IMMUNOPHENOTYPES, CYTOKINE PRODUCTION, AND CYTOTOXIC ACTIVITY OF NATURAL KILLER CELLS IN HIV-1 INFECTED CASES

SUJIN ASSAWAWITOONTIP

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IMMUNOPHENOTYPES, CYTOKINE PRODUCTION, AND CYTOTOXIC ACTIVITY OF NATURAL KILLER CELLS IN HIV-1 INFECTED CASES

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ABSTRACT

This research emphasized the investigation of NK cell numbers and their function in HIV-1 infected cases. In addition, the lymphocyte immunophenotype reference ranges of $CD3^+$, $CD4^+$ and $CD8^+$T cells, $CD19^+$B cells, $CD16^+$, $CD56^+$ and $CD16^+/CD56^+$NK cells and CD4/CD8 ratios were established in a total of 125 HIV seronegative healthy Thai adults. Flow cytometric analyses demonstrated relatively lower $CD4^+$T counts, but a higher NK cell count in Thais as compared to Caucasians. Our study was supported by other groups of investigators on Thai and other Mongoloid people. Statistical analyses showed that females had a significantly higher total $CD3^+$T cells, but lower NK cell counts as compared to males ($p<0.05$); and approximately 86% of NK cells carried both CD16 and CD56 molecules on the same cell. Regarding age variation, an increase of 1.1% of $CD4^+$T cells per decade was seen. We also studied the changes in lymphocyte subsets in correlation with the level of $CD4^+$T cell count in HIV seropositive subjects with $CD4^+$T cells<14 or $\geq$14%. The results showed that a decrease in $CD4^+$T cell count had resulted in an increase in number of $CD8^+$T cells and NK cells. Meanwhile, the change on $CD4^+$T cell count had no effect on level of $CD19^+$B cell count.

A three color flow cytometric analysis was used to detect intracellular cytokine/chemokine production in 10 HIV seronegative and 28 HIV seropositive subjects. It was revealed upon stimulation with PMA and ionomycin that both NK and $CD8^+$T cells were important sources of IFN-γ and MIP-Iβ ; whereas RANTES and MIP-Iα were mainly produced by $CD8^+$T cells. Moreover, the study demonstrated that HIV seronegative and seropositive subjects are not different in the ability to produce chemokine/cytokine.

NK cell cytotoxic activity assay was performed in 69 HIV seronegative and 107 HIV seropositive subjects by using a standard ^{51}chromium release assay against K-562 target cells and its cytotoxic activity was expressed as lytic unit (LU20) per 10^6PBMCs. The results showed that NK cell cytotoxic activity in HIV seronegative subjects had no difference by sex with an average LU20 of 10.5 ± 8.4. A decrease in NK cytotoxic activity was observed in the late stage of HIV infection i.e., in subjects with $CD4^+$T cells<14% as compared to HIV seronegative subjects. However, level of $CD4^+$T cell count in HIV infected cases had no correlation with level of NK cell cytotoxic activity. Collective data from this study suggested that NK cells may be an alternative to $CD8^+$T cells and neutralizing antibodies in the prevention against HIV disease progression. A longitudinal study is needed in order to explore this point of view.

KEY WORDS: IMMUNOPHENOTYPES/ CYTOKINE/ CYTOTOXIC ACTIVITY/ NK CELLS/ HIV
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IMMUNOPHENOTYPES, การผลิตสารไซโตไคน์, และการทำลายเซลล์แปลกปลอม ของเซลล์ NATURAL KILLER ในผู้ติดเชื้อเอชไอวี ไทป์ 1 (IMMUNOPHENOTYPES, CYTOKINE PRODUCTION, AND CYTOTOXIC ACTIVITY OF NATURAL KILLER CELLS IN HIV-1 INFETED CASES)

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บทคัดย่อ

โครงการวิจัยนี้ เน้นการศึกษา ชนิด จำนวนและการทำงานของเซลล์เอ็นเคในผู้ติดเชื้อเอชไอวี และการศึกษานี้ยังได้สร้างค่าอ้างอิงมาตรฐานของเซลล์ลิมโฟไซต์ชนิดอื่น ๆ อีกด้วย ได้แก่ เซลล์$CD3^+$, $CD4^+$ และ $CD8^+$T cells, $CD19^+$B cells, $CD16^+$, $CD56^+$ และ $CD16^+/CD56^+$NK cells และอัตราส่วน CD4/CD8 ในคนไทยที่มีสุขภาพแข็งแรง ไม่มีการติดเชื้อเอชไอวี จำนวน 125 คน การศึกษา lymphocyte immunophenotypes โดยวิธี flow cytometry พบว่าคนไทยมีปริมาณ $CD4^+$T cells น้อยกว่าชาวคอเคเชียน ซึ่งคล้ายกับที่มีผู้เคยรายงานไว้ในคนไทยหรือชาวมองโกลอยด์ในประเทศอื่น ในแง่การศึกษาเซลล์เอ็นเค ได้พบว่าชาวไทยมีจำนวนเซลล์นี้มากกว่าชาวคอเคเชียน และเพศหญิงมีปริมาณ $CD3^+$T cells สูงกว่าในเพศชาย แต่ในเพศชายมีปริมาณ เซลล์เอ็นเค สูงกว่าในเพศหญิง และเซลล์เอ็นเคประมาณร้อยละ 86 มีโมเลกุลของ CD16 และ CD56 อยู่บนเซลล์เดียวกัน และการศึกษาความผันแปรทางอายุ พบว่าจำนวนของเซลล์CD4 จะเพิ่มขึ้นร้อยละ 1.1 เมื่ออายุมากขึ้นทุก10 ปี

งานวิจัยได้ทำการวิเคราะห์หาการเปลี่ยนแปลงจำนวนของ immune cells ชนิดต่างๆ เมื่อจำนวน $CD4^+$T cells เปลี่ยนแปลงไป โดยทำการศึกษาในผู้ติดเชื้อเอชไอวี 2 กลุ่ม คือกลุ่มที่มี $CD4^+$T cells<14% และ$\geq$14% และพบว่าเมื่อจำนวน $CD4^+$T cells ลดลง จำนวน$CD8^+$T cells และเซลล์เอ็นเค จะเพิ่มขึ้น แต่จำนวน B cells จะไม่มีการเปลี่ยนแปลง

การศึกษาความสามารถของเซลล์ในการสร้างสารไซโตไคน์ และคีโมไคน์ โดย flow cytometry ในเม็ดเลือดขาวของผู้ไม่ติดเชื้อเอชไอวี จำนวน 10 คน และผู้ติดเชื้อจำนวน 28 คน ด้วยการกระตุ้นด้วย PMA และ ionomycin แสดงให้เห็นว่า ทั้งเซลล์เอ็นเค และ$CD8^+$T cells เป็นแหล่งสำคัญในการผลิตสารอินเตอร์เฟอรอนแกมม่า และ MIP-Iβ ในขณะที่เซลล์ซึ่งสร้างสารRANTES และ MIP-Iα ส่วนใหญ่เป็น $CD8^+$T cells และเซลล์ของคนปกติ และผู้ติดเชื้อเอชไอวี ไม่มีความแตกต่างกันในด้านความสามารถในการผลิตสารไซโตไคน์ และคีโมไคน์

จากการศึกษาการทำลายเซลล์แปลกปลอม โดยเซลล์เอ็นเค (NK cell cytotoxicity assay) ในคนปกติจำนวน 69 ราย และผู้ติดเชื้อเอชไอวี 107 ราย ด้วยวิธีการปลดปล่อยสารโครเมียม-51 จากเซลล์เป้าหมาย K562 โดยวัดเป็นค่า lytic unit (LU20)/10^6PBMCs พบว่าค่าเฉลี่ย LU20$\pm$ค่าเบี่ยงเบนมาตรฐานในคนปกติคือ 10.5$\pm$8.4 และค่าที่ได้ไม่มีความแตกต่างทางด้านเพศ และการศึกษานี้แสดงว่าการทำงานของเซลล์เอ็นเค ในผู้ติดเชื้อเอชไอวีลดลงเมื่อเปรียบเทียบกับคนปกติ อย่างไรก็ตาม การศึกษาในผู้ป่วยเอชไอวี ไม่พบความสัมพันธ์ระหว่างปริมาณ $CD4^+$T cells และการทำงานของเซลล์เอ็น เค ในการทำลายเซลล์แปลกปลอม

การศึกษานี้ชี้แนะว่าเซลล์เอ็นเค น่าจะเป็นเซลล์ที่ดีในการป้องกันและควบคุมการดำเนินโรคในผู้ติดเชื้อเอชไอวี นอกเหนือไปจากเซลล์ $CD8^+$และนิวทรัลไลซิ่ง แอนติบอดี การศึกษาติดตามผู้ป่วยอย่างต่อเนื่อง จะให้ข้อมูลที่ชัดเจนขึ้น

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LIST OF ABBREVIATIONS

%	Percentage
^{51} Cr	Chromium-51 radioisotope
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AIDS	Acquired immunodeficiency virus
ARC	AIDS related complex
BFA	Brefeldin A
bp	Base pair
CA	California
CD	Cluster of differentiation
CDC	Center for Disease Control
Ci	Curie
cm	Centimeter
CO_2	Carbon dioxide
cpm	Count per minute
CRF	Circulating recombinant form
CT	Connecticut
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Effector
e.g.	exampli gratia (for example)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
Env	Envelope glycoprotein
env	Envelope gene
et al.	et alii (and others)

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LIST OF ABBREVIATIONS (Continued)

F	Female
FACS	Fluorescence activated cell sorter
Fc	Fragment crystallizable
FcγR	Fc gamma receptor
FCS	Fetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
g	Gram
GMCSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HEPES	N-2 hydroxyethylpiperazine-2-ethane sulfonic acid
HIV	Human immunodeficiency virus
I	Ionomycin
i.e.	id est (that is)
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
Kb	Kilobase
KCl	Patassium chloride
KD	Kilo Dalton
lbs	Pound (load balance system)
LGL	Large granular lymphocyte
LTR	Long terminal repeat
LU	Lytic unit
M	Male
MA	Matrix
mAb	Monoclonal antibody

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LIST OF ABBREVIATIONS (Continued)

MCP	Macrophage chemoattractant protein
MD	Maryland
MHC	Major histocompatibility complex
Min	Minute
MIP-Iα	Macrophage inflammatory protein-I alpha
MIP-Iβ	Macrophage inflammatory protein-I beta
ml	Milliliter
mM	Millimolar
MO	Missouri
mRNA	Messenger ribonucleic acid
$Na_2{}^{51}CrO_4$	Sodium chromate (Radioactived isotope)
NaCl	Sodium chloride
NaOH	Sodium hydroxide
N-CAM	Neural cell adhesion molecule
Nef	Negative factor
NJ	New Jersey
NK	Natural killer cell
NY	New York
nm	Nanometer
No	Number
NS	Not significant
O.D.	Optical density
^oC	Degree celsius
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridin chlorophyl protein

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LIST OF ABBREVIATIONS (Continued)

PFA	Paraformaldehyde
PGL	Persistant generalized lymphadenopathy
pH	Potential of hydrogen
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulation-upon activation, normal T-cell expressed and secreted
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT	Room temperature
SD	Standard deviation
SDF-1	Stromal-derived factor 1
SDS	Sodium dodecyl sulfate
sIg	Surface immunoglobulin
T	Target
T_H	T helper
U	Unit
μg	Microgram
μl	Microliter
USA	United States of America
UV	Ultraviolet
Vpr	Viral accessory gene product
WBC	White blood cell

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CHAPTER I
INTRODUCTION

Determination of the distribution of immunoregulatory cells in the peripheral blood is important in the management of diseases that involve alterations in lymphocyte subpopulations. Changes in the immunoregulatory lymphocyte subsets have been found in many pathological conditions such as immunodeficiency including AIDS (1, 2), sarcoidosis (3), measles (4) and leukemia (5). The process of measuring the number of lymphocyte subsets in the whole blood sample is referred to as immunophenotyping and is usually accomplished by flow cytometric analysis. Profound changes of immune cell number occur throughout the HIV disease course. Therefore, lymphocyte immunophenotypes can be used as markers of immune status which is important to aid in monitoring of HIV disease progression and the response to therapy (6).

The high rate of antigenic variation renders HIV the ability to escape from both humoral and cell-mediated immune response. There are clear evidences that rapid mutation in the cytotoxic T lymphocyte (CTL) epitopes can lead to an escape from CTL recognition (7, 8). Moreover, during the course of HIV infection, the Nef protein downregulates MHC class I expression and may thus reduce the role of CTLs which results in viral persistence. HIV antigenic variation has no affect on NK cell recognition of the infected cells. Moreover, the downregulation of MHC class I molecules on the infected cells may render themselves more sensitive to NK cell-mediated cytotoxicity (9). Previous studies had shown that Thai people had higher frequency of NK cells as compared to the Caucaseans (10). So in this study, we focused on activities of NK cells, the cell which is an important component of innate and adaptive immune system.

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NK cells were defined by the capacity to bind and kill certain tumor, parasitic and virus-infected cells without prior sensitization. They can function nonspecifically in mediating lytic activity in term of antigen recognition; they also function specifically through the mechanism of antibody dependent cell cytotoxicity (11-14). After NK cells are activated by the infection, they release cytokines and chemokines that induce inflammatory responses, modulate hematopoiesis, control monocyte and granulocyte cell growth and function, and influence the type of adaptive immune responses that follow. Peripheral blood NK cell number and functional activity are in general, stable for a given individual overtime (15). However, NK cell number and function are altered in many diseases, including HIV, cancers, viral infections and autoimmune disease. In particular, NK cells are thought to be important in early host defense against viral infection, including HIV.

Variable levels of deficiency in both NK cell number and function has been shown in adult patients with AIDS (16, 17). Rapid disease progression after HIV infection also has been correlated with a decrease in NK cell activity (18). Furthermore, NK cells are important sources of cytokines and CC-chemokines which regulate immune response and suppress HIV entry or replication *in vitro* (19, 20). Vitale *et al,* (20) found a higher percentages of IFN- γ producing cells in NK cell population than in T cells in both healthy and HIV infected subjects. Moreover, Kottilit *et al*, demonstrated that NK mediated suppression of HIV replication is as potent as that of $CD8^{+}$T cells; The suppression is mediated predominantly by secretion of CC-chemokines (19, 21).

This current research studied the lymphocyte immunophenotype subpopulations in HIV seronegative subjects and the HIV seropositive cases with different disease stages. In addition, NK cell functions: both cytolytic activity and the cytokine/ chemokine production were compared between the HIV seronegative and the HIV seropositive cases.

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CHAPTER II
OBJECTIVES

This study was conducted to investigate the various properties of NK cells including lymphocyte immunophenotypes, cytokine/ chemokine production and cytotoxic activity in HIV seronegative and HIV seropositive subjects. The aims of this studies were:

1. To determine lymphocyte immunophenotype reference ranges of $CD3^+$T, $CD4^+$T, $CD8^+$T, $CD19^+$B as well as NK cells in HIV-1 seronegative, healthy Thais
2. To determine the changes in lymphocyte immunophenotype subsets in HIV seropositive subjects in accordance to level of $CD4^+$ T cell count
3. To determine cytokine and chemokine production in NK cells and $CD8^+$T lymphocytes
4. To measure NK cell cytotoxic activity in HIV seronegative, healthy subjects and HIV-1 seropositive subjects by using ^{51}Cr–release assay

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CHAPTER III
LITERATURE REVIEW

1. Human immunodeficiency virus (HIV)

HIV is devided into two groups denoted type 1 (HIV-1) and type 2 (HIV-2). As viewed by electron microscopy, HIV has the characteristics of a lentivirus with a cone-shaped capsid core of 80-100 nm in diameter. The surface of the HIV is characteristically made up of 72 knobs containing trimers of the envelope glycoproteins. The envelope structures are derived from a 160 kd precursor, gp160, which is cleaved into gp120 external surface envelope protein and a gp41 transmembrane protein (22, 23). The virion gp120, located on the virus surface, contain the binding site(s) for the cellular receptor(s) and the major neutralizing domains (24). The capsid core is composed of the viral p24 Gag protein. Inside this capsid are two identical RNA strands of approximately 9.2 kb in length, with which the viral RNA-dependent DNA polymerase or reverse transcriptase (RT) and the nucleocapsid proteins are closely associated (Fig. 1) (25). Diagram of HIV virion is shown in Figure 1. The HIV genome includes the structural genes *gag, pol,* and *env* genes and six regulatory genes, namely *tat*, *rev*, *vif*, *nef*, *vpr* and *vpu* (found in only in HIV-1) or *vpx* (found only in HIV-2) of which their products regulate the replication cycle. The *gag* sequence encodes the viral core; *pol* sequence encodes RT, integrase, and viral protease enzymes required for viral replication; and the *env* sequence encodes the envelope glycoprotein gp120 and gp41, which are required for infection of cells (26, 27). HIV genomic organization and the encoded proteins are shown in Figure 2.

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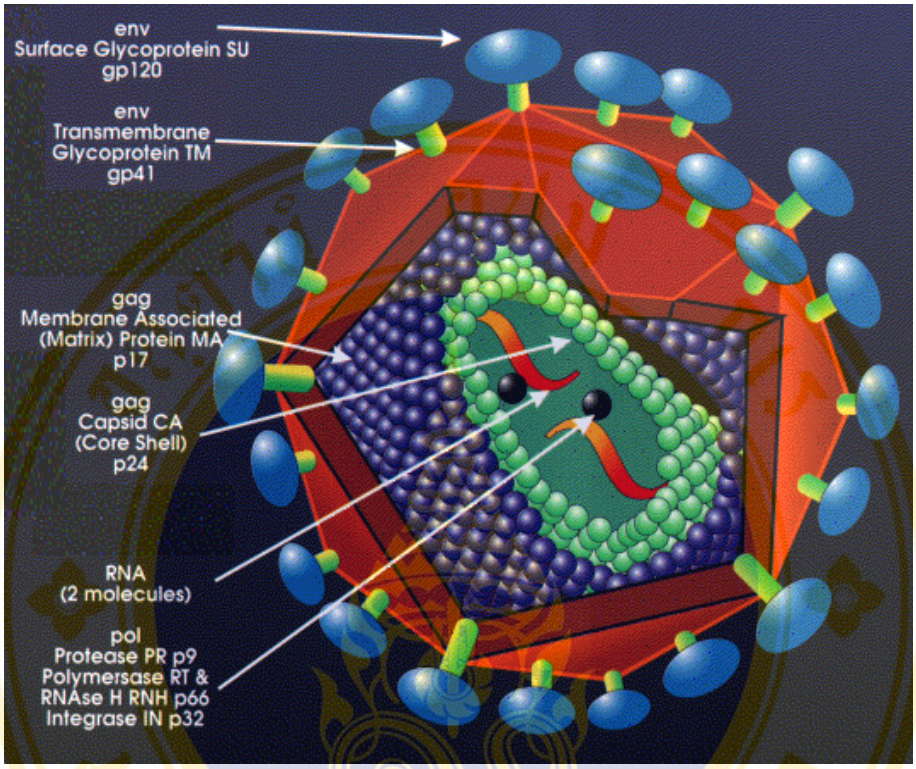

Figure 1. The basic structure of HIV particle
(http://www.critpath.org/aric/library/img002.htm)

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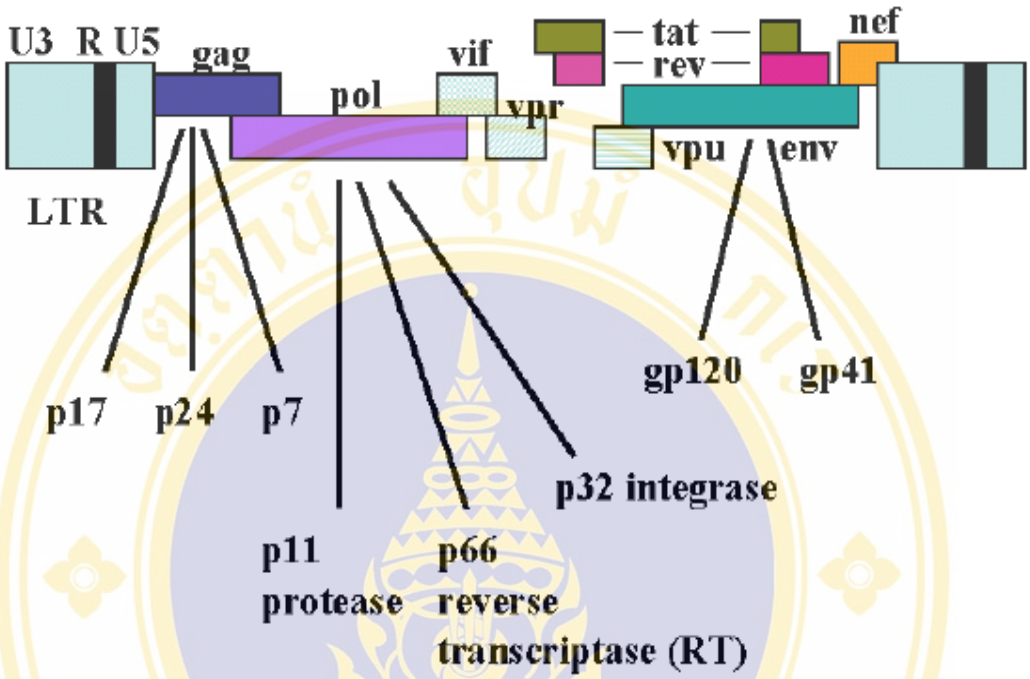

Figure 2. Genomic organization and feature of the HIV genome (http://www.hivmedicine.com)

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Phylogenetic analysis of *env* gene sequences of HIV-1 isolates obtained from various countries led to the classification of HIV-1 into three distinct groups: M, N and O. The predominant M or major group contains 9 subtypes or clades: A-D, F-H, J and K. Similary, six subtypes of HIV-2 (A-F) have been identified. Moreover, there is extensive variability within each subtype (28). Subtype C is found in the highest frequency among all subtypes, nearly all the identified HIV-1 subtypes (A-E, G, H, J and O) can be found in sub-Saharan Africa (25, 29, 30). Subtype B was responsible for the first homosexual epidemic, but also shown to be capable for spreading parenterally and heterosexually. Subtype B is the most common subtype found in the United States and Western Europe. While subtype E predominates in Thailand and Southeast Asia where its primary mode of transmission is heterosexual. The subtype predominated in Thailand are B' and E. Subtype E takes part of more than 90% of the cases. The Thailand subtype B' is different from the subtype B viruses in North America by the amino acids in its V3 loop (GPGQ or GPGH *versus* GPGR) (25). The subtype E in Thailand is currently renamed as circulating recombinant form (CRF) 01_ AE . CRF 01 A_E is the mosaic virus of which most of the genome is derived from subtype A, but *env* gene and assignment of the LTR are devired from subtype E (31, 32).

