functional quality, those responded with full 5 main functions: production of IL-2, TNF-α, IFN-γ, MIP1-β and degranulation of cytotoxic granule (CD107a) [19]. Here, we also demonstrate that discordance in HIV-1 control between individuals carrying the same protective alleles is a result of the functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses against the same immunodominant epitopes of HIV-1 Gag p24 protein.

Two decades of intensive studies have strongly demonstrated the impacts of HIV-1-specific-$CD8^+$ T cells on HIV-1 infection [7, 8, 10, 11, 13]. Among all evidences, association between some certain HLA-I and HIV-1 clinical outcome is the most unequivocal. Some HLA-I associates with good clinical outcomes (low HIV-1 set-point, low plasma HIV-1 load and slow disease progression), namely "protective alleles" while several others with bad clinical outcomes [21]. HLA-I association is a result of the particular HIV-1 epitopes presented on HLA-I molecule since epitope binding specificity differ between each HLA-I and $CD8^+$ T cells recognize specific

antigen in a form of peptide-MHC (HLA) complex (pMHC) [139]. However, we did not find any associations between any specific HLA-I allele, not even the three frequently reported protective HLV-I alleles: HLA-B*27, -B*57 and –B*58, and plasma HIV-1 load in this study.

In HLA-B*27 positive carriers, low plasma HIV-1 load has been observed in those who mounted strong $CD8^+$ T cell responses against an immunodominant HLA-B*27-restricted epitope KRWIILGLNK (KK10, gag 263-272) [14, 168-170]. On the other hand, HLA-B*27 positive carriers who did not respond against KK10 and those with an immune escape mutation within this epitope were observed with significantly higher plasma HIV-1 load [14, 168]. In this study, HLA-B*27 positive individuals were equally composed of those with lower plasma HIV-1 load, "viraemic controllers (VC)" and those with higher, "typical-progressors (TP)". Lose of HIV-1 control in HLA-B*27 positive carrying TP was not due to the weakness in KK10-specific-T cell responses since both TP and VC mounted the same magnitude of responses. Also, this could not be explained by lacking of KK10-specific responses since of 2 KK10 non-responders in this study, each was VC and TP. Lacking of KK10-specific-T cell response observed in this 2 individuals might be explained by their specific HLA-I protein binding specificity since they both carried HLA-B*2706. From several previous studies, HLA-B*27 that was associated with HIV-1 control is HLA-B*2705 [14, 27, 151, 168-170]. There are 4 amino acid polymorphisms between HLA-B*2705 and HLA-B*2706, at residue 77, 114, 116 and 151 [171]. Residue 77 and 116, both are the key residues determining F pocket binding specificity [172]. Changing from acidic aspartic acid to nucleophilic serine at residue 77 might decrease binding affinity between lysine-residue at C-terminal of KK10 epitope. Fortunately, this polymorphism seem to put a minor impact since other donors with HLA-B*2704 (2 VC and 3 TP), which also contain serine at position 77, were able to mount a strong KK10-specific-T cell response. This is not the same in the case of tyrosine at position 116, bulky aromatic side chain of tyrosine residue might confer some physiochemical difficulties in binding with a large basic lysine residue of KK10 which

result in very low binding affinity or, the worse case, lack of binding. Since this study used a fixed concentration of peptides, we were unable to address this speculation.

Due to the limited number of only 1.3% HLA-B*5701 positive carriers in Thai population [173] and since both HLA-B*5701 or HLA-B*5801 are members of HLA-B*58 supertype which share the same epitope binding specificity [172], study of these HLA-I restricted $CD8^+$ T cell responses were considered as a whole group of HLA-B*57/58 positive individuals in this study. Unlike HLA-B*27, HLA-B*57/58 present more than 1 HIV-1 Gag p24 epitope, most of them are located in functional constrained region of the mature capsid protein [25, 28, 29, 174-177]. Hence, a strong association observed between HLA-B*57/58 and HIV-1 control come from an ability to focus $CD8^+$ T cell response toward several high fitness cost epitopes presented by these 2 HLA-I alleles [21]. On the contrary, multiple escape mutations of these epitopes which resulted in narrowing of breadth of T cell responses have been shown to be associated with lose of control [28, 29]. In this study, which the difference in plasma HIV-1 load (pVL) was strongly significant between HLA-B*57/58 positive TP and VC (p value = 0.0091), both breadth and magnitude of T cell responses as assayed by IFN-γ ELIspot were similar. The similar frequency of IFN-γ responding T cells observed between VC and TP is in agreement with several previous studies suggesting that another property of HIV-1 Gag p24-specific-$CD8^+$ T cells which might contribute to or at least, associate with HIV-1 control are needed to be address [177].