2. Pathogenesis of HIV infection

Transmission of HIV occurs through direct contact with infected body fluids including blood products, semen, vagina and cervical secretion, amniotic fluid and breast milk via three major routes : sexual transmission, parenteral transmission and vertical transmission from mother to newborn. Sexual transmission is the great majority of HIV infections worldwide. HIV has been reported to infect a wide range of cells *in vitro*, including $CD4^+$T bone marrow cells, precursor $CD4^+$ bone marrow cells, $CD8^+$ T cells, B cells, NK cells, eosinophils, peripheral blood dendritic cells, follicular dendritic cells, immature thymic precursor cells, Langerhans cells, megakaryocytes, astrocytes, oligodendroglia, renal epithelial cells, cervical cells, rectal and bowel mucosal cells, goblet, and columnar epithelial cells, trophoblastics cells, as well as cells and tissues from organs such as liver, lungs, salivary glands, eyes, prostate gland, testes, and adrenal gland (33, 34). Because the only cell types

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that are consistently found to be infected with HIV *in vivo* are $CD4^+T$ lymphocytes and macrophage-lineage cells, the relevance of *in vitro* viral replication in other cell types to HIV disease is unclear at present. It is now accepted that the major viral reservoir during asymptomatic phase of HIV infection is lymph node tissue. There are two sources of HIV in lymphoid tissue, the productively infected cells (mononuclear cells, including both $CD4^+T$ lymphocytes and monocyte/macrophages) and the virions trapped as immune complexes on the follicular dendritic cell network of germinal center. It has been postulated that these trapped virions could act as a source of infectious virus (35-37).

The life cycle of HIV-1 can be divided into early and late phases. The early phase of HIV replication begins with the attachment of mature virion to the target cell. The entry of HIV-1 into the target cell is mediated by interaction between the viral envelope glycoprotein, the CD4 receptor, and the HIV-1 coreceptor. The primary HIV-1 coreceptors are the chemokine receptor CCR5, used by R5 HIV-1 (M-tropic, nonsyncytial inducing HIV-1), and CXCR4 used by X4 HIV-1 (T-tropic, syncytial inducing HIV-1) (38, 39). The entry process is a two-step event where an initial interaction between gp120 on envelope and CD4 molecules on cell surface elicits conformational changes in gp120 to bind the chemokine receptor binding site. Subsequent interaction of the gp120-CD4 complex with the relevant chemokine receptor molecule results in further conformational changes in the gp41 subunit which in turn facilitates the fusion event between the viral and host cell membranes, respectively, and then entry and uncoating in cytoplasm (40). Once inside the cytoplasm, the conversion of the viral RNA into double-stranded DNA provirus commences as the viral reverse transcriptase becomes active. Reverse transcriptase synthesizes a double-stranded DNA copy from the single-stranded viral RNA and generating a DNA provirus. The viral DNA migrates to and enters the host cell nucleus (a process facilitated by the HIV proteins Vpr and MA) and integrates into the host genome by the catalytic activity of integrase enzyme. Provirus can then remain latent or be active. When the host cell divides, the provirus is transmitted to daughter cells as part of host cell chromosomes. During the late phase of replication cycle, proviral DNA will be transcribed into species of viral mRNAs and later into

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the viral RNA genomes . The viral components assembly into nucleocapsid which will bud from cytoplasmic membrane and generate hundred of progeny virions (41, 42).

3. Natural history of HIV infection

The immunopathogenesis of HIV infection is extremely complex. A variety of virologic and immunologic mechanisms contribute to the progressive deterioration of immune function and to progression of HIV disease to AIDS. It is now recognized that the typical case may take 8-10 years or more for AIDS to develop after seroconversion (43). A dramatic increase in the knowledge of the natural history of HIV infection has yielded insights into mechanisms of HIV disease pathogenesis. Although the course of HIV infection may vary somewhat among individual patients, a common pattern of development has been recognized (Fig. 3). The typical course of HIV infection is devided into 3 phases as follows;

Primary infection : This period is followed by the development of detectable humoral and cellular immune responses. After exposure to HIV, an infected individual becomes acutely viremic and HIV is widely disseminated during this early stage of infection, particularly in lymphoid organs (44). Moreover, within one week to three months, there is an immune response to the HIV infection and the HIV-specific immunity initiates during this stage which is associated with a dramatic decline in viremia (43, 45). However, this immunity is apparently inadequate to suppress viral replication completely, since the viruses can persist in lymph node (46).

Clinical latency : The period of inapparent infection may last for years. All patients have a gradual deterioration of the immune system, and manifest by the depletion of $CD4^{+}$T lymphocytes (47).

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Clinically apparent disease : The inevitable outcome of the progressive deterioration of the immune system that occur in most patients with HIV infection is clinically apparent disease or an acquired immunodeficiency syndrome (AIDS)-defining illness, either severe with persistent constitutional sign and symptoms or an opportunistic infection or neoplasm (48).

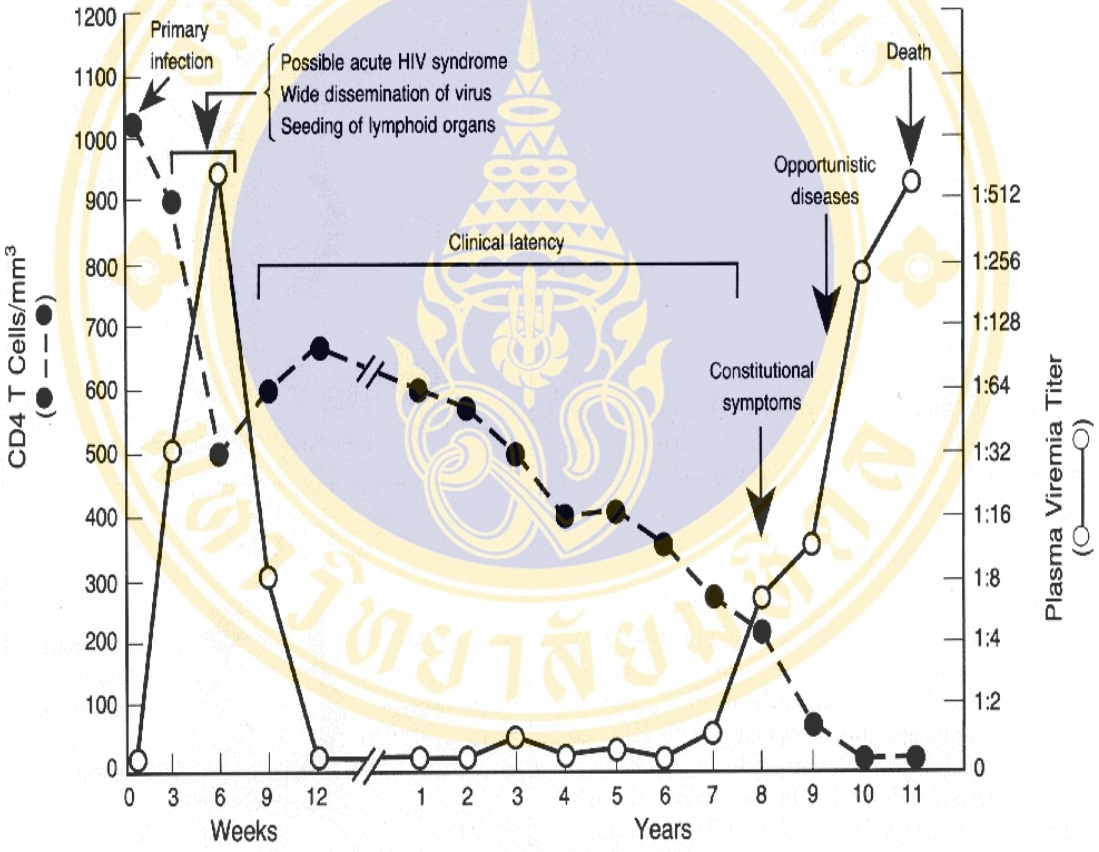

Figure 3. Typical course of HIV infection

(Pantaleo G *et al*. N Eng J Med 1993; 328: 327-35)

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On the basis of the duration of HIV infection and the kinetics of virologic and immunologic events, three dominant patterns of evolution of HIV disease have been described as follows (45):

a) Typical progressors : 80 to 90 % of HIV infected persons are "typical progressors". Progression from clinically apparent disease to AIDS occurs within 8 to 10 years as the results from the continuous replication of virus in the lymphoid tissue and severe impairment of immune function (45).

b) Rapid progressors : This category occurs in 5 to 10% of HIV-infected persons. Rapid progression to AIDS occurs within 2 to 3 years after seroconversion (49). Immune response is usually defective, level of antibodies against HIV proteins and neutralizing antibody are low to absent in this group of patients (50).

c) Long-term nonprogressors : This group comprises a small percentage (5%) of infected persons who do not experience clinical progression of HIV infection, and have stable $CD4^{+}$T cell count, absence of symptoms, and both HIV specific humoral and cell-mediated immune responses are very strong for many years (7 or more years) despite lack of therapy (51).

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4. Lymphocyte immunophenotypes

Lymphocyte subpopulations comprising T, B and natural killer (NK) cells have been sucessfully enumerated by flow cytometry employing a panel of monoclonal antibodies to immunophenotypic surface markers. Determination of lymphocyte immunophenotypes may be useful in the disease diagnosis, prognosis, assessing the efficacy of treatment and/or searching for clues to pathogenesis. However, normal values of certain lymphocyte subsets can vary according to age (1, 10, 52-61), sex (1, 10, 53, 56, 57, 61), ethnic group (1, 10, 53, 56, 58, 60, 61), circadian rhythm (62) and methodologies (2, 63-65). Changes of lymphocyte immunophenotypes from the reference ranges were reported in several diseases of infectious and noninfectious origins, e.g. AIDS (2, 66), measles (4), cancer (67) and diabetes mellitus (68). In persons infected with HIV, level of CD4 cell count is established as a guideline for AIDS case definition or disease staging, starting anti-retroviral drugs and prophylaxis for opportunistic infections as well as a marker of treatment success (69-71).

Collective data from Asian countries including Malaysia, Hong Kong and Thailand suggested that Mongoloid people have less $CD4^+$ T cells, but higher NK cells as compared to the Caucaseans (Table 1)(1, 10, 56, 58). High NK cell number, therefore, may be a compensation for low CD4 cell count. Thai clinicians often come across with AIDS cases who have low CD4 cell count, but are still capable to sustain normal life activity. The published data on lymphocyte immunophenotypes in healthy adults of Caucasean and Mongoloid origins was shown in Table 1 (1, 10, 53-56, 72, 73).

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Table 1. Published data on lymphocyte immunophenotypes in healthy adults of Caucasean and Mongoloid origins

Lymphocyte subset	Reichert *et al.* [a] (n=261)	Erkeller Yuksel *et al.* [a] (n=101)	Hulstaert *et al.* [a] (n=85)	Choong *et al.* [b] (n=212)	Webster *et al.* [b] (n=217)	Kam *et al.* [b] (n=208)	Vithayasai *et al.* [b] (n=150)	Webster *et al.* [b] (n=143).	Lee *et al.* [b] (M=33,F=38)
	Number of cells by % and ranges; and absolute number and ranges								
$CD3^+$	73±6.2%	72 (67-76)% 1.4 (1.1-1.7) [c]	75 (71-79)% 1.3 (1.0-1.5) [c]	67.5±8.5%	M (n=131); 65.3±8.4% F (n=86); 70.2±6.4%	69±7.7% (1.37±0.4) [c]	64.0±8.8% (1.63±0.6) [c]	69±8.5% (1.36±0.43) [c]	M; 67.5(61-73)%, 0.8(0.7-1) [c] F; 71(67-76)%, 1.1(0.9-1.3) [c]
$CD4^+$	43±7.5%	42 (38-46) % 0.8 (0.7-1.1) [c]	48 (43-54)% 0.7 (0.6-.98) [c]	35.5±7.8%	Both (n=154); 37.4±6.8%(0.84±0.240) [c] M (n=90); 36.1±6.7% (0.82±0.25) [c] F (n=64); 40.4±6.5% (0.93±0.22) [c]	36.4±7.5% (0.73±0.23) [c]	36.1±6.4% (0.91±0.3) [c]	40±8.0% (0.78±0.28) [c]	M;33.5(27-39)%,0.4(0.4-0.5) [c] F;37(30-43)%, 0.5(0.4-0.7) [c]
$CD8^+$	33±7.5%	35 (31-40) % 0.7 (0.5-0.9) [c]	32 (28-37) %	36.8±8.5%	M (n=131); 27.6±6.6% F (n=86); 30.2±6.4%	29.7±7.2% (0.59±0.21) [c]	25.7±7.3% (0.67±0.35) [c]	26±6.9% (0.51±0.20) [c]	M; 28(24-32)%, 0.4(0.3-0.4) [c] F;30(26-35)%, 0.4(0.4-0.8) [c]
CD4/CD8	1.4±0.6	1.2 (1.0-1.5)	1.8 (1.4-2.4)	1.1±0.6	1.35±0.48	1.0±0.4	1.5±0.6	1.7±0.7	M; 1.2 (0.9-1.5) F; 1.2(1.0-1.6)
$CD19^+$	14±4.2%	13 (11-16) % 0.3 (0.2-0.4) [c]	13 (11-16) % 0.21 (0.16-0.27) [c]	12.4±4.5%	14.7±4.5%	11.1±3.9% (0.22±0.11) [c]	-	14±4.8% (0.28±0.14) [c]	M; 10(9-14)%, 0.1(0.1-0.2) [c] F;12(10-15)%, 0.2(0.1-0.2) [c]
NK	14±6.1%	14(10-19) % 0.3 (0.2-0.4) [c]	11 (8-15) %	17.9±8.1%	M (n=131); 20.6±8.5% F (n=85); 15.3±6.4%	19.8±8.1% (0.39±0.19) [c]	-	17±8.7% (0.34±0.21) [c]	M; 20(17-28)%, 0.3(0.2-0.4) [c] F;14(10-19)%, 0.2(0.1-0.3) [c]
WBC	5.7-5.9 [c]	5.9 (4.6-7.1) [c]	5.3 (4.4-6.3) [c]	-	-	-	7.8±2.3 [c]	5.8±1.5 [c]	M; 4.8 (4.1-6.7) [c] F; 5.1(4.4-6.7) [c]
Lymphocyte	30-33%	32 (28-39) %	31 (27-34) % 1.6 (1.3-1.9) [c]	-	2.4±0.5%	30.6±7.7% (1.98±0.53) [c]	33.8±8.1%	34±7.7% (1.97±0.59) [c]	M; 30(23-32)%, 1.3 (1.1-1.5) [c] F; 27(24-33)%, 1.5(1.2-1.9) [c]

a = Caucaseans ; b = Mongoloids ; c = absolute number (x 10^3 cell/ µl)

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5. Lymphocyte immunophenotypes in HIV infected subjects

Profound changes of immune cellular activation occur after infection with HIV and persist throughout the disease course. Prospective studies of the natural history of HIV infection have demonstrated that immunological deterioration often begins soon after HIV-1 seroconversion (74). Subsequently, most infected individuals experience a progressive loss of immunologic capacity and a decline in the number of circulating $CD4^+$lymphocytes with a corresponding increase of $CD8^+$T lymphocytes, leading to a drastically reduced CD4/CD8 ratio (74, 75-77). Sampalo *et al*. reported that HIV-infected patients had a striking increase in amount of B lymphocytes (78, 79). Regarding NK cell number, there are several studies with conflict results. One study had reported on the reduction of $CD4^+$T cell count in associated with a decrease in number of circulating NK cells (80, 81); however, the other studies had demonstrated that numbers of circulating NK cells did not differ significantly between HIV seropositive and HIV seronegative subjects (82, 83). De Souza *et al*. (84) reported that there was no significantly different in NK cell number as compared between the HIV-seronegative and seropositive Thais, while the significant difference was demonstrated as compared between HIV-seronegative and seropositive North Americans. Moreover, Jason *et al*. (85) demonstrated an increase in the percentage of NK cells in HIV seropositive Thais as compared with HIV-seronegative subjects. Additionally, extensive prospective study by Ullum *et at*. in Europe demonstrated that the amount of NK cells was of no prognostic value for HIV disease progression (86).

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6. HIV classification system for adolescents and adults

CDC has revised the classification system for HIV infection to emphasize the clinical importance of the $CD4^+$T lymphocyte count in the categorization of HIV related clinical conditions. The classification system establishes mutually exclusive subgroups for which the spectrum of clinical conditions is integrated with the $CD4^+$T lymphocyte count. The 1993 revised classification system for HIV infection is based on three ranges of $CD4^+$T lymphocyte counts and three clinical categories and is represented by a matrix of nine mutually exclusive categories (Table 2) (87, 88).

The three $CD4^+$ T lymphocyte categories are defined as follow :

Category 1 : $\geq$ 500 cells/ μl

Category 2 : 200-499 cells/ μl

Category 3 : < 200 cells/ μl

The clinical categories of HIV infection are defined as follow:

Category A

Category A consists of more of the conditions listed below in an adolescent or adult (≥13 years) with documented HIV infection. Conditions listed in categories B and C must not have occurred.

- Asymptomatic HIV infection
- Persistent generalized lymphadenopathy
- Acute (primary) HIV infection with accompanying illness or history of acute HIV infection

Category B

Category B consists of symptomatic conditions in HIV infected adolescent or adult that are not included among condition listed in clinical category C and that meet at least one of the following criteria: a) the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity; or b) the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection. Examples of conditions in clinical category B include, but are not limited to:

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- Bacillary angiomatosis
- Candidiasis oropharyngeal (trush)
- Candidiasis vulvovaginal
- Cervical dysplasia/ cervical carcinoma *in situ*
- Constitutional symptoms such as fever (38.5^{o} C) or diarrhea (lasts >1 month)
- Hairy leucoplakia, oral
- Herpes zoster (shingles), involving at least two distinct episodes
- Idiopathic thrombocytopenic purpura
- Listeriosis
- Pelvic inflammatory disease, peripheral neuropathy.

Category C

Category C includes the clinical conditions listed in the AIDS surveillance case definition. For classification purpose, once a category C condition has occurred, the person will remain in category C. The conditions included in AIDS surveillance case definition as follow;

- Candidiasis of bronchi, trachea, esophageal, or lungs
- Cervical cancer, invasive
- Coccidiodomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestine (>1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV-related
- Herpes simplex: chronic ulcer (>1 month's duration); or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis disseminated or extrapulmonary
- Isosporiasis, chronic intestine (1 month's duration)
- Kaposi's sarcoma
- Lymphoma, Burkitt's (or equivalent term)
- Lymphoma, primary of brain

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- *Mycobacterium avium* complex or *M. kansasii*, disseminated or extrapulmonary
- *Mycobacterium tuberculosis*, any site (pulmonary or extrapulmonary)
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
- *Pneumocystis carinii*
- Pneumonia, recurrent
- Progressive multifocal leukoencephalopathy
- *Salmonella* septicemia, recurrent
- Toxoplasmosis of brain,
- Wasting syndrome due to HIV.

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Table 2. Summary of CDC 1993 revised classification system for HIV infection for adolescents and adults

$CD4^+$T cell categories	**Clinical categories**		
	(A) Asymptomatic, acute HIV or PGL	**(B) Symptomatic, not (A) or (C) conditions**	**(C) AIDS-indicator conditions**
1) >500 cells /μl	A1	B1	C1
2) 200-499 cells /μl	A2	B2	C2
3) < 200 cells /μl	A3	B3	C3

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7. Equivalences for $CD4^+$T lymphocyte count by absolute numbers and percentages

Enumeration of $CD4^+$T lymphocytes in term of absolute number is more accurate than that presented in term of percentage upon repeated measurements. However, data correlating natural history of HIV infection with the $CD4^+$T cell percentages have not been as consistently available as the data on absolute $CD4^+$T lymphocyte count. Therefore, the CDC classification system emphasizes the use of $CD4^+$T lymphocyte counts by absolute number, but the use of $CD4^+$T lymphocyte by percentages are also accepted. Estimation for an equivalence between absolute number and percentages of $CD4^+$T lymphocyte count is shown in Table 3 (87).

Table 3. Equivalence between absolute numbers and percentages

$CD4^+$T cell category	$CD4^+$T lymphocytes / μl	% $CD4^+$ T lymphocyte
(1)	≥ 500	≥ 29
(2)	200-499	14-28
(3)	< 200	<14

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8. Immune response to HIV infection

The immune system in HIV infection has been divided into innate and adaptive component, each with a different function and role (89, 90). Innate immunity is the first line of defense against invading infection. The mechanism of innate immunity exist before an encounter with the viruses and are rapidly activated by the viruses before the development of adaptive immune response. The innate immune system consist of epithelial barriers, circulating effector cells (granulocytes, macrophages, NK and dendritic cells) and circulating effector protiens (e.g. complements) (26). The NK cells display at least two effector functions that can contribute to control of infection : first, they can kill infected cells ; second, they are a rich source of inflammatory cytokines, especially IFNγ and chemokines such as RANTES, MIP-1α and MIP-1β with antiviral activity. Macrophages and dendritic cells also play a key role in the early control of infection by secreting IL-2 and IL-18 that can induce IFNγ , to elicit direct and indirect antiviral effects (91).