Several previous studies have suggested that quality, not the quantity, of HIV-1-specific-$CD8^+$ T cell response is important in a natural HIV-1 control, as high quality HIV-1-specific-T cells responses have been frequently observed in both HIV-1 slow-progressors (those with stable $CD4^+$ T cell count over a decade) and elite controllers (those with undetectable plasma HIV-1 load) maintain [5, 19, 156, 167]. Facing with a unique scenario that HIV-1 infected individuals carrying the same protective HLA-I alleles, mounting the similar magnitude of responses against the same high fitness cost rendering epitopes of HIV-1 Gag p24 protein, yet still with significantly different plasma HIV-1 load, have provided the best opportunity to study the role of

$CD8^+$ T cell response functional quality in natural HIV-1 control. In this study, the functional quality of HIV-1 Gag p24-specific $CD8^+$ T cell responses was determine whether the discordances in HIV-1 clinical outcome observed in the protective HLA-I matched individuals were associated with qualitative differences in their $CD8^+$ T cell responses. Instead of cells proportion in total lymphocytes, this study investigated HIV-1 Gag p24-specific-$CD8^+$ T cells as absolute number in order to point out that cell adequacy is also an important factor in real-life control of HIV-1. In general, whole HIV-1 Gag p24-specific responses in VC were with higher functional quality than that of TP regarding both percentage and absolute number. Similar to several previous studies, most TP's HIV-1 Gag p24-specific-$CD8^+$ T cells responses were with 2 or 3 functional combinations (CD107a, MIP1-β and IFN-γ) [19, 156]. Though some TP also responded with higher quality, their numbers were significantly lower than that of VC. This finding suggest that actual number of $CD8^+$ T cells responded with high functional quality are important in HIV-1 control since both VC and TP were with the same number of total $CD8^+$ T cells. Moreover, higher functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell responses of VC was not attributed from any particular protective-alleles since limited functional quality of responses were also observed in both HLA-B*27 and HLA-B*57/58 positive TP. This protective alleles-independency was also shown in a previous study [178].

Several factors have been reported to be in association with high functional quality of $CD8^+$ T cell responses in HIV controllers, for instance loss of antigenic stimulus, antigen sensitivity, proliferative capacity, cellular senescence and diversity of repertoire [141, 151, 155, 179, 180]. Decreased number of specific antigen due to escape mutation has been shown to be associated with loss of high functional $CD8^+$ T cell clone and HIV-1 control [151, 179]. This is not the case in our study since comparison between VC and NC were conducted in a specific response matching manner, thus only responding individuals were compared. Recent study has shown that functional quality of specific-$CD8^+$ T cell clone is governed by their antigen sensitivity [155]. Evidently, $CD8^+$ T cell responses against HIV-1 infection are mostly

oligoclonal, composing of high and low antigen sensitivity clones [155, 181]. In the presence of strong antigenic stimulation, $CD8^+$ T cell clones with high avidity were either driven toward proliferative senescence or were selectively depleted [182]. Thus, poor quality of immunodominant epitope-specific responses in TP could be partially explained by losing of high antigen sensitive $CD8^+$ T cell clones. The true question is how VC maintains their high quality of responses since they are also facing with strong antigenic stimulation. One explanation is a strong proliferative capacity observed in VC [141]. Though this study did not directly investigate proliferative capacity, exclusive presence of IL-2 producing $CD8^+$ T cells in VC specific responses suggesting their preserved capacity to proliferate [183, 184]. Though, immunodominant $CD8^+$ T cells of VC are also selectively depleted, HIV control is continuously maintained by renewal of similarly high sensitivity clones. This constantly renewal of dominant $CD8^+$ T cells is reflected by the high clonal-turnover rate in previous study [151]. Size and diversity of specific-$CD8^+$ T cell repertoire are other important factors. Larger repertoire has more chance to provide high antigen sensitivity $CD8^+$ T cells than narrower one. An association observed between HLA-B alleles and high quality of $CD8^+$ T cell responses partially strengthen this speculation since HLA-B are the largest and the most diverse allele of all 3 HLA-I alleles [180, 185]. However, the difference in $CD8^+$ T cell repertoire is beyond the scope of this study.