The adaptive immune response consists of cellular and humoral immunity which are important in controlling the HIV infection. Humoral immunity directs antibody response within 6 to 9 weeks after infection to a variety of HIV antigens. However, little evidences show that the antibodies have any beneficial effect in limiting the disease (26). The cellular immune response play a vital role in HIV pathogenesis. The studies of the long-term nonprogressors, and the exposed yet uninfected persons have suggested that certain aspects of cellular immunity may protect against HIV infection or slow disease progression (41). Two aspects of cellular immune response are the cytotoxic T-cells and the response that release cytokines (92-94) and lyse target cell expressing foreign antigens; and T-helper cells which is responsible directly or indirectly for induction of a wide array of lymphoid and lymphoid cell functions. These effects include activation of macrophages; induction of cytotoxic T cells; NK cells and B cells; and secretion of a variety of soluble factors that induce growth and differentiation of lymphoid cells and affect hematopoietic cells (95). Cellular immunity generally occurs in parallel with the humoral (antibody) response, although the two can be separated in certain circumstances. The infection usually evoke both arms of immune, which broadly differ in their function: the cellular immune response

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controls the infection and the humoral response prevents further infection with the same agent (96). There is clear evidence that both during acute and chronic phases of HIV infection, rapid mutation in epitopes which are recognized by HIV-specific CTL may occur. In addition, the Nef protein can down regulate the molecules of MHC class I expressing on the surface of infected cell, thus lead to a reduction in the role of CTLs (97). These events result in HIV escape from the immune response. However, HIV antigenic variation has no affect on NK cell activity and MHC Class I down regulation may render themselves more sensitive to NK cell-mediated cytotoxicity.

9. Cytokines and chemokines

Cytokines are important mediators generated during immune response and are produced by a variety of activated cells, such as $CD8^+$T cells, T_H0 and T_H1 $CD4^+$T cells and NK cells (98, 99). They play an important role in regulating the immune response and determining resistance or susceptibility to disease. Chemokines constitute a large family of small cytokines with four conserved cysteins linked by disulfide bonds. When chemokines bind to HIV coreceptors, they are potent and selective inhibitors of HIV infection. Cytokines are a family of proteins that mediated many of responses of innate and adaptive immunity (26). The same cytokines may be produced by many cell types and individual cytokine often acts on diverse cell types. They are synthesized in response to inflammation or antigenic stimuli and usually act locally, in an autocrine or paracrine fashion, by binding to high affinity receptors on target cells (27). Thus, cytokines serve many functions that are critical to host defense against the pathogens and provide link between innate and adaptive immunity (26). Depending on the nature of the infecting virus, the infected cells may be triggered to produce anti-viral cytokines and chemokines that inhibit one or more steps in the viral life cycle (90), thereby limiting the extent of the infection. The complex network of cytokines involved in inflammatory and immunoregulatory responses has an important role in several components of the pathogenesis of HIV infection and AIDS (100).

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Recently, several cytokines, e.g., IFN-γ, IFN-α, IFN-β, IL–2, IL–12 and IL-16 have been demonstrated to profoundly affect the state of HIV expression, overall spreading, and suppress HIV infection. In addition, some chemokines e.g., RANTES, MIP-Iα, MIP-Iβ, SDF-1, MCP-1, MCP-2, MCP-3 and IP-10 function as the HIV-suppressive factors by interacting with chemokine receptors. When viruses infect cells, production of IFN is induced, and consequently, viral replication is inhibited. Interferon is believed to be part of the body's first line of defense against viral infection and to be the first cytokine to be recognized. Interferon regulate humoral and cellular immunity and has broad cell growth regulatory activities (27, 98, 100-102).

IFNs are cytokines that play a complex and central roles in the resistance of mammalian host to pathogens. IFNs, although best known for their anti-viral properties, are potent regulators of cell growth and have immunomodulatory activity (103-105). IFN is a family of related proteins that may be grouped into two types, I and II. Type I IFNs are composed of two distinct groups of proteins called IFN-α and IFN-β which are synthesized by many cell types after viral infection. Double-stranded RNA is a potent inducer of IFN synthesis (26, 104). There are two distinct families (I and II) of IFN-α. IFN-α (I) is 166 amino acid long whreas IFN-α (II) has 172 amino acids. Major sources of IFN-α are lymphocytes, monocytes, macrophages. IFN-β is related to IFN-α with 30% amino acid sequence homology and source of IFN-β are fibroblast and some epithelial cells. IFN-α and IFN-β seem to share the same receptor and have very similar biological activities (26, 106).

The action of type I IFNs are to protect cells against viral infection and promote cell-mediated immunity against intracellular microbes by inhibiting viral replication, increase expression of class I MHC molecule, stimulates the development of T_H1 cells and inhibit the proliferation of many cell types (26, 106). IFN-α is the first cytokine for which a role as an endogenous suppressor factor of HIV replication has been reported. Addition of the neutralizing antibody to IFN-α into the PBMC culture derived from HIV infected cases has been shown to increase both the levels of HIV replication and the frequency of positive viral (107, 108). Defective production of

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IFN-α *in ex vivo* has been described in HIV infected individuals (109). While some reports demonstrated high level of IFN-α during HIV infection (110, 111). Regarding IFN-β, significant changes in the number of HIV proviral copies PBMCs have not been found after treatment of HIV infected patients with this agent (112). It implies that IFN-β may not play significant role in HIV infection.

Type II IFN or IFN-γ is the principle macrophage-activating cytokine and serves critical functions in innate immunity and in specific cell-mediated immunity. It has some anti-viral activity, but it is not a potent anti-viral IFN, and it functions mainly as an effector cytokine of immune response. IFN-γ is a homodimeric protein produced by NK, $CD4^{+}T_H0$, and T_H1, and $CD8^{+}T$ cells. In innate immunity, NK cells secrete IFN-γ in response to recognition of unknown components of microbes or in response to IL-12; in this setting IFN-γ functions as a mediator. In adaptive immunity, T cells produce IFN-γ in response to antigen recognition, and this is enhanced by IL-12. The sequence of reactions involving IL-12 and IFN-γ is central to cell-mediated immunity against intracellular microbes. IFN-γ functions by ;1) providing the T lymphocytes and NK cells to activate macrophages to kill phagocytosed microbes (104, 113) stimulation of expression of class I and class II MHC molecules (114); 3) acting as costimulator on antigen presenting cells (26); 4) promoting the differentiation of naïve $CD4^{+}$ T cells to the T_H1 subset and inhibiting the proliferation of T_H2 (114, 115); 5) acting on B cells to promote switching to certain IgG subclasses; 6) activating of neutrophils (104); and 7) stimulating to the cytolytic activity of NK cells (116).

Several studies have described an elevation of IFN-γ production in HIV-infected individual; however, there are conflicting reports on the status of IFN–γ production by cells isolated from HIV infected patients. Highly increased constitutive production of IFN-γ has been observed in PBMCs of asymptomatic HIV-infected patients with CDC group II/III as well as patients who have progressed to CDC group IV, as compared to healthy subjects (117, 118). However some studies demonstrated that PBMCs from HIV-infected individuals produce less IFN-γ in response to the stimuli (119, 120). Vitale *et al.* demonstrated that both in HIV uninfected and HIV-infected subjects, IFN-γ was produced from NK cells in a higher percentages than T cells (20).

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Chemokines are 8-10 kd peptides that are the product of distinct genes clustered to human chromosomes 4 and 17. Chemokines regulate to selectively promote the rapid adhesion, chemotaxis and activation of leukocyte effector subpopulations (121, 122). In addition, some are able to regulate the proliferative potential of hematopoietic progenitor cells, endothelial cells, and certain types of transformed cells (123, 124). Chemokines act via a family of G-protein-coupled receptors and these types of receptor are usually large ligand (60 amino acids). Chemokine can be divided into two groups, α and β by the arrangement of the first two of four conserved cysteins. Members of the β chemokines posses the first two conserved cysteins which are adjacent. The gene for the β chemokines is on human chromosome 17, and they generally activate monocytes, lymphocytes, basophils and eosinophils. The major human chemokine β family includes MIP-1α, MIP-1 β and RANTES which have been identified as the major M-tropic HIV suppressive factors (125). The biological properties of MIP-1 α, MIP-1 β and RANTES have been characterized in Table 4 (104, 106, 123).

β Chemokines act by competitive binding to a coreceptor, CC chemokine receptors (CCR5), that is also required by macrophage tropic HIV-1 strains as a coreceptor for entry into the host cell. This receptor-ligand binding renders CCR5 unavailable to the virus either by competitively blocking virus-cell interactions or by causing down-regulation from host cell surface ((126-130).

The α chemokine, also known as the CXC chemokines, contain a single amino acid between the first and second cystein residues. The gene that instructs synthesis of the α chemokine is on human chromosome 4. The major α chemokine is stroma cell-derived cofactor (SDF-1) which is an extremely efficacious chemoattractant for T lymphocytes and play a prominent role in B cell development. SDF-1 is a 352 amino acid proteins, mostly closely related to the receptor for IL-8 (131, 132). In addition, α chemokine (SDF-1) was shown to specifically inhibit CXCR4 mediated infection by T cell line tropic HIV-1. SDF-1 has been found to be responsible for competitive binding to a coreceptor CXCR4 which was identified as a specific coreceptor essential for entry of T-tropic HIV-1 into target cells(132).

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The accumulating data have suggested that HIV infection that augmented production of RANTES, MIP-1 α and MIP-1 β may limit viral replication *in vivo* (133) and provided evidence for a significant inverse correlation between level of these chemokines and a reduction of virus burden *in vivo*. A specific immune response involving a high production of β chemokine by T cells seems to play role of protection in exposed uninfected individuals (134) and may contribute to the control of viral replication in long-term nonprogressors (135). Several studies demonstrated that PBMCs from HIV-1 infected, long term nonprogressors often produce higher levels of β-chemokines upon antigen stimulation *in vitro* than do PBMCs from rapid progressors (136, 137); and higher production of MIP-1β by PBMCs has been associated with an asymptomatic status and a decreased risk of disease progression (138). Infection without disease progression for longer than 10 years, even though occurs in a minority of HIV-infected people, may be attributable from chemokine production, chemokine receptor polymorphisms including host factors (139).

The previous studies have demonstrated that $CD8^+$ T cells, $CD4^+$ T cells and macrophages, as well as several other cell types, secrete cytokines and CC-chemokines productions (140, 141). In addition, a few studies also showed the roles of NK cells for cytokine and chemokine production.

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Table 4. Biological properties of chemokines

Chemokine	Percent identity to MIP-Iα	Receptor	Source	Targets/ actions	Major effect
β Chemokine; MIP-Iα	100	CCR1, 3, 5	B, T and NK lymphocytes, mast cells, fibroblasts, macrophages, monocytes	Neutrophil and monocyte chemotaxis, neutrophil and macrophage activation, stem cell suppression, potentiation of GM-CFU stimulation by GMCSF, PGE-independent endogenous pyrogens Monocyte, NK, T, basophil and dendritic cell chemoattraction, monocytes, NK, T, and dendritic cell chemoattraction	Competes with HIV-1 for binding to coreceptors, anti-viral defense, promotes T_H1 immunity
MIP-Iβ	67	CCR1, 3, 5	B, T and NK lymphocytes, macrophages, monocytes, neutrophils	Inhibits MIP-Iα action on stem cells, potentiates GM-CFU stimulation by GMCSF, inhibits macrophage activation by MIP-Iα , monocytes, NK, T, and dendritic cell chemoattraction	Competes with HIV-1 for binding to coreceptors
RANTES	46	CCR1, 3, 5	T and NK lymphocytes, platelets,endothelial cells	Monocyte, granulocytes, memory T lymphocytes, NK, basophil, eosinophil, and dendritic chemoattraction	Competes with HIV-1 for binding to coreceptors, degranulates basophils, activate T cells, chronic inflammation
α Chemokine; SDF-1		CXCR4	T and NK lymphocytes, dendritic cells bone marrow stroma cells	B cell maturation and lymphopoiesis, hematopoiesis, transendothelial chemotaxis on lymphocyte, monocyte but not monocyte, potent mononuclear cell attractant	Competes with HIV-1 for binding to coreceptors

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10. Cytokine and chemokine assays

Several novel methods have been developed which facilitate the detection of cytokine and chemokine production by immune cells. Cytokine and chemokines can be assayed by: 1) enzyme-linked immunosorbent assay (ELISA) for quantifying the amount of secreted cytokine and chemokine in serum or cell culture supernatant; 2) determination of specific mRNA in chemokine and cytokine producing cells using polymerase chain reaction (PCR) (142); 3) enumeration of activated cells secreting cytokine by using enzyme-linked immunospot (ELISpot) (143, 144). However, these methods are time consuming by requiring long activation times and they are labour intensive (93). In addition, these methods can not specify cell type which produce cytokine/ chemokine because the same cytokine/ chemokine may be produced from several cell types. For instance, IFNγ may be produced from $CD8^+$, $CD4^+$ and NK cells.

A highly sensitive flow cytometric technique, that is based on the ability to quantitate and phenotypically characterize cytokine expressing cells simultaneously without prior sorting has been developed (145). This technique has the advantage of rapid determining intracellular cytokine production by activating the cells with mitogen in a short incubation time. Subsequently, addition of brefeldin A (BFA) to block the secretary pathway, a process that disrupts intracellular Golgi-mediated transport and allows the cytokines to accumulate and yield an enhanced signal for the detection by flow cytometry. This method has been applied to detect several types of cytokines in every discreted cellular population, and also phenotypically characterize the cytokine producing cells in various diseases such as HIV disease, autoimmunity and allergy. It is also possible to detect intracellular cytokine production for evaluation of human immune response, vaccine efficacy, drug and therapeutic modalities.

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11. Characteristics of NK cells

NK cells are one component of the innate immune system and have the ability to both lyse target cells and provide an early source of immunoregulatory cytokines. Human NK cells are defined phenotypically by their expression of CD16, CD56 and lack of expression of CD3; and comprise about 15% of all lymphocytes (146). However Thai people have higher number of NK cells as compared to the Caucaseans (10). NK cells are central in the innate immune response against tumor, parasites and cells infected with virus and intracellular bacteria (147, 148).

Previous studies have provided strong evidence that NK cells belong to the lymphocyte lineage. NK cells originated and differentiated in bone marrow (149, 150). Most of the characteristic of NK cells are summarized in Table 5 (86). NK cells are more closely related to T cells than B cells, because neither T nor NK cells express surface immunoglobulin (sIg), and they share expression of many cell surface molecules and functional attributes, including effector machanism (151). NK cells can develop independently from mature antigen specific T or B lymphocytes and do not productively rearrange or express $\alpha\ \beta$ or $\gamma\ \delta$ T-cell receptor on their cell surface (147). NK cells were originally identified by their ability to spontaneously mediate lysis of certain susceptible tumor cells (148).

Morphologically, NK cells are characterized as large granular lymphocytes (LGLS) with a kidney-shaped nucleus and prominent azurophillic cytoplasmic granules. NK cells are members of lymphoid lineages. They are larger than most B or T cells with ranging in diameter from 12 to 15 μm. The majority of human NK cell activity is mediated by $CD3^-$, $CD16^+$, $CD56^+$ lymphocytes. Due to their expression of CD16 (immunoglobulin Fc receptor, FcγRIII) these cells ($CD16^{bright}$) exhibit high level of antibody-dependent cellular cytotoxicity (ADCC) activity which is an arm of adaptive immunity. However $CD16^-$ NK cells also exist (152).

Approximately, 90% of human NK cells have low-density expression of CD56 ($CD56^{dim}$) and express high level of Fcγ receptor III (FcγRIII, $CD16^{bright}$), whereas about 10 % of NK cells are $CD56^{bright}$ $CD16^{dim}$ or $CD56^{bright}$ $CD16^-$ (151). CD56 is a 140 kd molecule, an isoform of the neural cell adhesion molecule (N-CAM),

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found on neural tissue and some tumor cell. The CD56 is not expressed by other hematopoietic cells or lymphocytes (151, 153). The CD16 is a 70-kd glycoprotein of Ig superfamily and is expressed as a transmembrane protein on the majority of human peripheral blood NK cells (154). CD16 is the low affinity FcγRIII on the surface of NK cells, which binds to antibody-coated (opsonized) targets and signals through associated subunits containing an immunoreceptor tyrosine-based activation motif (ITAM) on target cell to direct ADCC (155). Now, there are ample evidence to suggest that these NK-cell subsets have unique functional attributes and therefore, distinct roles in the human immune response. The $CD56^{dim}$ $CD16^{bright}$ NK cell subset is more naturally cytotoxic and express higher levels of Ig-like NK receptors and Fcγ RIII than the $CD56^{bright}$ NK cell subset. By contrast, the $CD56^{bright}$ subset has the capicity to produce abundant cytokines following activation of monocytes, but has low natural cytotoxicity and is $CD16^{dim}$ or $CD16^{-}$ (146).

NK cells are derived from pluripotent hematopoietic stem cells. A committed precursor for T and NK cells expressing FcγRIII has been demonstrated both in human and mice (156). NKT cells are a subset of lymphocytes that are characterized by the co-expressing of the NK cell receptor, CD161 and a single large invariant TCRα chain (Vα24JαQ in the human and Vα14Jα28 in the mouse). In contrast to conventional T lymphocytes, the NKT TCR does not interact with peptide antigen presented by classical MHC class I or II molecule, but instead it recognizes glycolipids presented by CD1d (non classical-MHC-class-I like molecule). They have the unusual property of being readily stimulated through their TCR as a result of presentation of the nonpeptide antigen, α-gallactosylceramide (α-GalCer), by CD1d molecules (157-160). Phenotypically, NKT cell are either $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ and this T cell population represents a major fraction of the mature T cell in thymus, nearly 50 % of α /β TCR^{+} T cells in liver and up to 5% of splenic T cells, but are rare in lymph node (161). As yet, little information is available on the tissue distribution of human invariant NKT cells. They constitute approximately 0.02-0.2 % of peripheral blood T cell compartment in both males and females (162).

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NKT cells are capable of rapidly producing both T_H1 (*e.g.* IFN-γ) and T_H2 type cytokine (*e.g.* IL-4, IL-5, IL-10) upon triggering suggesting a potential role of NKT cells in immunoregulation (163). Nevertheless, NKT cells have now been shown to control various immune responses, including autoimmune, allergic, antitumor, and antimicrobial immune response. In HIV infection, depletion of NKT cells upon infection with HIV-1 results from the high susceptibility to infection of CD4 and CCR5 coexpressing NKT cells, potentially in combination with activation-induced cell death during persistent immune activation (162-164). Since a subset of NKT cells is $CD4^+$T and also highly express level of the chemokine receptor CCR5 and CXCR6 but lower level of CXCR4 and CCR7. Unutmas *et al*. found that $CD4^+$NKT cells are indeed higher susceptible to R-tropic but less so to X-4 tropic virus and caused rapid destruction of this subset *in vitro* (165, 166). Recent findings have shown that NKT cells in PBMCs of HIV-1 infected individuals are dramatically reduced, compared with healthy donors and they established a positive correlation with high viral load and lower $CD4^+$ NKT numbers (164, 166).

Innate immunity effector cells, including NK cells recognize the presence of different pathogens mainly through a recently identified family of 10 genetically invariant receptors, the Toll-like receptors (TLR 1-10), capable of recognizing distinct molecular components of microbes (167). In particular, TLR3 is the specific receptor for dsRNA, a common intermediate in the reproductive cycle of many viruses. TLR3 expression on human NK cells and the ability of dsRNA to augment NK cell functions has been reported (168), but the mechanisms enabling NK cells to functionally respond to this product of viral infections are mostly unknown. Sivori *et al*. demonstrated that NK cells are able to respond to stimuli acting on different TLRs expressed by myeloid dendritic cells. Thus, after culture in the presence of the microbial products such as dsRNA (virus-derived) as well as CpG (of bacterial origin) can induce peripheral blood NK cells activation characterized by; 1) de novo expression of activation markers such as CD69 and CD25; 2) release of various cytokines including IFN-γ and TNF-α; 3) up-regulation of antitumor cytotoxicity, and; 4) acquisition of cytotoxicity against immature dendritic cells (169).

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Table 5. Phenotypic characteristics of human NK cells (170)

	Characteristics
1. General morphology	
Size	Large, 12-15 μm (mouse, 8-10 μm)
Cytoplasmic granules	+
Kidney-shaped nucleus	+
Adherence	-
Phagocytosis	-
2. **Histochemical properties**	+
Acid phosphate	+
Non-specific esterase	+
β-Glucuronidase	+
Peroxidase	-
TdT	-
3. **Cell surface antigens**	
CD2	≥ 75% (rat 30%)
CD3	≤ 5%
CD4	≤ 1%
CD5	≤ 5%
CD7	80% (Not tested in rodents)
CD8	25% (1% in mouse, >90% in rat)
CD11	>90% (Not tested in rodents)
CD16 (FcγRIII)	50-90% (ADCC is functionally weak in rodents)
CD56	10 % of human NK (not reliable marker for mouse)
FcμR	<1%
sIg	<1%
Ia	10% (<1% in mouse)
Asialo GM1	>90%

Discordance between spieces indicated by parenthesis +, >90% of the cells are positive for this characteristic; -, negative for the indicated characteristics

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12. NK cell activity

The principal physiologic role of NK cells is to get rid of cells harbouring foreign antigens. NK cells are activated by recognition of three types of targets: 1) antibody coated cells (ADCC); 2) cells infected by viruses and some intracellular bacteria; and 3) cell lacking class I MHC molecules (Fig. 4) (26).

ADCC process requires three components: 1) target cell expressing pathogen's antigens on the surface; 2) antibodies of the IgG isotype to the target antigen, and 3) effector cells bearing Fc gamma receptor (FcγR) (13). Therefore, ADCC occurs only when the target cell is coated with antibody, and free IgG in plasma neither activates NK cells nor competes effectively with cell-bound IgG for binding to FcγRIII (13, 26, 152, 156, 171, 172). Engagement of FcγRIII by antibody-coated target cells activates the NK cells to synthesize and secrete cytokines such as IFN-γ, as well as discharge the content of their granules, which contain several proteases and nuclease as well as perforin, a molecule which polymerizes to form pores in the membrane of the target cell (171, 173). If NK cells are to mediate direct cytotoxicity against cells infected with viruses or other pathogens, they must have some mechanism for distinguishing the infected from the uninfected cells (27). Since all nucleated cells normally express MHC class I molecules, many viruses have evolved means to inhibit the expression of these molecules in infected cells (26, 27). The downregulation of MHC class I on surfaces of the infected cells can protect themselved from lysis by virus spectific $CD8^+$cytotoxic T lymphocytes, but these viral infected cells may render themselves more sensitive to NK cell mediated cytotoxicity (Fig. 4) (148, 172, 174).

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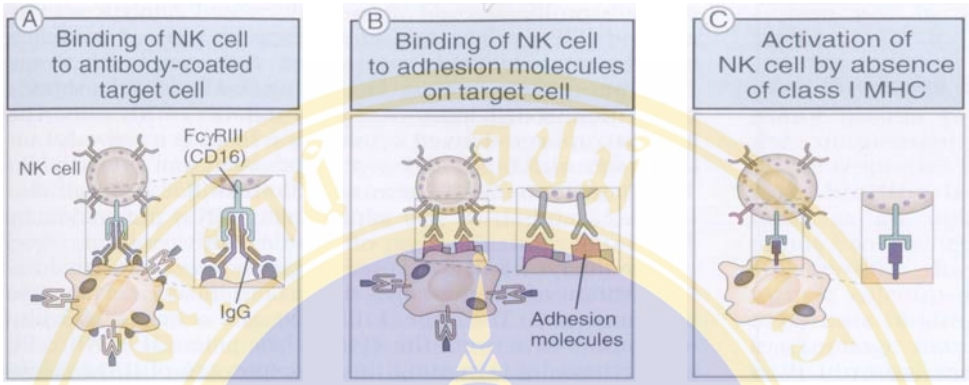

Figure 4. Recognition of target cells by NK cells. (A) NK cell recognizes antibody-coated targets by the FcγRIII receptor; (B) NK cell binds to target by adhesion molecules and other unknown ligands; (C) NK cell is activated by target cell lacking class I major histocompatibility complex (MHC) molecules; the activating receptors involved in the response to class I-deficient target are not known (26)

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The possible mechanisms whereby NK cells distinguish infected from non-infected cells was shown in Figure 5. A proposed mechanism of NK cell recognition is shown. NK cells can use several different receptors that signal them to kill infected cells, including lectin-like receptors that recognize carbohydrate on self cells. However, another set of receptors, called Ly49 in the mouse and killer inhibitory receptors (KIR) in the human, recognize MHC class I molecules and inhibit killing by NK cells by overruling the actions of the killer receptors. This inhibitory signal is lost when host cells do not express MHC class I and perhaps also in cells infected with virus, which might inhibit MHC class I expression or alter its conformation. Normal cells respond to IFN-α and β by increasing levels of MHC class I expression, making them resistant to activated NK killing. The infected cells can fail to increase MHC class I expression, making them targets for activated NK cells.

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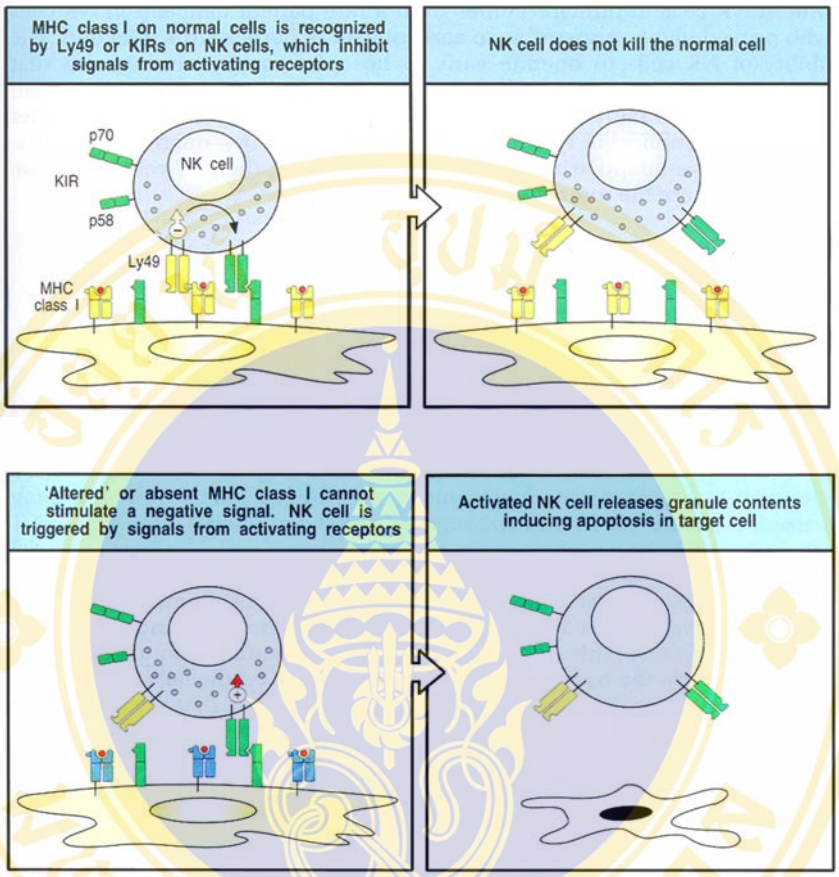

Figure 5. Mechanism by which NK cells can distinguish infected from non-infected cells (27)

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13. NK cell response to HIV infection

The role of NK cells in controlling HIV replication and spread of the viruses has not been fully delinated. Several studies have correlated high NK cell activity with the reduced susceptibility of certain individuals to HIV infection (175); and loss of NK cell activity and frequency had been correlated with HIV disease progression, particularly in individuals with opportunistic infections (14, 176, 177). As the result of the rapid mutation in HIV-specific CTL epitopes, HIV escape mutants were discovered. Moreover, Nef protein downregulates MHC class I molecules expression on HIV infected cells and escape from CTL destruction (9, 86, 178). Antigenic variation of HIV may render themselves more sensitive to NK cells than CTL mediated cytotoxicity. So NK cell may thus serve as a second line of defense against HIV-infected cells evading the T cell mediated immune response as well as a protective mechanism against an array of secondary opportunistic infections and malignant manifestation of HIV infection.

Pheripheral blood mononuclear cells (PBMCs) with or without AIDS or AIDS related complex (ARC) exhibit poor NK cytotoxic activity in the ^{51}Cr release assay (80, 85, 179-182). While lronson *et al*., demonstrated that there was no difference in NK cell cytotoxic activity between the HIV seronegative subjects and asymptomatic HIV seropositive cases with low $CD4^{+}$T cell count (83). In particular, NK cells are thought to be important in early host defense against viruses, including HIV. Studies of NK cells in HIV infection have shown variable levels of deficiency in both NK cell number and function. Ullum *et al.,* demonstrated rapid disease progression after HIV infection also has been correlated with decrease NK cell activity (86).

Scott-Algara *et al*. reported that the defective mechanism underlying the observed defective NK cytotoxic activity could be the results from lack or decreased frequency of effector cells, inability to recognize and bind the target cells, failure to be activited for the release and/or synthesize of NK cytotoxic factors (182). The HIV specific ADCC mechanism also declined with disease progression due to both a defect in the ability to generate anti-HIV antibodies required for ADCC as well as the diminished lytic capacity of the NK cells (13, 149).

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14. Measurement of NK cell cytotoxic activity

Several studies on NK cytotoxic activity in HIV infection were based on the standard protocol ^{51}Cr-release assay. NK cell cytotoxic activity was expressed as number of lytic unit (LU20) / 10^6 PBMCs. The LU20 /10^6 PBMCs is defined as lytic activity of effector cells which are required to lyse 20% of $5x10^3$ target cells. LU20 /10^6 PBMCs was calculated based on the equation (183) :

$$\text{Number of lytic units}/10^6 \text{ PBMC} = 10^6 / T \cdot X_p$$

T = number of target cells i.e., 5000 cells/well

X_p = E : T ratio required to lyse p% of target cells

p = reference lysis level (i.e., 20%)

As 5 E : T ratios were performed, it will be easier to make calculation by LU computer program (184).

Determination of NK cytotoxic activity was performed by using fresh PBMCs without prior *in vitro* sensitization. The K562 (erythroleukemic cell line) is used for the standard target cells for measuring in this assay. Cytotoxicity of NK cells can be measured by assaying the release of ^{51}Cr from labeled target cells. ^{51}Cr is thought to bind to cytoplasmic proteins of the cell. Following NK cell attack on the target cell, these proteins are released to the surrounding area, providing a marker for the cytoplasmic events of apoptosis. PBMCs usually are used as a source of immune effector (NK) cells. After incubation of the effector and tumor target cells, the radioisotope was relatively release from the lysed of target cells into the supernatant. Measurement of releasing radioisotope is performed by using automated radioisotope counter (184).

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CHAPTER IV
MATERIALS AND METHODS

1. Subjects

The subjects comprised 3 major groups in 3 different studies as follows:

1) Determination of lymphocyte immunophenotype reference ranges : A total of 125 HIV seronegative healthy subjects including 67 males and 58 females at age range of 18-55 years (mean±SD = 30.9+10.1, median = 27.0 years)

2) Determination of intracellular cytokine/ chemokine production :

2.1 A total of 10 HIV seronegative healthy subjects including 2 males and 8 females at age range of 23-31 years (mean±SD = 26.0±2.5, median = 25.5 years)

2.2 A total of 28 HIV seropositive subjects with different ranges of $CD4^+$T lymphocyte count as follows:

2.2.1 nine HIV seropositive subjects with CD4<14% including 9 males and 1 female at age range of 24-40 years (mean±SD= 30.1±5.1, median= 28.0 years)

2.2.2 19 HIV seropositive subjects with CD4≥14% including 11 males and 8 females at age range of 21-53 years (mean±SD= 31.9±10.0, median= 28.0 years)

3) Determination of NK cell cytotoxic activity :

3.1 A total of 69 HIV seronegative, healthy subjects including 38 males and 31 females at age range of 21-55 years (mean±SD = 32.8±10.0, median = 30.0 years) and 66 HIV-1 subtype E and 3 HIV-1 subtype B

3.2 A total of 107 HIV seropositive subjects categorized on the percentages of $CD4^+$T lymphocyte count as follows:

3.2.1 46 HIV seropositive subjects with CD4<14% including 26 males and 20 females at age range of 18-50years (mean±SD= 29.7±6.9, median= 28.5 years)

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3.2.2 61 HIV seropositive subjects with CD4≥14% including 20 males and 41 females at age range of 19-53 years (mean±SD= 29.4±7.80, median= 27.0 years)

All of HIV infected subjects attended the HIV Clinic, Department of Preventive and Social Medicine, Faculty of Medicine Siriraj Hospital during the period between July 1999-June 2002. All were naïve for treatment with anti-retroviral drug at the time of enrollment. The subjects understood the objective of this study and signed in the consent form.

2. Blood specimen collection

For each subject, 25 ml of the venous blood were collected in EDTA vacutainer tubes for NK cell cytotoxicity assay; and the other 2 ml were collected in a sodium heparinized vacutainer tube (Becton-Dickinson Vacutainer System, Franklin Lakes, NJ). EDTA blood sample was further divided into 3 aliquots for the following purposes:

2.1 Determination for white blood cell count and differential cell count by automated machine (Beckman-Coulter STKS, CA, USA.) at the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital

2.2 Determination for lymphocyte subsets

2.3 Determination for intracellular cytokine and chemokine expressing cells

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3. Immunophenotype staining

Lymphocyte subpopulation present in EDTA blood sample were enumerated by two color staining of the surface markers using matched combination of fluorecein isothiocyanate and phycoerythrin conjugated mAbs (Becton Dickinson). The lymphocyte subsets determined were total $CD3^+$T lymphocytes, $CD3^-CD19^+$B lymphocytes, $CD3^+CD4^+$T helper/inducer lymphocytes, $CD3^+CD8^+$T suppressor/cytotoxic lymphocytes, $CD3^-$ $CD16^+$ and/or $CD56^+$ NK lymphocytes, $CD3^-$ $CD16^+$ and $CD3^-CD56^+$NK lymphocyte subpopulation. The characterization of lymphocyte immunophenotype was shown in Table 6. All lymphocyte subsets were measured by flow cytometer (FACScan; Becton Dickinson Immunocytometry System San Jose, CA, USA) using a panel of mAbs and reagent as follow:

1. Simultest LeucoGATE reagent (CD45/CD14, Anti-Hle-I/Leu-M3, Catalog No. 340040) which contains FITC labeled anti-CD45 mAb and PE labeled anti-CD14 mAb

2. Simultest Control reagent γ1/ γ1 (IgG_1/IgG_1, Catalog No. 349526) which contains FITC labeled anti-IgG_1 mAb and PE labeled anti-IgG1 mAb

3. Simultest CD3/CD19 reagent (Leu-4/12, Catalog No. 349211) which contain FITC label anti-CD3 mAb and PE labeled anti-CD19 mAb

4. Simultest CD3/CD4 reagent (Leu-4/3a, Catalog No. 340043) which contains FITC labeled anti-CD3 mAb and PE labeled anti-CD4 mAb

5. Simultest CD3/CD8 reagent (Leu-4/2a, Catalog No. 340044) which contains FITC labeled anti-CD3 mAb and PE labeled anti-CD8 mAb

6. Simultest CD3/CD16+CD56 reagent (Leu-4/11c+19, Catalog No. 340042) which contains FITC labeled anti-CD3 mAb and PE labeled anti-CD16 and CD56 mAb

7. CD3-FITC reagent (Leu-4 FITC, Catalog No. 349201) which contains FITC labeled anti- CD3 mAb

8. CD16-PE reagent (Leu-11c, Catalog No. 347617) which contains PE labeled anti-CD16 mAb

9. CD56-PE reagent (Leu-19, Catalog No. 347747) which contains PE labeled anti-CD56 mAb

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10. FACS Lysing Solution 10X concentration (Catalog No. 349202) which contains reagent for lysing the erythrocytes under gentle hypotonic condition and preserving the leucocytes

3.1 Staining procedure

1. Set up eight 12x75 mm tubes for one blood sample
2. Gently mix thoroughly the EDTA blood and place each 50 µl of EDTA blood into each tube
3. Add 5-10 µl of each pair of mAb into each tube as follow:
   - 3.1 Simultest CD45/14
   - 3.2 Simultest γ1/γ1 (IgG_1/IgG_1)
   - 3.3 Simultest CD3/CD19
   - 3.4 Simultest CD3/CD4
   - 3.5 Simultest CD3/CD8
   - 3.6 Simultest CD3/CD16+CD56
   - 3.7 CD3 and CD16
   - 3.8 CD3 and CD56
4. Vortex gently and incubate the tubes for 15 to 30 minutes at room temperature in the dark
5. Add 2 ml of 1X FACS Lysing solution into each tube. Immediately vortex gently and incubate for 10 minutes at room temperature in the dark
6. Centrifuge the tubes at 200xg for 5 minutes
7. Aspirate the supernatant by leaving approximately 50 µl of residual fluid; avoid disturbing the pellet
8. Add 2 ml of phosphate buffer saline (PBS) into each tube. If samples contain too many red blood cells, add 2 to 3 drops of distilled water before adding PBS immediately
9. Vortex the tubes gently and centrifuge at 200xg for 5 minutes
10. Aspirate the supernatant and then resuspend the cell pellet in 0.5 ml of 1.0% paraformaldehyde in PBS

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The stained cells were analyzed by a flow cytometer using SimulSET Software (Becton Dickinson), within 24 hours after staining.

Table 6. Characterization of lymphocyte immunophenotypes

Reagents (FITC/PE)	**CD number** (FITC/PE)	**Functional identity of relevant subsets**
LeucoGATE	CD45/CD14	Establishment of an optimal lymphocyte gate for immuno-phenotyping of erythrocyte lysed whole blood (lymphocyte monocytes and granulocytes)
Isotype control	IgG1/IgG1	Direct conjugate control for non-specific staining
Leu-4/Leu-12	CD3/CD19	T (CD3+CD19-) and B (CD3-CD19+) lymphocytes
Leu-4/Leu-3	CD3/CD4	CD4+ helper/inducer T cells
Leu-4/Leu-2	CD3/CD8	CD8+ cytotoxic/suppressor T cells
Leu-4/Leu-11+ Leu-19	CD3/CD16+CD56	NK cells (CD3-CD16+ and/or CD56+)
Leu-4/Leu-11	CD3/CD16	NK cell subsets (CD3-CD16+)
Leu-4/Leu-19	CD3/CD56	NK cell subsets (CD3-CD56+)

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4. Intracellular cytokine staining

This technique is used for determination of cytokine production in heterogenous cell population based on the ability to detect a single cytokine expressing cell. This rapid method determines the intracellular cytokine production by activating PBMCs with mitogen in a short incubation time (4 hours). Subsequently, addition of brefeldin A to block the secretory pathway, allows the accumulation of cytokines that can be detected by flow cytometer using a panel of mAbs. Reagents No.1-12 were purchased from Becton Dickinson, San Jose, CA, USA, and No.13-15 were purchased from Sigma Chemical Co., St Louis, MO, USA

1. IgG_1-PE (Isotype control, Catalog No. 349043) which contains PE labeled anti-IgG_1 mAb
2. CD3-FITC reagent (Leu-4 FITC, Catalog No. 349201) which contains FITC labeled anti-CD3 mAb
3. CD3-PerCP reagent (Leu-4 PerCP, Catalog No. 347344) which contains PerCP labeled anti-CD3 mAb
4. CD8-PerCP reagent (Leu-2a PerCP, Catalog No. 347314) which contains PerCP labeled anti-CD8 mAb
5. CD16-FITC reagent (Leu11a FITC, Catalog No. 347523) which contains FITC labeled anti-CD16 mAb
6. CD56-FITC reagent (NCAM16.2, Catalog No. 340410) which contains FITC labeled anti-CD56 mAb
7. Anti-human IFN-γ mAb-PE (Catalog No. 18905A) which contains PE labeled anti-human IFN γ mAb
8. Anti-human RANTES mAb-PE (Catalog No. 20975A) which contains PE labeled anti-human RANTES mAb
9. Anti-human MIP-1α mAb-PE (Catalog No. 20955A) which contains PE labeled anti-human MIP-1α mAb
10. Anti-human MIP-1β mAb-PE (Catalog No. 23855A) which contains PE labeled anti-human MIP-1β mAb
11. FACS Lysing Solution 10X concentration (Catalog No. 349202)
12. FACS Permeabilizing Solution 10X concentration (Catalog No. 340457)

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which contains the solution for the permeabilization of lymphocyte membranes prior to intracellular immunofluorescence staining with mAb

13. Ionomycin Calcium salt (I) 1 mg (Catalog No. I-0634)

14. Phorbol 12-myristate 13-acetate (PMA) 1 mg (Catalog No. P-8139)

15. Brefeldin A (BFA) 5 mg (γ_1 4-Dihydroxy-2[6-hydroxy-1-heptenyl]-4-cyclopentane-crotonic acid λ-lactone, Catalog No. B-7651)

4.1 Cell stimulation for cytokine or chemokine production

1. Dilute 500 µl of whole heparinized blood in 500 µl RPMI 1640 (GIBCO, Paisley Scotland, USA) in a 15 ml conical centrifuge tube

2. Add 25 µl of 25 ng/ml PMA, 20 µl of 1 µg/ml I and 10 µl of 0.5 mg/ml BFA for cell stimulation into each tube (Tubes of unstimulated samples are set up in parallel by adding BFA but without PMA and I)

3. Mix the tube by vortex and incubate at 37 °C in 5 % CO_2 humidified atmosphere for 4 hours

4. Add 100 µl of 20 mM EDTA into each tube

5. Vortex the tube and incubate at room temperature for 15 minutes

6. Add 4.5 ml of FACS Lysing solution into each tube

7. Gently mix the tube and incubate at room temperature for 10 minutes

8. Wash with 2% fetal bovine serum (FBS) (GIBCO) in PBS as washing buffer

9. Centrifuge at 500xg for 5 minutes

10. Discard the supernatant

11. Add 2.5 ml of FACS permeabilizing solution into each tube and incubate at room temperature for 10 minutes

12. Wash with washing buffer by centrifuge at 500 x g for 5 minutes and discard the supernatant

13. Resuspend the cell pellet with 0.5 ml of washing buffer

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4.2 Intracellular cytokine and chemokine staining

The stimulated cells were stained using triple colors as the following protocol:

1. Set up nine 12x75 mm tubes for 1 blood sample
2. Add 5 μl of each mAb into each tube as follow;
    1.1 CD16 plus CD56-FITC/IgG_1-PE/CD3-PerCP
    1.2 CD3-FITC/IFN-γ-PE/CD8-PerCP
    1.3 CD3-FITC/RANTES-PE/CD8-PerCP
    1.4 CD3-FITC/MIP-1α-PE/CD8-PerCP
    1.5 CD3-FITC/MIP-1β-PE/CD8-PerCP
    1.6 CD16 plus CD56-FITC/ IFN-γ-PE/CD3-PerCP
    1.7 CD16 plus CD56-FITC/ RANTES -PE/CD3-PerCP
    1.8 CD16 plus CD56-FITC/ MIP-1α-PE/CD3-PerCP
    1.9 CD16 plus CD56-FITC/ MIP-1β-PE/CD3-PerCP
3. Add 100 μl of stimulated cells into each tube
4. Incubate all samples in room temperature for 30 minutes in the dark
5. Wash the stained cell sample with washing buffer
6. Centrifuge at 500xg for 5 minutes
7. Aspirate the supernatant and resuspend in 1% paraformaldehyde in PBS
8. Analyse by flow cytometer using CellQuest software (Becton Dickinson)

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Table 7. Monoclonal antibodies to characterize immune cells expressing cytokine and chemokine

Tube No.	Mab conjugated with FITC or PE or PerCP	Functional identity of relevant subset
1	CD16+CD56/IgG_1/CD3	Isotype-matched negative control
2	CD3/IFN-γ/CD8	IFN-γ expressing CD3 and CD8 T cells
3	CD3/RANTES/CD8	RANTES expressing CD3 and CD8 T cells
4	CD3/MIP-1α/CD8	MIP-1α expressing CD3 and CD8 T cells
5	CD3/MIP-1β/CD8	MIP-1β expressing CD3 and CD8 T cells
6	CD16+CD56/ IFN-γ/CD3	IFN-γ expressing CD3 T and NK cells
7	CD16+CD56/ RANTES /CD3	RANTES expressing CD3 T and NK cells
8	CD16+CD56/ MIP-1α/CD3	MIP-1α expressing CD3 T and NK cells
9	CD16+CD56/ MIP-1β/CD3	MIP-1β expresing CD3 T and NK cells

5. Flow cytometric analysis

Samples were analysed using a FACScan or FACSort flow cytometer (Becton Dickinson) which had been calibrated by using CaliBRITE Beads and Auto Comp Software (Becton Dickinson). Two color immunophenotype analyses were accomplished with the SimulSET software (Becton Dickinson). The LeucoGATE mAb (CD45/CD14) identified lymphocytes, monocytes and granulocytes by light scatter gating; and the purity of at least 95% lymphocytes was obtained for immunophenotype analyses. The SimulSET software was programmed to enumerate 15,000 lymphocyte cells in a measurement.