In conclusion, this study supports the idea that functional quality of HIV-1 Gag p24-specific-$CD8^+$ T cell response is the main contributing factor leading to a natural HIV-1 control. Though some particular HLA-I alleles might help focusing immune response toward highly conserved, high fitness cost rendering epitopes on HIV-1 Gag p24 protein, without an adequate number of high functional quality $CD8^+$ T cells, an HIV controller status is not achieved. The significantly strong negative and positive correlation observed between absolute numbers of high quality HIV-1 Gag p24-specific-$CD8^+$ T cells with plasma HIV-1 load and $CD4^+$ T cell count, the 2 standard clinical readouts of HIV-1 infection, strongly supported the protective roles of these high quality HIV-1 Gag p24-specific-$CD8^+$ T cells. Moreover, this superior functionality

observed in HIV controllers is independent of age, sex, duration of HIV-1 infection or not restricted by any particular HLA-I type.

In order to develop an universally-effective disease-modifying HIV-1 vaccine, further studies should aim at elucidating the mechanisms conferring high quality of $CD8^+$ T cell responses against any HLA-I restricted conserved targets.

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APPENDICES

APPENDIX A

REAGENTS, MATERIALS, AND INSTRUMENTS

A. Reagents

Absolute ethanol	(Merck, Germany)
Agarose (molecular biology grade)	(Research Organics, USA)
Alkaline phosphatase substrate	(Biorad, USA)
Anti-human CD28	(Beckton Dickinson, USA)
Anti-human CD49d	(Beckton Dickinson, USA)
Anti-human interferon-gamma monoclonal antibody 1-D1K	(Mabtech, Sweden)
Anti-human interferon-gamma monoclonal antibody 7-B6-1-Biotin	(Mabtech, Sweden)
APC-conjugated anti-human TNF-α monoclonal antibody	(Biolegend, USA)
APC-H7 conjugated anti-human CD3 monoclonal antibody	(Beckton Dickinson, USA)
BCA protein assay kit	(Pierce Biotechnology, USA)
Brefeldin A	(Sigma-Aldrich, Germany)
Cytofix-Cytoperm reagent	(Becton Dickinson, USA)
Dimethyl sulphoxide	(Sigma-Aldrich, Germany)
DNA ladder mix (100 base pair)	(Fermentas, Canada)
DNAse	(Sigma-Aldrich, Germany)

DNAse-RNAse-free distilled water	(Invitrogen, USA)
dNTPs (molecular biology grade)	(Fermentas, Canada)
Ethylenediaminetetraacetic acid	(Research Organics, USA)
Fetal bovine serum	(Bio Whittaker, USA)
Ficoll Hypaque	(AmershamBioscience, Sweden)
FITC-conjugated anti-human IL-2 monoclonal antibody	(Biolegend, USA)
Isopropanol	(Merck, Germany)
Pacific blue conjugated anti-human CD8 monoclonal antibody	(Biolegend, USA)
Paraformaldehyde	(Sigma-Aldrich, Germany)
PE-conjugated anti-human MIP1-β monoclonal antibody	(Beckton Dickinson, USA)
PE-Cy5 conjugated anti-human CD107a monoclonal antibody	(Biolegend, USA)
PE-Cy7-conjugated anti-human IFN-γ monoclonal antibody	(Biolegend, USA)
Penicillin	(Invitrogen, USA)
Peptide	(Mimotope, USA)
Phytohemagglutinin	(Sigma-Aldrich, Germany)
Phosphate buffered saline	(Sigma-Aldrich, Germany)
RPMI 1640 medium	(Gibco, USA)
Sodium azide	(USB, USA)

Staphylococcus enterotoxin B	(Sigma-Aldrich, Germany)
Streptavidin-conjugated alkaline phosphatase	(Mabtech, Sweden)
Streptomycin	(Invitrogen, USA)
Sybergreen DNA gel stain	(Invitrogen, USA)
Trypan blue	(Sigma-Aldrich, Germany)
Tween-20	(Sigma-Aldrich, Germany)