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Three color flow cytometric analyses was performed with CELLQuest software (Becton Dickinson). The results were analysed by setting up the templates which used the plots and histrograms for each sample collection. Lymphocytes were initially gated by forward and side scatter, secondary gating was set on the basis of staining with isotypic control mAb. The quardrant markers used to determine the percentage of lymphocytes producing cytokine and chemokine were set by basing on unstimulated sample. The percentages of antigen specific cytokine and chemokine response were calculated by substracting the values of the stimulated sample with that of the unstimulated match sample.

6. Evaluation of natural killer cell cytotoxic activity

NK cell cytotoxic activity was measured by a standard chromium 51 (^{51}Cr) release assay. Determination of NK cell cytotoxic activity was performed by using fresh PBMCs without prior *in vitro* sensitization. The cell line K-562 (kindly provided by Dr. Chitraporn Karnasuta, Armed Forces Research Institute of Medical Sciences) is used as the standard target cells.

6.1 Preparation of effector cells

Effector cells were PBMCs isolated from EDTA blood samples by standard ficoll hypaque (Robbins Scientific, Sunnyvale, CA, USA) density gradient as follows:

1. Centrifuge the tube of EDTA blood at 200xg for 10 minutes at room temperature and remove the plasma
2. Dilute the packed blood with 2 volumes of steriled PBS, for example, resuspend 4 ml of blood in 4 ml of PBS
3. Slowly overlay 8 ml of diluted blood on to 4 ml of ficoll-hypaque in a 15 ml conical centrifuge tube and centrifuge at 500xg for 20 minutes at room temperature with the brake off
4. After centrifuging, carefully collect PBMCs in white band at interface. Upper part of the tube contains plasma, platelets, and PBS, and the lower part contains ficoll-hypaque, red blood cells and granulocytes
5. Wash the cells once with PBS and twice with RPMI 1640 with antibiotic by centrifuging at 200xg for 10 minutes at room temperature with brake on

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6. Resuspend separated PBMCs in growth medium (RPMI 1640 plus 10% fetal bovine serum) to the concentration 5x10 ^{6} cells in 5 ml. In order to enrich lymphocyte by depletion of the adherent macrophages from PBMC population, remove adherent macrophages by placing the PBMC suspension in a 25 cm^2 polystylene tissue culture flask (Costar, Corning, NY)

7. Incubate the cells at 37 C in 5% CO_2 incubator for overnight. The flask should be laid down flat in the incubator

8. After incubation, collect the nonadherent lymphocyte and dispense into 15 ml centrifuge tube

9. Centrifuge the tube at 1,500rpm for 10 minutes and decant the supernatant

10. Resuspend cell pellet in growth medium and adjust to the concentration of $5x10^6$ cells/ml

6.2 Preparation of target cells

The cell line K562, derived from a patient with chronic myelogenous leukemia in blast crisis, is the standard target cells for measuring NK cytotoxic activity. This target cells are the continuous cell line and could multiple indefinitely. The K562 cells were cultered in 10% FCS RPMI growth medium and incubated at 37°C in a CO_2 incubator. In the assay, the K562 target cells should be in log phase and more than 95% viable. The viability of the cells were determined by tryphan blue exclusion. They were fed with 10% FCS RPMI growth medium one day prior to the test. One million K562 cells are sufficient for setting up assay for 3-4 subjects.

The K562 cell lines should be subpassaged twice a week. The cell line could be continuous maintained or cryopreserved in the freezing medium, and stored in liquid nitrogen.

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6.3 Cryopreservation of cells

Cells should be frozen at concentration about $10x10^6$ cells in 1 ml freezing medium; and their viability should be more than 95%. RPMI 1640 containing 20% FCS and 10% dimethyl sulfoxide (DMSO)

1. Resuspend the cells to be frozen in 0.5 ml of cold RPMI 1640 supplemented with 40% FCS in a cryovial (Costar, Corning, NY)
2. Gradually add dropwise of 0.5 ml cooled RPMI 1640 containing 20% DMSO into the vial of cells with gently mix
3. Immediately place cryovials in a storage foam box in a -70 ^{0}C freezer for overnight, then transfer the vial into a liquid nitrogen tank

Notice: Cap of the vial should be the type of inner thread with O-ring. Volume of cell suspension added into the cryotube should not be higher than the indicated line on the vial wall.

6.4 Thawing frozen cells from liquid nitrogen tank

1. Transfer cryovial from the liquid nitrogen tank to a -70 ^{o}C freezer for 1 hour
2. Place cryovial into an ice box with closed lid for 1 hour in order to let liquid nitrogen evaporated. If the screw cap was immediately loosen while liquid nitrogen gas still remain at the joint between the cap and the body of vial, the cryovial might be bursted out.
3. After thawing, put the cell suspension into a tissue culture flask, and add with 10 ml of growth medium
4. Incubate the flask overnight at 37 ^{0}C in a CO_2 incubator
5. Replace with new growth medium and observe daily for growth

With this freezing-thawing protocol, viability of the reconstituted K562 cells is always higher than 95%.

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6.5 Labeling of K562 target cells

1. Place 10^6 K562 cells in log phase in a 15 ml conical tube
2. Centrifuge the tube at 1500 rpm for 10 minutes at 25 ^{0}C
3. Discard the supernatant and resuspend the cell pellet with 1 ml of growth medium
4. Add 200 μCi of $Na_2{}^{51}CrO_4$ (Amersham Phamacia Biotech UK limited, Buckingham shire, England) per $1x10^6$ K562 cells (the volume of ^{51}Cr needed is adjusted according to the decay of the isotype)
5. Incubate the target cells for 1-1.5 hour at 37 ^{0}C with shaking every 15 min to ensure uniform labeling
6. Wash the ^{51}Cr-labeled K562 target cells with 10 ml of 1x RPMI 1640 by centrifugation at 1500 rpm for 10 minutes for 3 times
7. Resuspend the cells in 1 ml of growth medium
8. Count cells by using a hemacytometer, and adjust to the cell concentration of 5 x 10^6 viable ^{51}Cr-labeled K562 cells/ml. This is the stock required for plating the target cells

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6.6 Plating of effector and target cells

The test was run in a tripicate in the microtiter U-shape (Costar, Corning, NY). Effector cells were mixed with target cells at the effector (E): target (T) ratios of 100:1, 50:1, 12.5:1 and 6.25:1

The U-bottom microtiter plate was set as below:

		Subject 1			Subject 2			Subject 3			Subject 4		
E : T		1	2	3	4	5	6	7	8	9	10	11	12
100 : 1	A												
50 : 1	B												
25 : 1	C												
12.5 : 1	D												
6.25 : 1	E												
	F	Spontaneous release											
	G												
	H	Maximum release											

6.7 Running NK cytotoxic activity assay

1. Add 100 µl of 10% FCS RPMI media into the wells B1-B3, C1-C3, D1-D3, E1-E3 and F1-F3

2. Add 200 µl of effector cells at concentration of 10^6 cells/ml in tripicate into wells A1-A3 of the first row

3. Mix cells in wells A1-A3 thoroughly, and perform serial two fold dilution by transfer 100 µl of the cell suspension from the wells A1-A3 to the wells B1-B3. The remaining amount of the effector cells in the wells A1-A3 is $5x10^5$ cells/well

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4. Mix and transfer 100 µl of the cell suspension from the wells B1-B3 to the wells C1-C3. The remaining amount of the effector cells in the wells B1-B3 is $2.5x10^5$ cells/well

5. Mix and transfer 100 µl of the cell suspension from the wells C1-C3 to the wells D1-D3. The remaining amount of the effector cells in the wells C1-C3 is $12.5x10^4$ cells/well

6. Mix and transfer 100 µl of the cell suspension from the wells D1-D3 to the wells E1-E3. The remaining amount of the effector cells in the wells D1-D3 is $6.25x10^4$ cells/well

7. Mix and discard 100 µl of the cell suspension from the wells E1-E3. The remaining amount of the effector cells in the wells E1-E3 is $3.125x10^4$ cells/well

8. Add 100 µl of 10% FCS RPMI media into the wells F1-F3, while add 100 µl of steriled 10% sodium dodecyl sulfate (SDS) (Sigma, MO, USA) into the wells H1-H3

9. Add 100 µl of ^{51}Cr -labeled K562 target cells at concentration of $5x10^4$ cells/ml into every well of rows A-F and H, column 1-3 (for one subject), to give effector: target cells ratios of 100:1 in row A, 50 :1 in row B, 25:1 in row C, 12.5:1 in row D and 6.25:1 in row E. The wells F1-F3 represent spontaneous release, while the wells H1-H3 represent maximum release

The experiment was done in tripicate and the component of the test wells were as below:

Row A; column 1-3 = Effector $5x10^5$ cells + target $5x10^3$ cells
Row B; column 1-3 = Effector $2.5x10^5$ cells + target $5x10^3$ cells
Row C; column 1-3 = Effector $1.25x10^5$ cells + target $5x10^3$ cells
Row D; column 1-3 = Effector $6.25x10^4$ cells + target $5x10^3$ cells
Row E; column 1-3 = Effector $3.125x10^4$ cells + target $5x10^3$ cells
Row F; column 1-3 = 100 µl of the growth medium + target $5x10^3$ cells
Row H; column 1-3 = 100 µl of 10% SDS (detergent) + target $5x10^3$ cells

10. Incubate the reaction plate for 4 hours at 37 °C in a CO_2 incubator to allow close contact between effector cells and the target cells

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6.8 Harvesting the assay plate

1. Carefully remove the plate from the incubator and carefully transfer 35 µl of culture supernatant from each well of the reaction plate to a scintillation fluid coated 96 well Luma plate (Packard Bioscience Company, CT, USA) in the corresponding well position

2. Decontaminate the infectiousness of the test wells by adding 15 µl of 100% commercial bleach to every well of the Luma plate and incubate overnight in order to let the fluid evaporated

3. Measure radioactivity present in the Luma plate by Beta-counter (Packard Bioscience Company, Model Top Count, CT, USA)

6.9 Data interpretation

Incubation of effector cells with ^{51}Cr-labeled K562 target cells will lead to target cell lysis and a release of the cytosol content together with ^{51}Cr into the culture supernatant. Measurement of releasing radioisotope is performed by using automated radioisotope Beta-counter. Percent lysis of each kind of effector cell was calculated based on the equation below.

$$\% \text{ Lysis} = \frac{\text{mean test cpm} - \text{mean spontaneous cpm}}{\text{mean max cpm} - \text{mean spontaneous cpm}} \times 100$$

cpm = counts per minute (average cpm of triplicate wells)

test cpm = cpm released by the target cells in the presence of the effector cells

spontaneous cpm = cpm released by the target cells in the absence of the effector cells

maximum cpm = cpm released by the target cells in the presence of 10% SDS

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NK cell cytotoxic activity was expressed as number of lytic unit (LU_{20}) / 10^6 PBMC. The LU_{20} /10^6 PBMCs is defined as lytic activity of effector cells which are required to lyse 20% of $5x10^3$ target cells (184). LU_{20} /10^6 PBMC was calculated by using computer software, kindly provided by Dr. Chitraporn Karnasuta.

7. Anti-HIV testing

Subjects enrolled in this study were tested for HIV-1 infection. Sera or plasma samples were assayed for anti-HIV antibody by ELISA (Organon Teknika BV, Netherland) and gel partical agglutination (Serodia-HIV-FUJIREBIO Inc, TOKYO, Japan). Reaction samples were confirmed by three screening test of different principles. The assay were performed as the diagnostic service of the Division of Virology, Faculty of Medicine Siriraj Hospital.

8. Statistical analysis

Data of each lymphocyte subset and percentage of cytokine and chemokine expressing cells and NK cytotoxic activity obtained from all subsets were analysed for difference between HIV-1 seronegative and seropositive subjects. Komogorov Smirnov test was used to verified the pattern of distribution of a dataset in order to determine the goodness of fit. When normal distribution of dataset was obtained ($p \geq 0.05$), parametric analyses would be employed, i.e., determination for sex difference of lymphocyte subsets would be performed by an independent t-test. On another hand, if distribution of dataset was not normal ($p < 0.05$), non-parametric assay would be employed, i.e., the difference of lymphocyte subsets would be determined by Mann whitney-U test. In addition, age variation was determined by simple linear regression test and the correlation between lymphocyte subset or lytic unit vs NK cell number was determined by pearson correlation coefficients.

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CHAPTER V
RESULTS

1. Leukocyte differential count in HIV seronegative, healthy subjects

A total of 125 HIV seronegative, healthy subjects were determined for the reference ranges of leukocyte differential counts by using an automated machine (Beckman-Coulter Counter STKS, CA, USA).

The results demonstrated that number of white blood cell (WBC) in males was significantly higher than in females (independent t-test; $p=0.005$); but there was no statistically significant difference in percentage of lymphocyte or monocyte count between both genders ($p \geq 0.05$) as analysed by independent t-test and Mann Whitney U-test, respectively (Table 8).

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Table 8. Leukocyte differential count of the HIV seronegative, healthy subjects

			Number of cell count		
Cell type	**Sex**	**N**	**Mean ± SD**	**Median**	**95% Reference range**
WBC x 10^3	M	67	7.3 ± 2.0*	7.1	6.8 - 7.8
cells/μl	F	58	6.4 ± 1.5* (p=0.005)	6.2	6.0 - 6.8
	Both	125	6.9 ± 1.9	6.6	6.6 - 7.2
%Lymphocyte	M	67	30.7± 7.7	30.0	28.8 - 32.6
	F	58	31.1± 8.0	30.5	29.0 - 33.2
	Both	125	30.9 ± 7.8	30.0	29.5 - 32.3
%Monocyte	M	67	7.2± 2.5	7.0	6.6 - 7.8
	F	58	7.2± 2.3	7.0	6.6 - 7.8
	Both	125	7.2± 2.4	7.0	6.8 - 7.6

* There was statistically significant difference as number of cell count of both sexes was compared (independent t-test; p=0.005)

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2. Reference ranges of lymphocyte immunophenotype subsets with respect to sex and age

Reference ranges for lymphocyte subsets were determined in 125 healthy Thai adults by flow cytometric analysis using a panel of monoclonal antibodies conjugated with either fluorescein isothiocyanate or phycoerythrin to identify cell-specific surface antigens. The reference range of each lymphocyte subpopulation as presented in term of percentage and absolute number are shown in Tables 9 and 10, respectively.

The result demonstrated that males had higher percentages and higher absolute numbers of NK cells than females (independent t-test; $p=0.008$ and 0.003, respectively). Discordant results between percentages and absolute number were observed with $CD3^+$T cell count. As absolute number was partly derived from WBC count, percentage value may better yield the actual result of comparison. Our study showed that females had significantly higher percentages of $CD3^+$T cells (independent t-test; $p=0.018$).

There was no discrepancy by sex as tested on various parameters: $CD4^+$T, $CD8^+$T, $CD19^+$B lymphocytes and CD4/ CD8 ratio (independent t-test; $p \geq 0.05$). Regarding the variation in values of all lymphocyte subsets by age as analysed by simple linear regression analysis, the difference were shown only in $CD3^+CD4^+$T cells with an increase of 1.1% and 53.8 cells/ μl per decade (Tables 9 and 10).

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Table 9. Lymphocyte immunophenotype reference ranges with respect to sex and age by percentages

Lymphocyte immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age variation
$CD3^+$ (total T cells)	M	67	63.4	8.4	64.0	61.3-65.4	3.4 % F > M (p=0.018)	NS
	F	58	66.8	7.4	66.0	64.9-68.7		
	Both	125	65.0	8.1	65.0	63.5-66.4		
$CD3^- CD19^+$ (B cells)	M	67	12.1	3.8	12.0	11.2-13.0	NS	NS
	F	58	13.0	4.9	13.0	11.7-14.3		
	Both	125	12.5	4.3	12.0	11.8-13.3		
$CD3^+CD4^+$ (T helper/inducer)	M	67	31.7	6.0	32.0	30.2-33.2	NS	1.1 % per decade
	F	58	32.6	5.8	32.5	31.0-34.1		
	Both	125	32.1	5.9	32.0	31.1-33.1		
$CD3^+ CD8^+$ (T suppressor/cytotoxic)	M	67	26.9	7.1	26.0	25.2-28.6	NS	NS
	F	58	28.7	7.5	29.0	26.8-30.7		
	Both	125	27.7	7.3	28.0	26.5-29.0		
CD4/CD8 ratios	M	67	1.3	0.5	1.2	1.2-1.4	NS	NS
	F	58	1.3	0.5	1.1	1.1-1.4		
	Both	125	1.3	0.5	1.2	1.2-1.4		
$CD3^- CD16^+$ and/or $CD56^+$ (Total NK cells)	M	67	23.2	9.1	21.0	21.0-25.5	4.0 % M > F (p=0.008)	NS
	F	58	19.2	7.5	19.0	17.2-21.2		
	Both	125	21.4	8.6	20.0	19.8-22.9		

NS = no significant difference

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Table 10. Lymphocyte immunophenotype reference ranges with respect to sex and age by absolute numbers

Lymphocyte immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age Variation
$CD3^+$ (total T cells)	M	67	1,355.5	401.9	1,307.0	1,257.4-1,453.5	NS	NS
	F	58	1,308.1	421.7	1,198.0	1,197.3-1,419.0		
	Both	125	1,333.5	410.2	1,269.0	1,260.9-1,406.1		
$CD3^- CD19^+$ (B cells)	M	67	268.9	124.5	252.0	238.5-299.3	NS	NS
	F	58	257.4	128.2	234.5	223.7-291.1		
	Both	125	263.6	125.9	245.0	241.3-285.8		
$CD3^+ CD4^+$ (T helper/inducer)	M	67	689.8	240.8	663.0	631.1-784.5	NS	53.8 cells/μl per decade
	F	58	633.8	198.7	584.5	581.6-686.1		
	Both	125	663.8	223.1	624.0	624.3-703.3		
$CD3^+ CD8^+$ (T suppressor/cytotoxic)	M	67	587.5	229.6	542.0	531.5-643.6	NS	NS
	F	58	570.3	277.4	527.5	497.4-643.3		
	Both	125	579.6	252.0	531.0	534.9-624.2		
CD4/CD8 ratios	M	67	1.3	0.5	1.2	1.2-1.4	NS	NS
	F	58	1.3	0.5	1.1	1.1-1.4		
	Both	125	1.3	0.5	1.2	1.2-1.4		
$CD3^- CD16^+$ and/or $CD56^+$ (Total NK cells)	M	67	533.3	352.1	455.0	447.4-619.1	152.3 cells/μl M > F (p=0.003)	NS
	F	58	381.0	202.3	366.5	327.8-434.1		
	Both	125	462.6	301.0	403.0	409.3-515.9		

NS = no significant difference

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3. Determination for the influence of sex on the number of NK cells and their subpopulations

In addition to determination of the total number of NK cells (cells which expressed either $CD3^{-}CD16^{+}CD56^{+}$ or $CD3^{-}CD16^{+}CD56^{-}$ or $CD3^{-}CD16^{-}CD56^{+}$), we also enumerated NK cell subset which expressed CD16 molecules (either with or without co-expression of the CD56 marker), and NK subset which expressed CD56 molecules (either with or without co-expression of CD16 marker). Based on he reference ranges for both sexes of 19.8-22.9% for total NK cells, 18.1-20.9% for the CD16 NK cell subset and 17.0-19.8% for the CD56 NK cell subset, we can estimate by calculation that approximately 86% of NK cells carried both CD16 and CD56 molecules on the same cells (Table 11). Generally, males have a higher number of NK cells than females (independent t-test; p=0.008). In addition, age variation as analysed by simple linear regression analysis, has not been found with NK cell number.

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Table 11. NK cell subpopulations by percentages and absolute numbers

NK cells Immunophenotype	Sex	N	Mean	SD	Median	95% Reference range	Sex difference	Age Variation
Immunophenotypes by percentages								
$CD3^-$ $CD16^+$and/or $CD56^+$	M	67	23.2	9.1	21.0	21.0-25.5	4.0 %	
	F	58	19.2	7.5	19.0	17.2-21.2	M > F	NS
	Both	125	21.4	8.6	20.0	19.8-22.9	(p=0.008)	
$CD3^-$ $CD16^+$	M	65	21.0	7.9	20.0	19.0-22.9	3.1 %	
	F	56	17.9	7.3	18.0	15.9-19.8	M > F	NS
	Both	121	19.5	7.8	19.0	18.1-20.9	(p=0.028)	
$CD3^-$ $CD56^+$	M	65	19.6	8.1	17.0	17.6-21.6		
	F	56	17.0	7.3	17.5	15.0-18.9	NS	NS
	Both	121	18.4	7.8	17.0	17.0-19.8		
Immunophenotypes by absolute numbers								
$CD3^-$ $CD16^+$and/or	M	67	533.3	352.1	455.0	447.4-619.1	152.3	
$CD56^+$	F	58	381.0	202.3	366.5	327.8-434.1	cells/μl	NS
	Both	125	462.6	301.0	403.0	409.3-515.9	M > F (p=0.003)	
$CD3^-$ $CD16^+$	M	65	481.7	302.8	429.0	406.7-556.7	124.5	
	F	56	357.2	192.9	349.5	305.5-408.9	cells/μl	NS
	Both	121	424.1	264.2	377.0	376.5-471.6	M > F (p=0.009)	
$CD3^-$ $CD56^+$	M	65	447.7	289.7	351.0	375.9-519.5	106.9	
	F	56	340.8	200.5	328.0	287.1-394.5	cells/μl	NS
	Both	121	398.2	257.0	340.0	352.0-444.5	M > F (p=0.022)	

NS = no significant difference

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4. Reference ranges of $CD4^+$T cells in healthy Thai adults based on absolute number of $CD4^+$T cell count

Absolute number of $CD4^+$T cells of healthy subjects were classified into three ranges of ≤399, 400-499, ≥500 cells/μl. The results demonstrated that 26.4 % of our subjects had $CD4^+$T cell count below 500 cells / μl; and 13.6% had $CD4^+$T cell count below 400 cells/μl (Table 12). $CD4^+$T cell count of these subjects ranged between 281 and 1,458 cells/ μl (data not shown), and with 95 % reference range of 624.3-703.3 cells/ μl (Table 10).

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Table 12. Distribution of subjects with different ranges of $CD4^+$ T cells

Sex	Number of subjects with $CD4^+$ T cells of			Total
	≤ 399 cells/μl	400-499 cells/μl	≥ 500 cells/μl	
Male	9	9	49	67
Female	8	7	43	58
Both	17 (13.6%)	16 (12.8%)	92 (73.6%)	125 (100%)

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5. Correlation between level of $CD4^+$T cell count and changes in level of $CD19^+$B, $CD8^+$T or NK lymphocytes in HIV seropositive subjects

It has been well accepted that number of $CD4^+$T cells decreases as the HIV disease progress. And as number of $CD4^+$T cell decrease, number of some lymphocyte subset(s) should increase in order to reach the total count of 100 %. The present study assayed for number of $CD8^+$T cells, $CD19^+$B cells and NK cells in HIV infected cases with $CD4^+$T cells < 14% or $\geq$ 14% in order to determine number of lymphocyte subset (s) which was affected when number of $CD4^+$T cells changes during course of the disease.

Statistical analyses using Pearson correlation and linear regression demonstrated that change on $CD4^+$T cell count had statistically significant effect on level of $CD8^+$T cell and NK cell counts in term of inverse correlation. Meanwhile, the change on $CD4^+$T cell count had no statistical effect on level of $CD19^+$B cell count (Figs. 6-8 and, Table 13).

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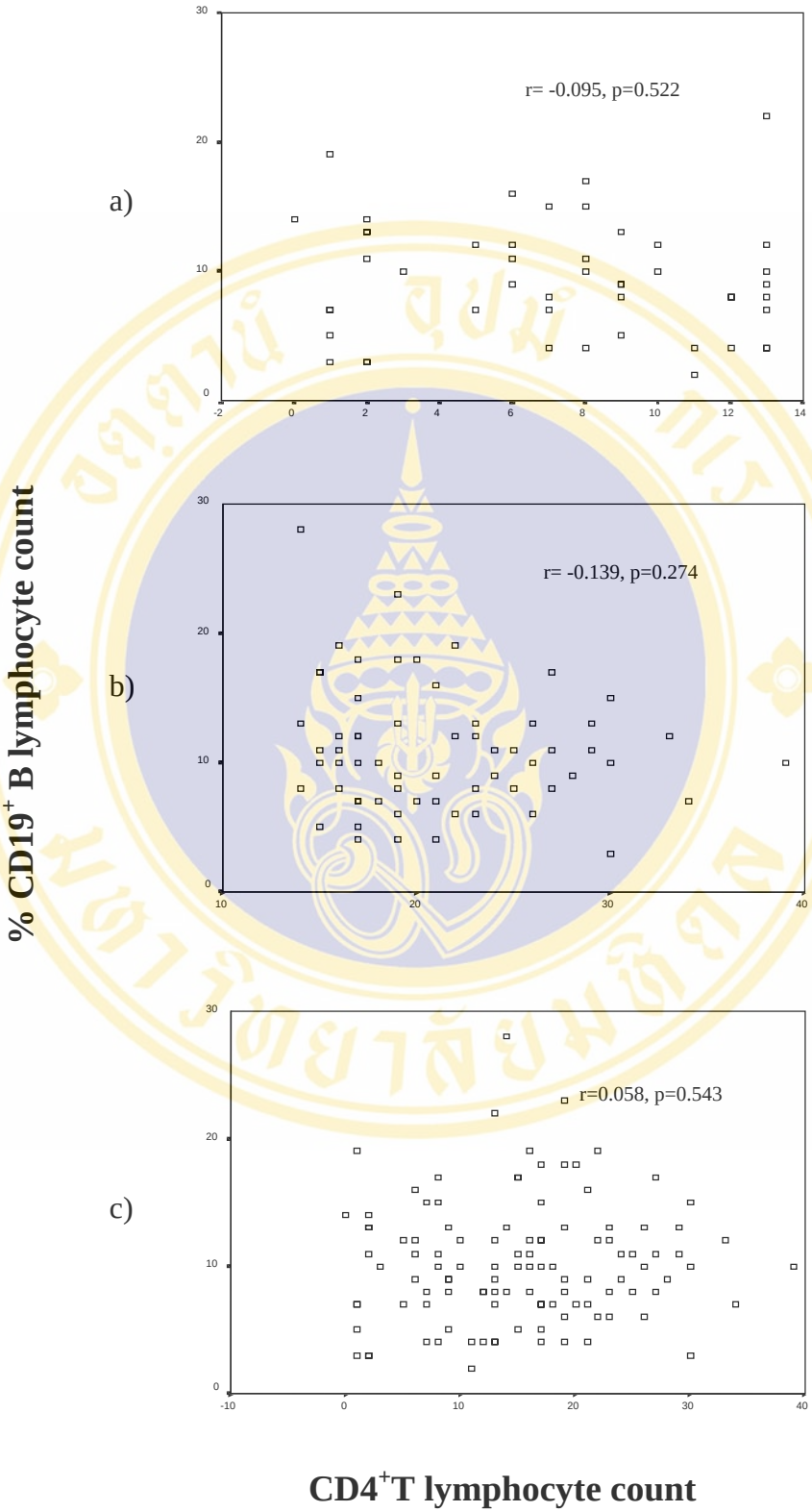

Figure 6. Correlation between level of $CD4^+$T lymphocyte and $CD19^+$B lymphocyte count in HIV seropositive subjects with ;
a) CD4 <14% (n=48) b) CD4 ≥ 14% (n=64) c) total subjects (n=112)

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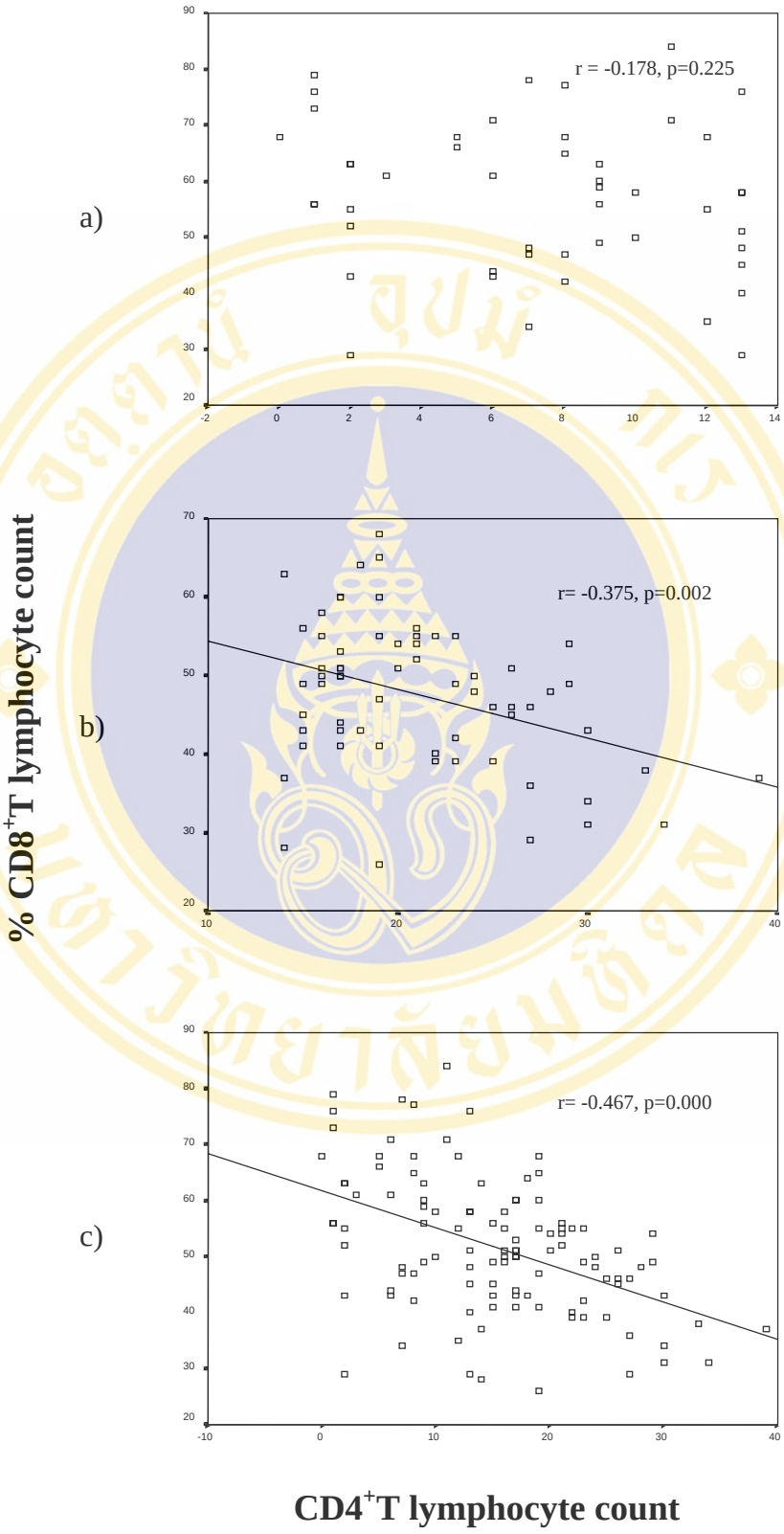

Figure 7. Correlation between level of $CD4^{+}$T lymphocyte and $CD8^{+}$T lymphocyte count in HIV seropositive subjects with ;
a) CD4 <14% (n=48) b) CD4 ≥ 14% (n=64) c) total subjects(n=112)

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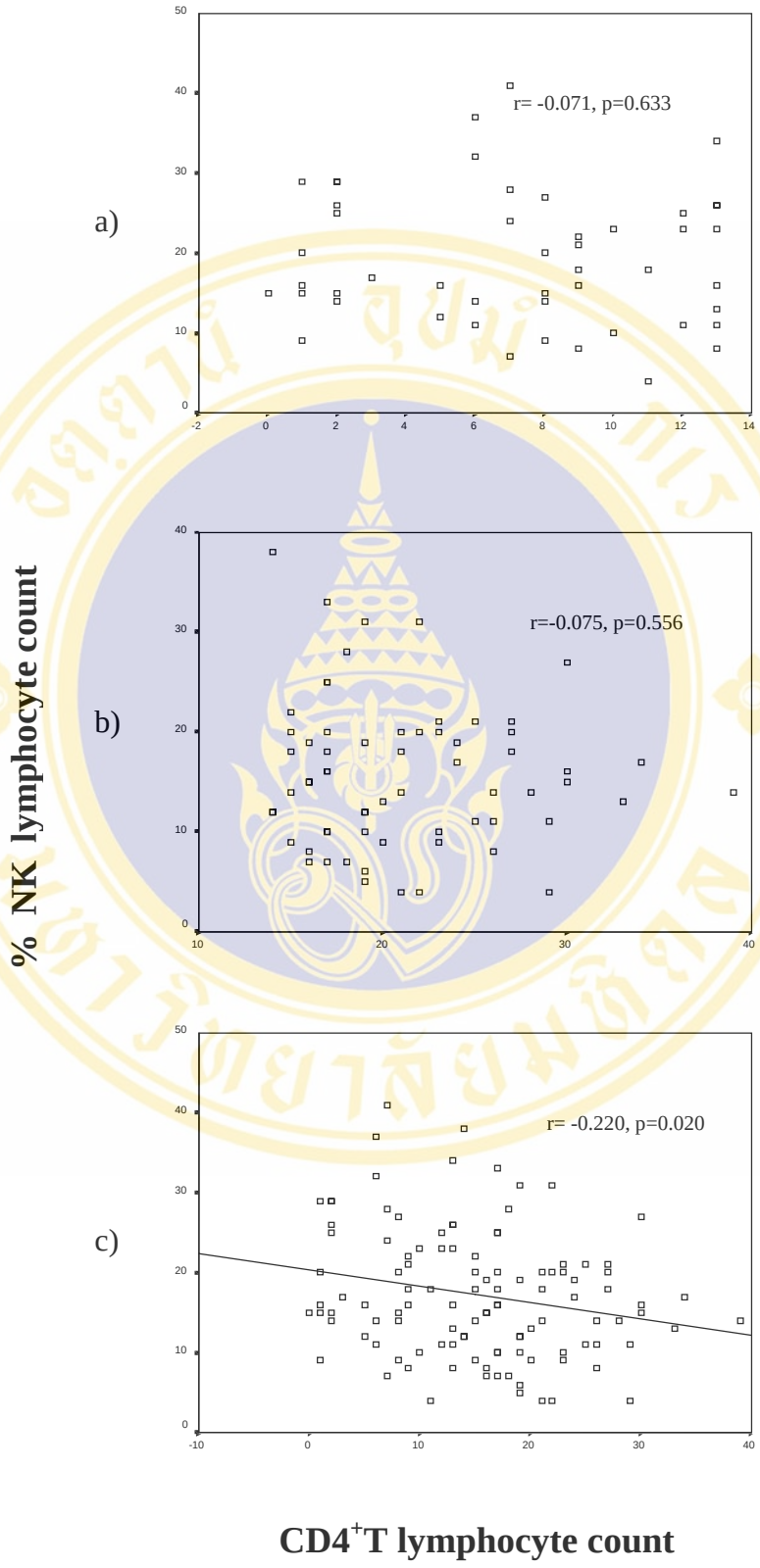

Figure 8. Correlation between level of $CD4^{+}$T lymphocyte and NK lymphocyte count in HIV seropositive subjects with ;
a) CD4 <14% (n=48) b) CD4 ≥14% (n=64) c) total subjects (n=112)

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Table 13. Correlation between level of $CD4^+$ T cells and $CD19^+$B, $CD8^+$ T cells or NK lymphocytes in HIV seropositive subjects

Parameter	Correlation with level of $CD4^+$T cells		
	CD4<14% (n=48)	**CD4 ≥ 14%** (n=64)	**All** (n=112)
$CD19^+$ B lymphocyte count	r = -0.095 p = 0.522	r = -0.139 p = 0.274	r = 0.058 p = 0.543
$CD8^+$ T lymphocyte count	r = -0.178 p = 0.225	r = -0.375 p = 0.002	r = -0.467 p = 0.000
NK lymphocyte count	r = -0.071 p = 0.633	r = -0.075 p = 0.556	r = -0.220 p = 0.020

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6. Synthesis of IFN γ, RANTES, MIP-I α and MIP-I β in different lymphocyte subsets of HIV seronegative and HIV seropositive subjects

Flow cytometry was used to analyse for expression of cell surface markers and intracellular cytokine and chemokine production in $CD3^+$ and $CD8^+$T cells and NK cells upon activation with PMA and ionomycin. The study included 10 HIV seronegative subjects and 28 HIV seropositive subjects (nine had $CD4^+$T cells<14%; and 19 had $CD4^+$T cells ≥14 %). Freshly heparinized whole blood specimens were stimulated with PMA and ionomycin for 4 hours in the presence of BFA. Unstimulated control (without the stimulation of PMA and ionomycin) were included to verify the staining specificity, as a guide for setting markers to delineate positive and negative populations and to assess the level of background of cytokine synthesis.

The stimulated and unstimulated control blood samples were stained by panel of monoclonal antibodies that determined lymphocyte subset and intracellular cytokines : IFN γ, RANTES, MIP-Iα, MIP-Iβ. The frequencies of cytokine expressing cells were enumerated by 3-color flow cytometric analysis. Gating of lymphocyte subsets and fluorescence intensity of the stained cells are shown in Figures 9-12.

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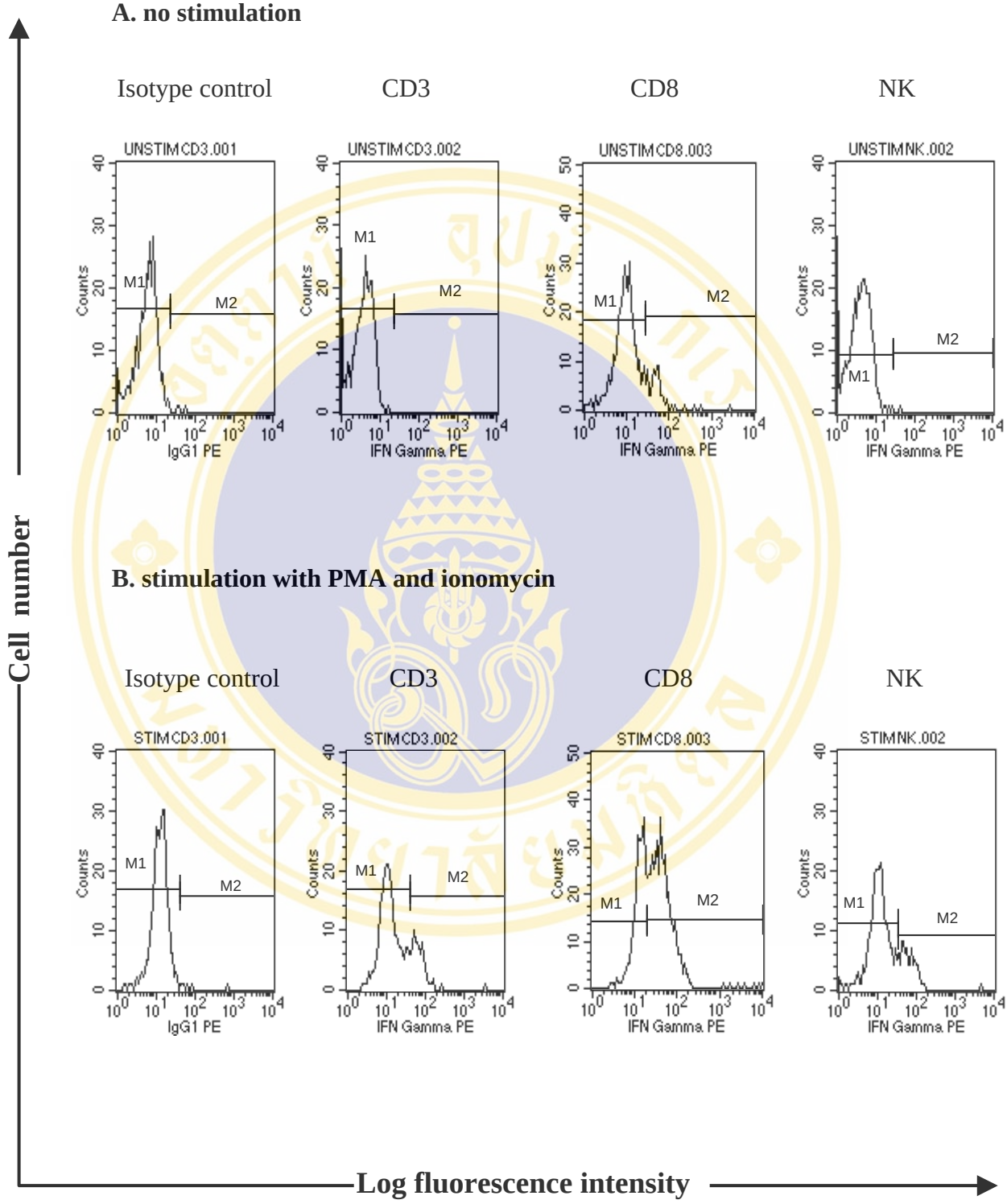

Figure 9. Flow cytometric three-color analysis of IFN γ expressing CD3, CD8 and NK cells from A: unstimulated control; and B: PMA and ionomycin stimulation