B. Materials

Barrier tip (10, 200, and 1000 µl)	(Sorenson, USA)
DNAse-RNAse-free clear tube (1.5 ml)	(Axygen, USA)
EDTA blood collection tubes	(Beckton Dickinson, USA)
Electrophoresis chamber	(MiniRun GE-100, China)
Freezing container	(Nalgene, USA)
Neubauer hemocytometer	(Boeco, Germany)
Ninety-six-well plate	(Corning, USA)
Pipette gun	(Accu-jet, USA)
Pipette tip (10, 200, and 1000 µl)	(Bioscience, USA)
Polyvinylidene difluoride plate	(Millipore, USA)
Sterile Combitips® (10 ml)	(Eppendorf, Germany)

Sterile cryo-tube (2 ml)	(Sarstedt, Germany)
Sterile serological pipette (1, 2, 5, 10, 25 ml)	(Corning, USA)
Sterile polypropylene tube (15, 50 ml)	(Becton Dickinson, USA)
Thin-wall flat-cap DNAse-RNAse-free tube (200 µl)	(Axygen, USA)
Twenty four-well plate	(Corning, USA)

C. Instruments

Automatic pipette (0.2-2.5, 2-20, 20-200, and 100-1000 µl)	(Eppendorf, Germany)
Centrifuge (Beckman Coulter Allegra X-15R)	(Beckman Coulter, USA)
Centrifuge (5418)	(Eppendorf, Germany)
Flow cytometer (FACS Aria II)	(BD Biosciences, USA)
Freezer (-80 °C)	(Thermo scientific, USA)
Gel-Doc	(Biorad, USA)
ELIspot reader	(Karl Zeiss, USA)
Incubator	(Thermo scientific, USA)
Liquid nitrogen tank	(Giss, USA)
Multichannel pipette (20-200 µl)	(Brand, Germany)
NanoDrop spectrophotometer	(Thermo scientific, USA)
Pipette controller (Accu-jet pro)	(BrandTech, USA)

Thermocycler (Eppendorf Mastercycler S)	(Eppendorf, Germany)
Vortex	(Brand, Germany)
Waterbath	(Grant, UK)

APPENDIX B

REAGENTS PREPARATION

Reagent preparation for sequencing

1. Sybergreen DNA gel stain

Sybergreen DNA gel stain	5 µl
1X TBE buffer	50 ml

2. 1% agarose gel

Agarose	0.3 g
1X TBE buffer	30 ml

Reagent preparation for determination of T cell response

1. ICS wash solution

0.02% Na azide	80 µl
FBS	1 ml
1X PBS	100 ml

2. 1x Perm/wash solution

10X Perm/wash solution	10 ml
Sterile DDW	90 ml

3. 1% Paraformaldehyde

10% Paraformaldehyde	1 ml
1X PBS	9 ml

4. Trypan blue

Trypan blue	3 ml
1X PBS	10 ml

5. Freezing medium

DMSO	1 ml
FBS	4 ml

6. Thawed medium

2 mg/ml DNAse	100 µl
FBS	2 ml
RPMI 1640 medium	8 ml

7. R10

FBS	50 ml
RPMI 1640 medium	450 ml

8. PBS with Tween-20

Tween-20	500 µl
1X PBS	1 L

BIOGRAPHY

Mr. Navapon Techakriengkrai was born on January 26th, 1983. He graduated his Doctor of Veterinary (DVM) degree from Chulalongkorn University, Thailand in 2007. He had been trained in general equine veterinary practice at Faculty of Veterinary Medicine, Chiangmai University during 2007-2008. During his DVM study, he had 2 research projects on antimicrobial agent combination testing against pathogenic swine respiratory bacteria, which had been presented at 2 international veterinary conferences. He had received funding from CHULALONGKORN UNIVERSITY GRADUATE SCHOLARSHIP TO COMMEMORATE THE 72nd ANNIVERSARY OF HIS MAJESTY KING BHUMIBOL ADULYADEJ for his Master degree study. This study had been presented at Keystone Symposia "Protection from HIV: Targeted Intervention Strategies" at Whistler conference center, Whistler, British Columbia, Canada, during March 20-25, 2011.