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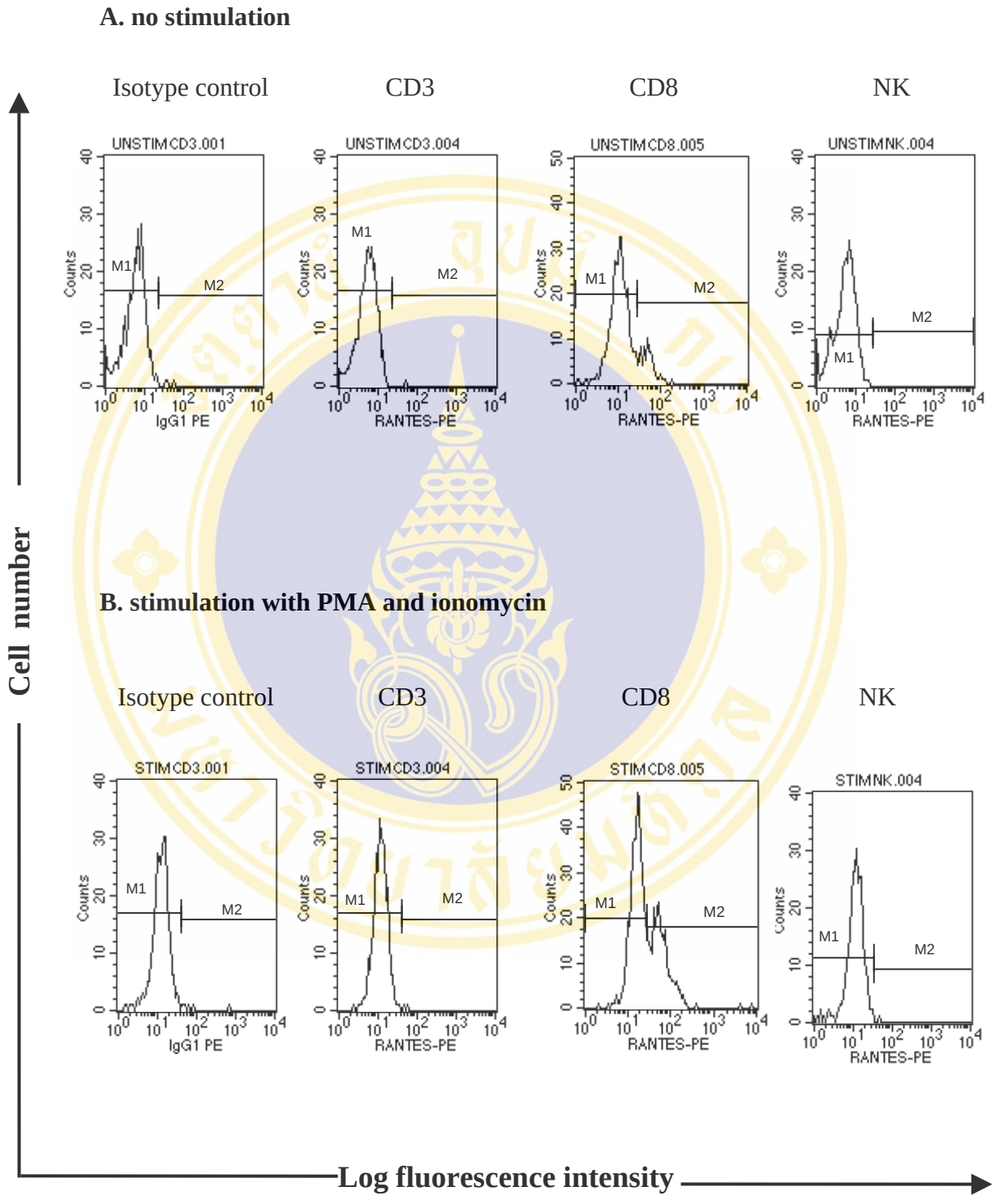

Figure 10. Flow cytometric three-color analysis of RANTES expressing CD3, CD8 and NK cells from A): unstimulated control; and B): PMA and ionomycin stimulation

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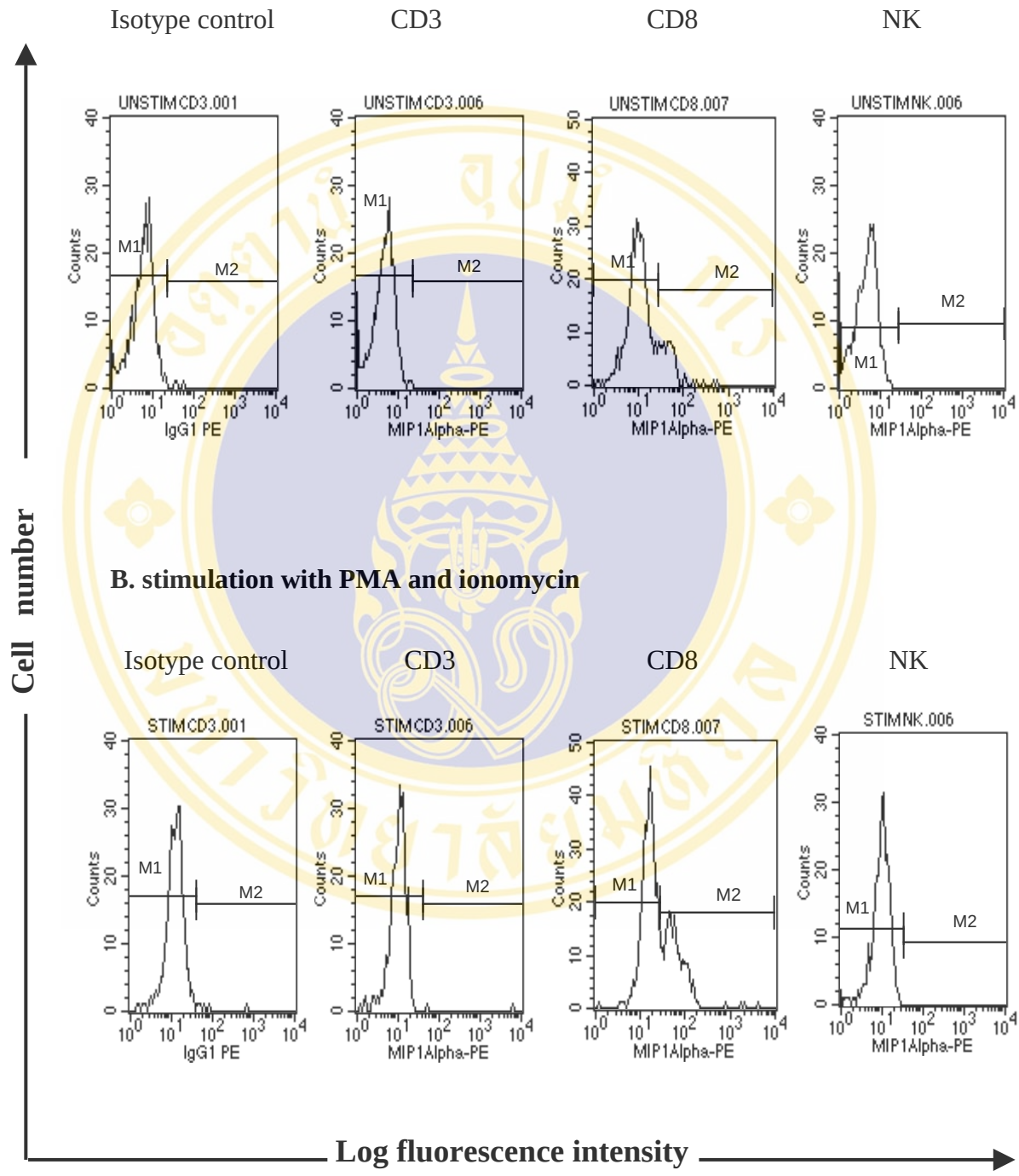

Figure 11. Flow cytometric three-color analysis of MIP-1α expressing CD3, CD8 and NK cells from A): unstimulated control and; B: PMA and ionomycin stimulation

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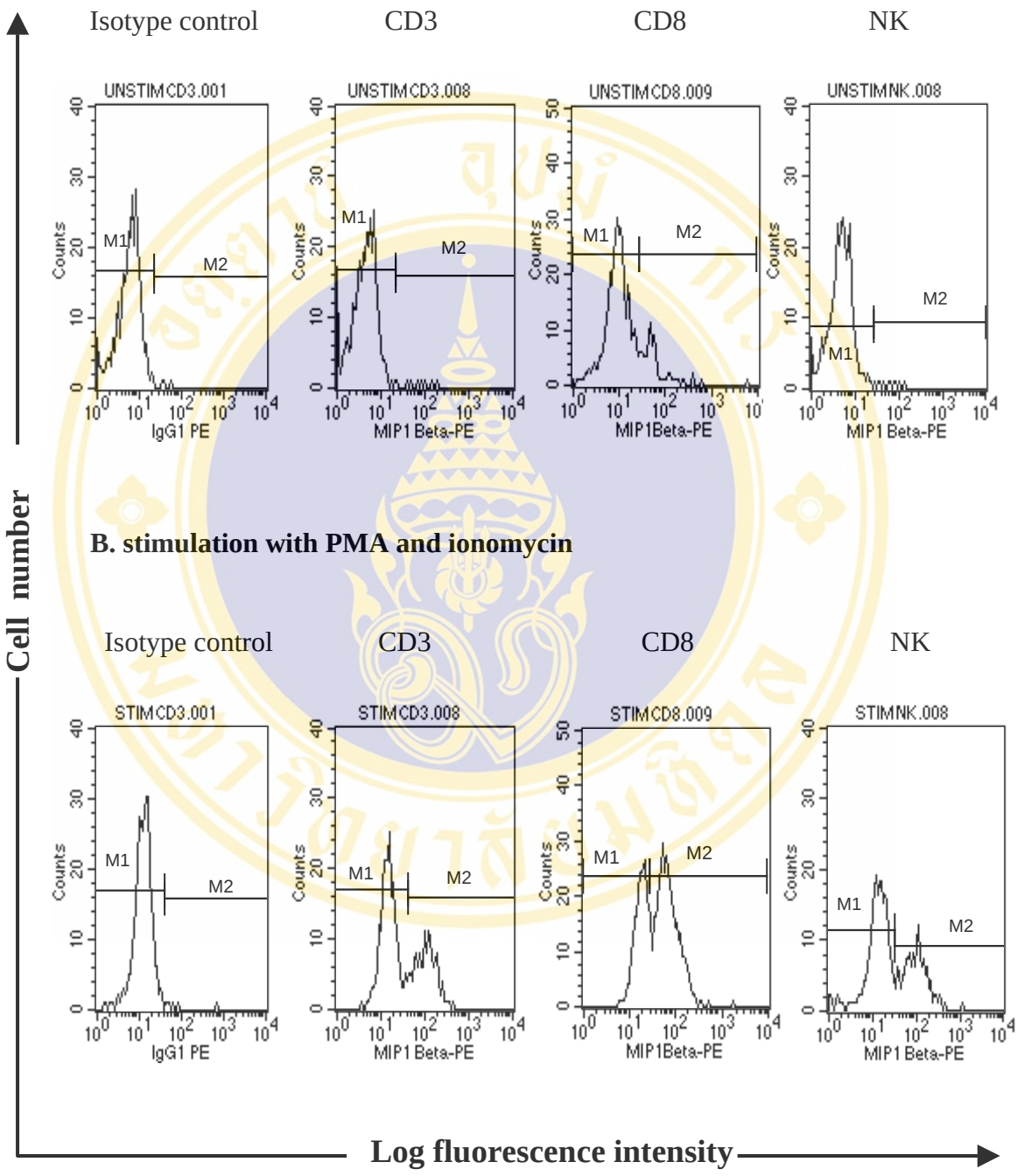

Figure 12. Flow cytometric three-color analysis of MIP-1β expressing in CD3, CD8 and NK cells from A): unstimulated control and; B): PMA and ionomycin stimulation

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6.1 Spontaneous production of cytokine/ chemokine without antigenic stimulation

Staining of the unstimulated cultures showed that spontaneous synthesis of IFN γ, RANTES, MIP-Iα, MIP-Iβ was clearly observed in $CD8^+$T cells, but not so in $CD3^+$T cells and NK cells (Figs. 13-15 and Table 14). Percentages of cells spontaneously synthesized cytokine/ chemokines was the background value which will be substracted from the percentages of stained cells of the stimulated culture to yield the corrected percentages of cells synthesizing cytokine/ chemokines as the result of PMA and ionomycin activation.

Table 14. The background value of cytokine expressing cell without antigenic stimulation

	Study groups	% of Mean ± SD		
		CD3 producing cells	CD8 producing cells	NK producing cells
IFN γ	HIV seronegative subjects (n=10)	0.6±1.2	15.5±20.4	0.1±0.1
	HIV seropositive subjects (n=28)	0.8±1.9	7.6±7.7	0.0±0.0
RANTES	HIV seronegative subjects (n=10)	1.7±2.3	6.8±6.0	1.0±2.2
	HIV seropositive subjects (n=28)	1.7±2.8	10.5±8.4	0.4±0.7
MIP-Iα	HIV seronegative subjects (n=10)	0.5±0.1	5.4±5.3	0.0±0.1
	HIV seropositive subjects (n=28)	1.0±2.4	8.3±8.1	0.0±0.0
MIP-Iβ	HIV seronegative subjects (n=10)	0.8±1.2	6.7±5.8	0.3±0.4
	HIV seropositive subjects (n=28)	1.5±3.4	8.7±7.6	0.3±0.4

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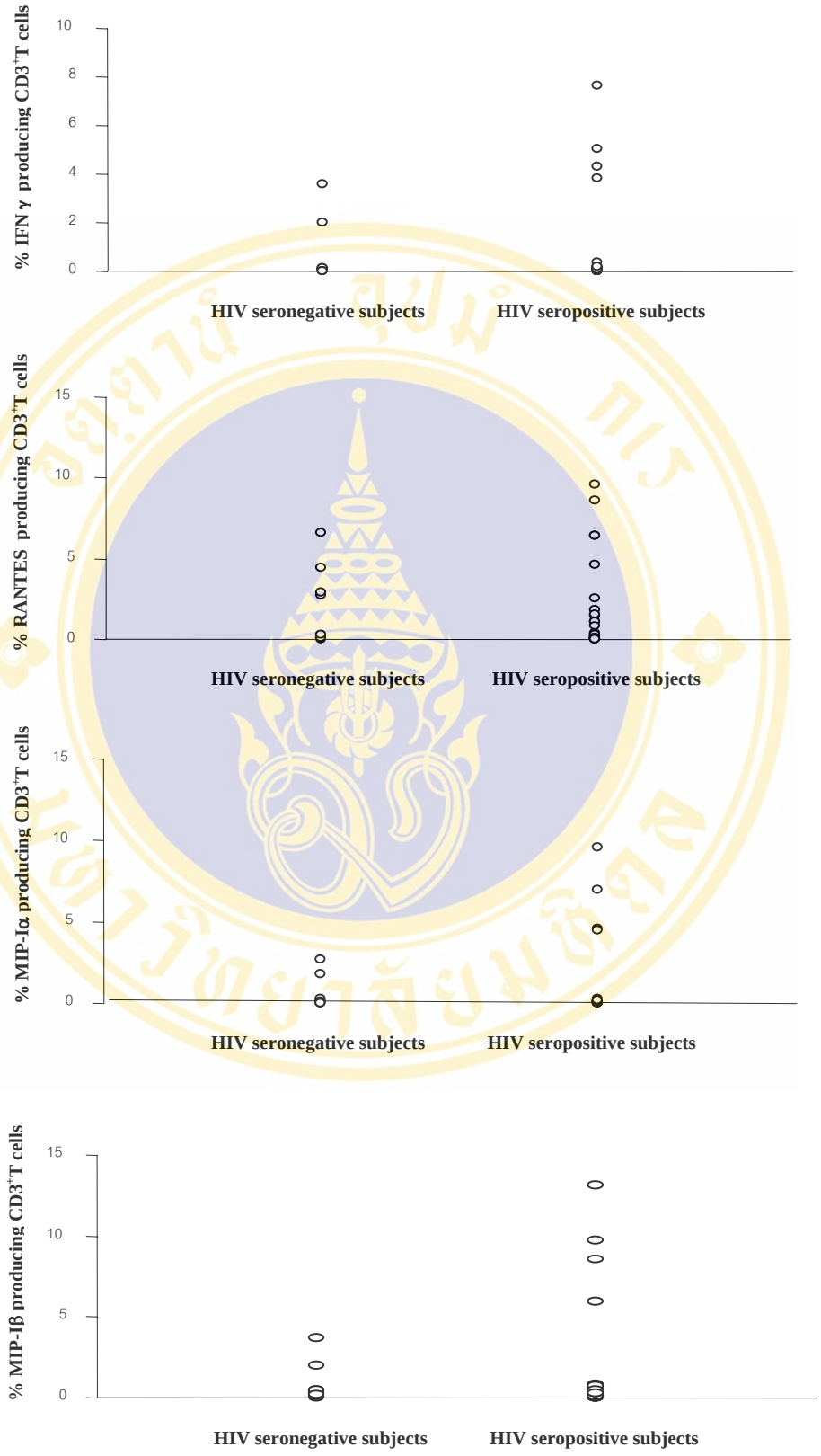

Figure 13. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated $CD3^+$T cells from HIV seronegative (n=10) and seropositive subjects (n=28)

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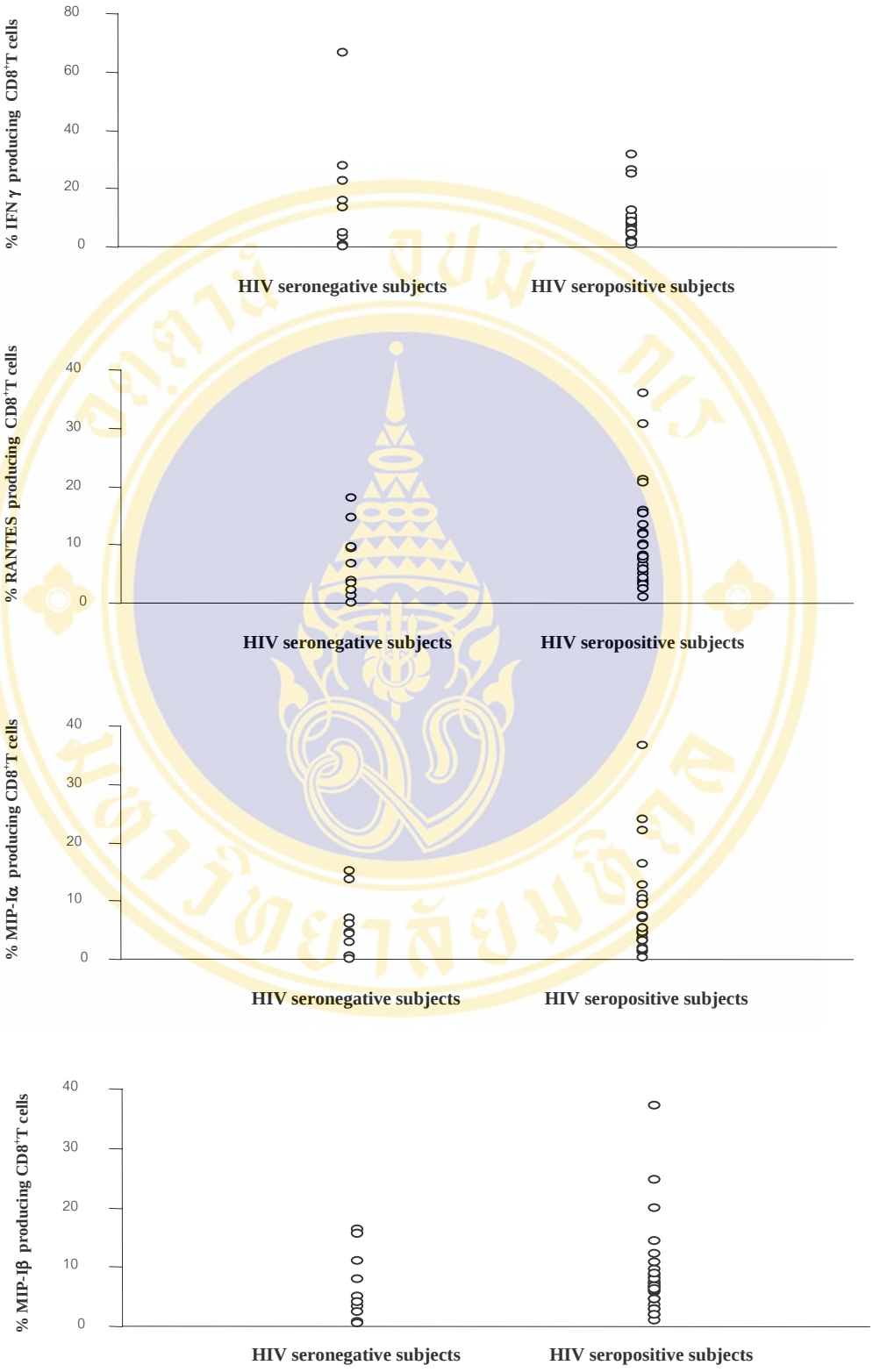

Figure 14. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated $CD8^+$ T cells from HIV seronegative (n=10) and seropositive subjects (n=28)

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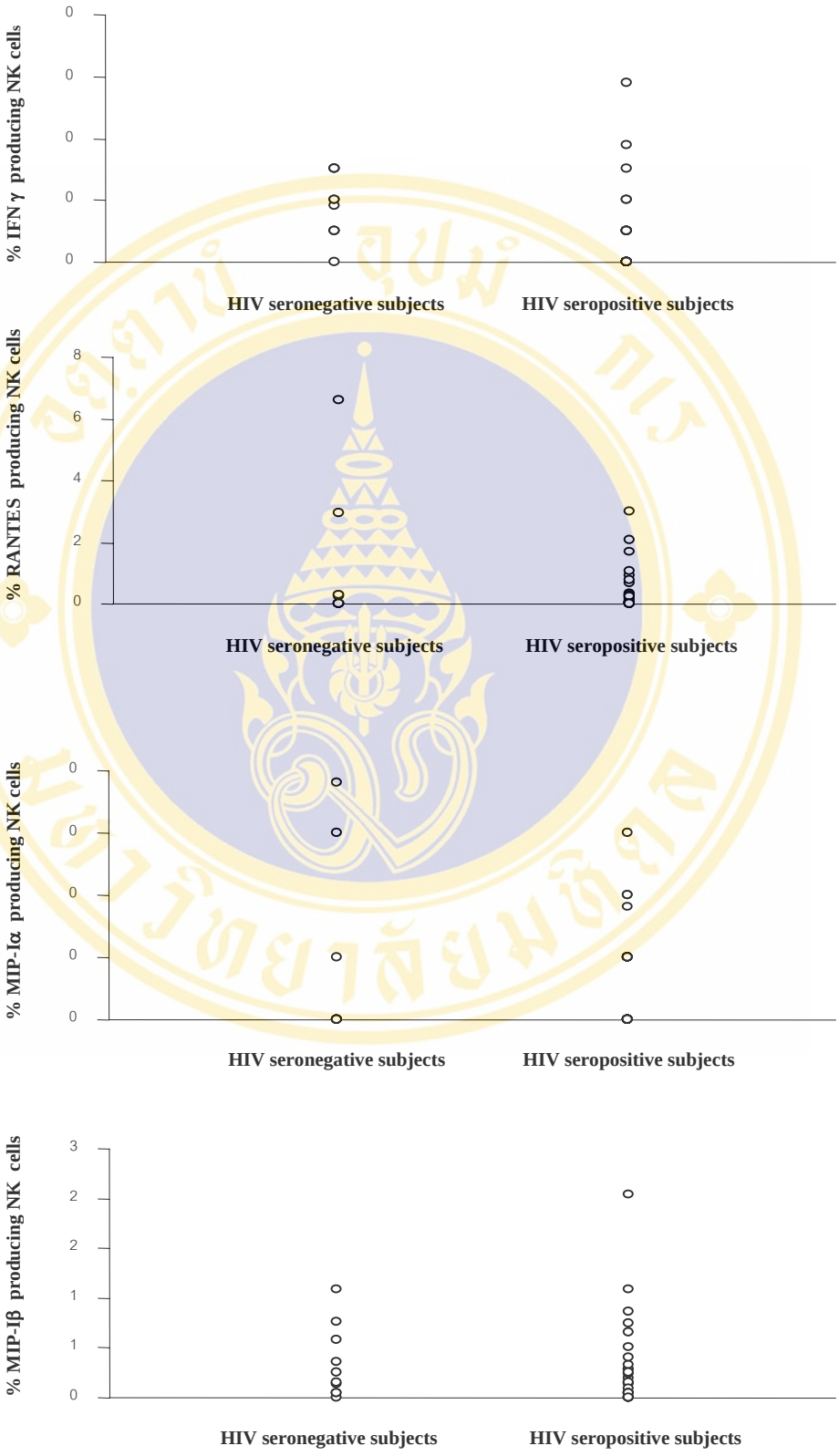

Figure 15. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated NK cells from HIV seronegative (n=10) and seropositive subjects (n=28)

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6.2. Production of cytokine/ chemokine upon stimulation with PMA and ionomycin

Among $CD3^+$T, $CD8^+$T and NK cells from three groups of subjects: HIV seronegative subjects, HIV seropositive cases with $CD4^+$ T cells < 14% and HIV seropositive cases with $CD4^+$T cells $\geq$14%, it has been shown upon stimulation that $CD8^+$T cells and NK cells were good sources of IFN γ and MIP-Iβ ; whereas MIP-Iα and RANTES were mainly produced from $CD8^+$T cells. Comparison was also conducted between the HIV seronegative subjects and HIV infected subjects among IFN γ, RANTES, MIP-Iα and MIP-Iβ expressing in $CD3^+$T, $CD8^+$T and NK cells. The study demonstrated that IFN γ, RANTES, MIP-Iα and MIP-Iβ expressing $CD8^+$T and NK cells were not significantly different as compared between the HIV seronegative subjects and HIV infected subjects. (as analysed by independent t-test and Mann Whitney U-test).

The results of cytokine/ chemokine production in $CD3^+$T cells, $CD8^+$T cells and NK cells of HIV seronegative and HIV seropositive subjects are shown in Figure. 16 together with the summarization in Table 15.

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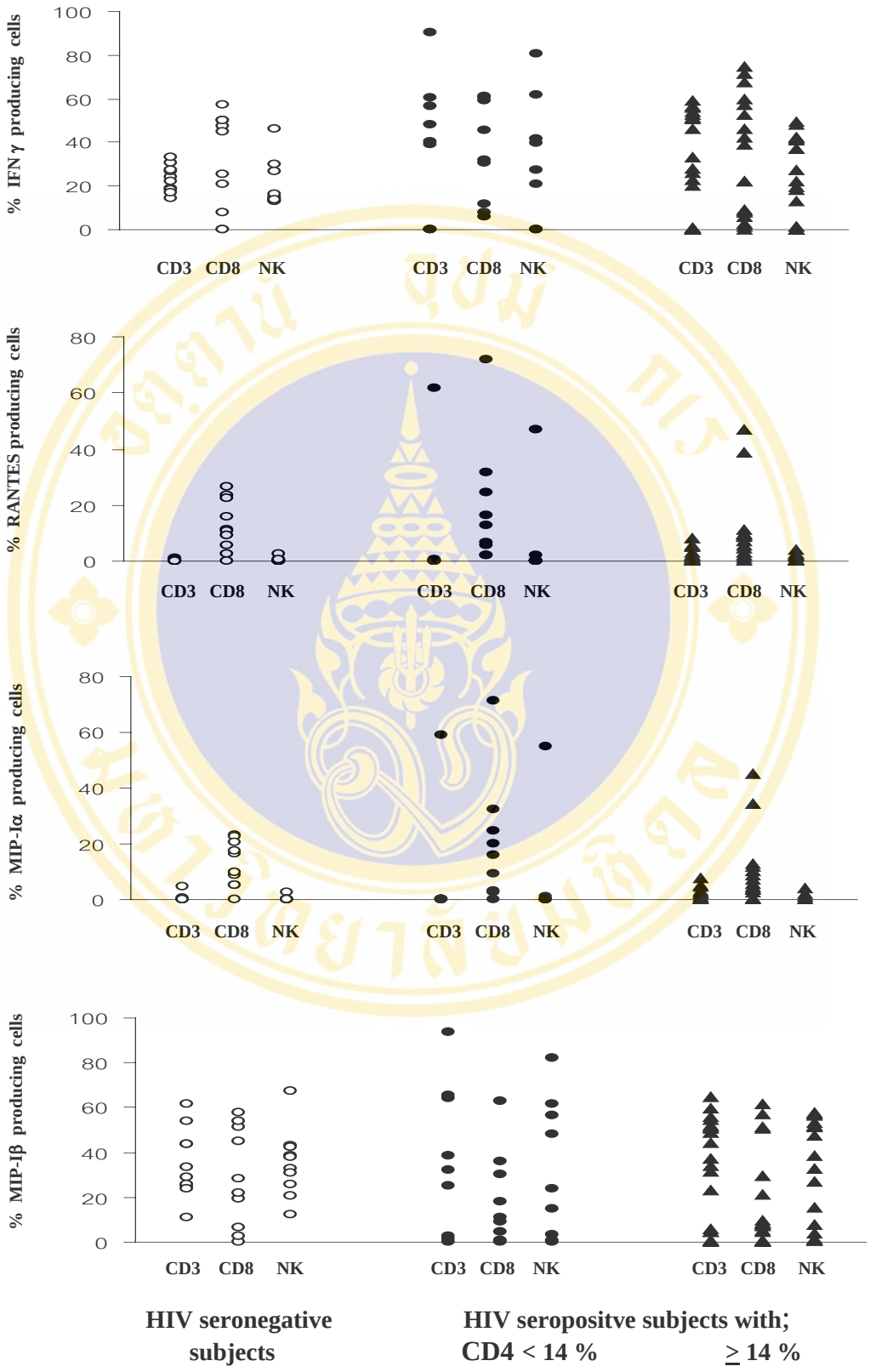

Figure 16. Corrected percentages of IFN γ, RANTES, MIP-Iα and MIP-Iβ expressing $CD3^{+}$T, $CD8^{+}$T and NK lymphocytes in HIV seronegative (n=10) and seropositive subjects (n=28)

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Table 15. Flow cytometric analyses on cytokine and chemokine producing cells upon stimulation by PMA and ionomycin

	Study groups	CD3 producing cells	CD8 producing cells	NK producing cells
IFN γ	HIV seronegative subjects (n=10)	23.2±6.2 [b]	30.3±22.2 [b]	20.1±10.8 [b]
	HIV seropositive subjects with ; CD4<14% (n=9)	37.2±31.5 [b]	34.9±22.7 [b]	30.1±28.7
	CD4≥14% (n=19)	29.3±23.4 [b]	31.2±26.9 [b]	23.1±18.7 [b]
RANTES	HIV seronegative subjects (n=10)	0.2±0.3[a]	12.8±9.1 [b]	0.4±0.8 [a]
	HIV seropositive subjects with ; CD4<14% (n=9)	6.9±20.5 [a]	19.2±22.2 [b]	5.5±15.6 [a]
	CD4≥14% (n=19)	1.6±2.3 *	8.2±12.9 [b]	0.8±1.2 [a]
MIP-Iα	HIV seronegative subjects (n=10)	0.6±1.5 [b]	12.8±8.1 [a]	0.3±0.8 [b]
	HIV seropositive subjects with ; CD4<14% (n=9)	6.7±19.7 [b]	19.9±22.2 [a]	6.3±18.2 [b]
	CD4≥14% (n=19)	1.5±2.2 [b]	8.2±12.0 [a]	0.5±1.0 [b]
MIP-Iβ	HIV seronegative subjects (n=10)	35.0±15.5 [b]	28.5±22.1 [a]	35.1±15.0 [b]
	HIV seropositive subjects with ; CD4<14% (n=9)	35.7±33.0 [b]	19.1±20.7 [b]	32.3±30.5 [b]
	CD4≥14% (n=19)	32.9±23.3 [b]	17.5±21.4 [a]	32.1±23.5 [b]

* = There was statistically significant difference as compared between HIV seronegative and seropositive subjects ($p<0.05$)

a = data was analysed by Mann Whitney U-test; b = data was analysed by independent t-test

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8. NK cell cytotoxicity assay

NK cell cytotoxicity assay was performed in 69 HIV seronegative (38 males, 31 females with age-range of 21-52 , 21-55 years; and mean age of 34.05+8.8, 31.3±11.1, respectively), and 107 HIV seropositive subjects (46 males, 61 females with age-range of 21-53, 18-50 years and mean age of 32.9±8.13, 26.97±5.63, respectively). Fresh, unsensitized PBMCs from the subjects was used as source of NK effector cells (E) and K562 cells labeled wih ^{51}Cr were used as the target cells (T). The NK cell cytotoxic activity was measured by chromium release from the radiolabeled target cells. Five different E: T ratios were used in a test assay and the value of NK cytotoxic activity was expressed in term of $LU_{20.}$

The result on NK cell cytotoxic activity in HIV seronegative subjects showed no sex difference (independent t-test; $p \geq 0.05$) (Table 16) with an average $LU_{20}/10^6$PBMC of 10.5±8.4. Among HIV seropositive subjects, the study did not find significant difference in NK cell number and NK cell cytotoxic activity as compared between the group with $CD4^+$T cell count<14% ($LU20/10^6$PBMC = 6.4±5.8) and the group with CD4>14% ($LU20/10^6$PBMC = 8.1±7.1) (independent t-test; $p \geq 0.05$). However, the study has demonstrated a decrease in NK cell cytotoxicity in both groups of HIV infected cases as compared either one with HIV seronegative controls (Mann Whitney U test; $p<0.05$). Moreover, a decrease in NK cell number was found in HIV infected cases with CD4≥14% as compared to HIV seronegative controls (independent t-test; $p=0.000$) (Table 17).

Similarly, when the mean values of $LU20/10^6$ NK cells from all subjects was taken into account, a decrease in NK cytotoxic activity could be observed in the late disease stage of HIV infected subjects with CD4<14% as compared to HIV seronegative subjects (Table 17). This study also showed the correlation between number of NK cells or their subpopulation ($CD16^+$ and $CD56^+$ NK cells) and NK cytotoxic activity (Figures 17-19). The result suggested that both NK cell subpopulations had cytotoxic function. Moreover, NK cells in late disease stage could retain their function, even though, not as good as NK cells from HIV seronegative subjects. On the other hand, our study had shown an inverse correlation between NK

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cell cytotoxic activity and $CD4^+T$ cell count only in HIV seronegative subjects, whereas, in HIV seropositive subjects, this correlation could not be demonstrated.

Table 16. Determination for the amount of NK cells and their function in HIV seronegative, healthy Thais as classified by sex

Subjects	N	Mean percentages of NK cells ± SD	Cytotoxic activity ± SD ($LU20/10^6$ PBMC)
Total subjects	69	20.8 ± 7.8	10.5 ± 8.4
Males	38	21.8 ± 8.8	10.7 ± 9.2 [a]
Females	31	19.5 ± 6.4	10.1 ± 7.5 [a]

a = There was no statistically significant difference in NK cell activity as compared between sex (independent t-test; $p \geq 0.05$).

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Table 17. Correlation between NK cell number and NK cytotoxic activity in HIV seronegative and HIV seropositive subjects with different ranges of $CD4^+$T lymphocyte count

Subjects	N	percentages of NK cells ± SD	Cytotoxic activity ± SD (LU20/ 10^6 PBMCs)	Mean LU20 /10^6NK cells
HIV seronegative subjects	69	20.8 ± 7.8	10.5 ± 8.4	52.3±42.9
HIV-1 seropositive subjects with ;				
CD4 ≥ 14 %	61	16.0 ± 7.3 [a,b] (p=0.000)	8.1 ± 7.1 [a,b*] (p=0.049)	52.1±46.8 [c*,d] (p=0.006)
CD4 < 14 %	46	19.0 ± 8.5 [c, a]	6.4 ± 5.8 [a, b*] (p=0.002)	32.2±26.0 [b*,d] (p=0.005, 0.006)

a, a* = There was no statistically significant difference as compared between HIV seropositive subjects with CD4<14% and ≥14% (p≥0.05).

b, b* = There was statistically significant difference as compared between HIV seronegative and HIV seropositive subjects (p<0.05).

c, c* = There was no statistically significant difference as compared between HIV seronegative and HIV seropositive subjects (p≥0.05).

d = There was statistically significant difference as compared between HIV seropositive subjects with CD4<14% and ≥14% (p<0.05).

a, b, c, d = analysed by independent t-test; a*, c* = analysed by Mann Whitney U-test

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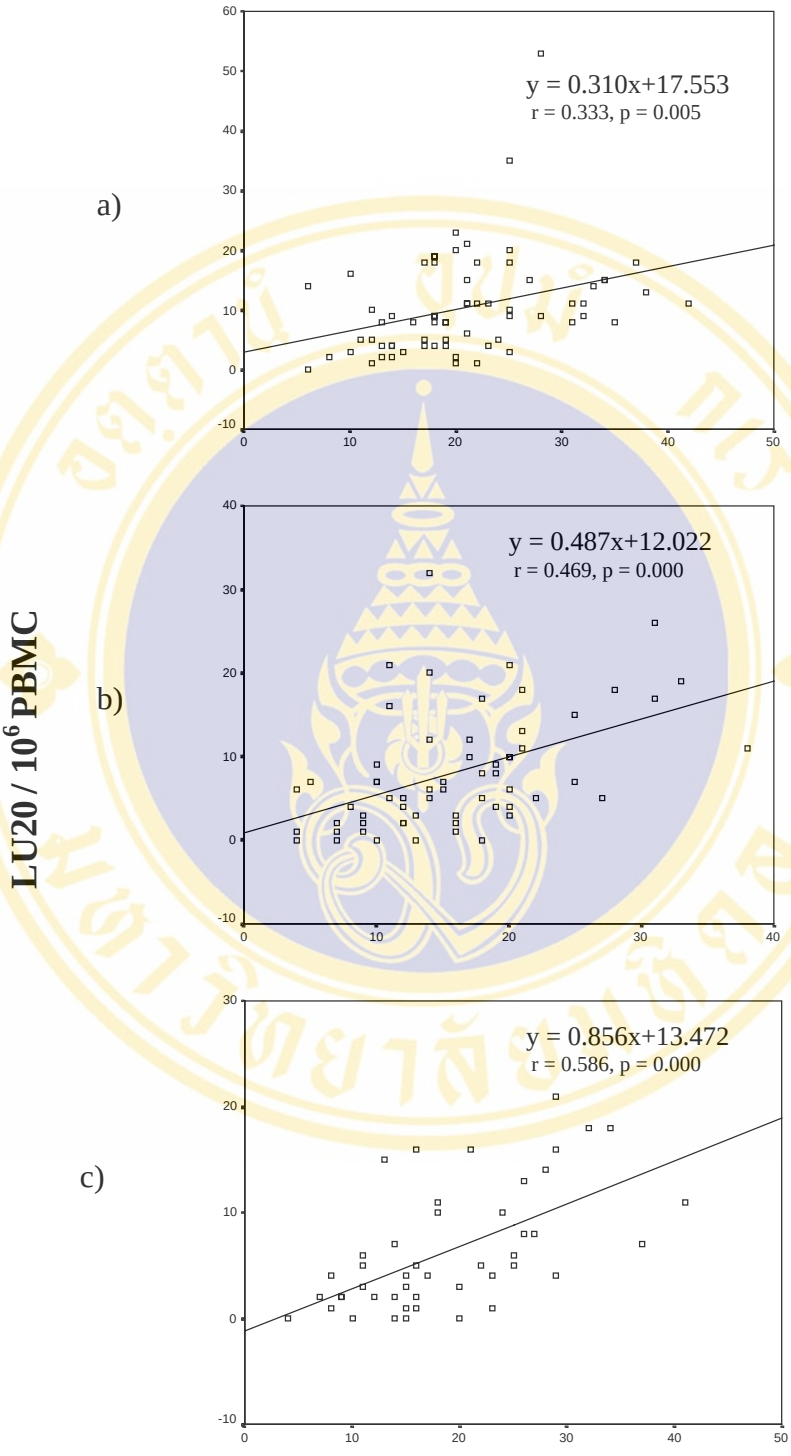

Figure 17. Correlation between LU20/ 10^6 PBMC and NK cell numbers in; a) HIV seronegative subjects; b) HIV seropositive subjects with CD4≥14% and; c) HIV seropositive subjects with CD4<14%

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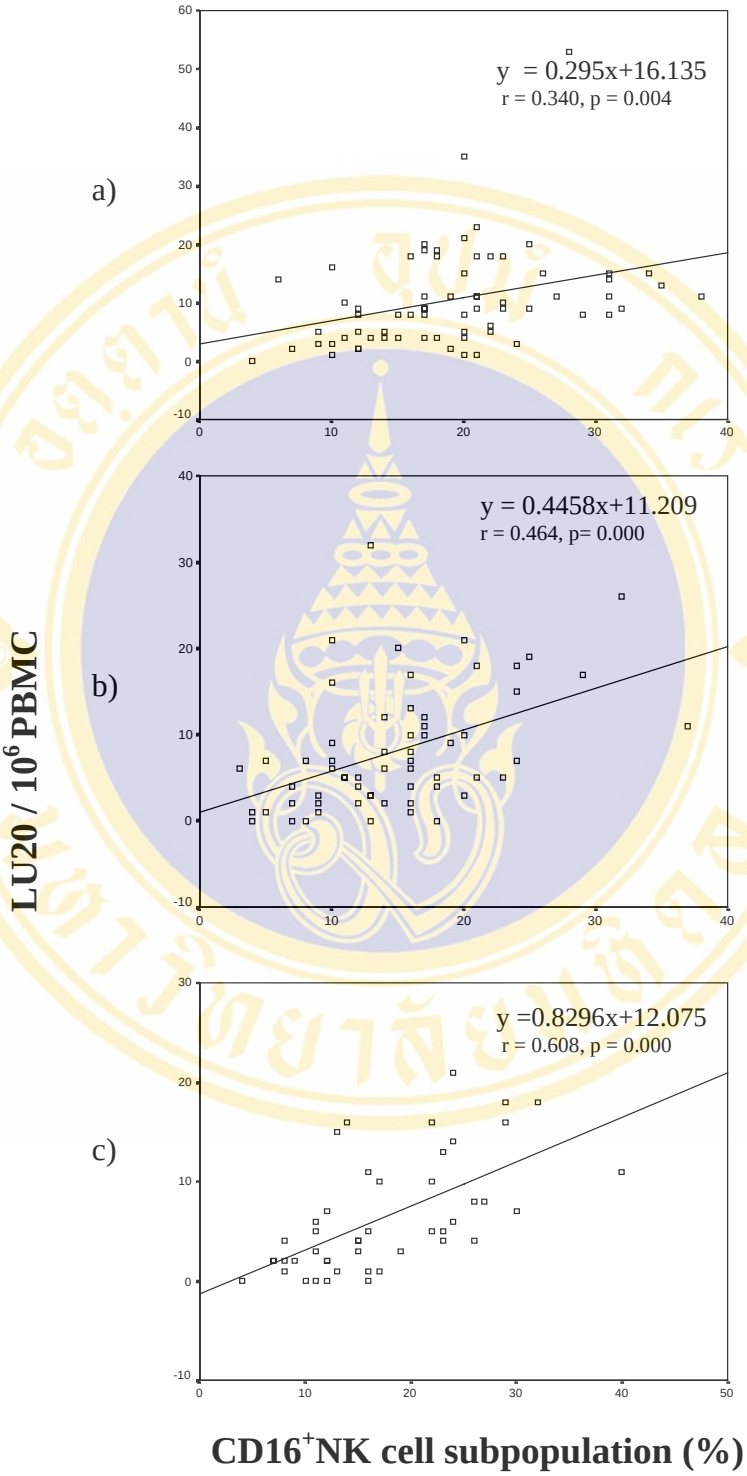

Figure 18. Correlation between LU20 / 10^6 PBMC and %$CD16^+$NK cell subpopulation in; a) HIV seronegative subjects ; b) HIV seropositive subjects with CD4≥14% and; c) HIV seropositive subjects with CD4<14%

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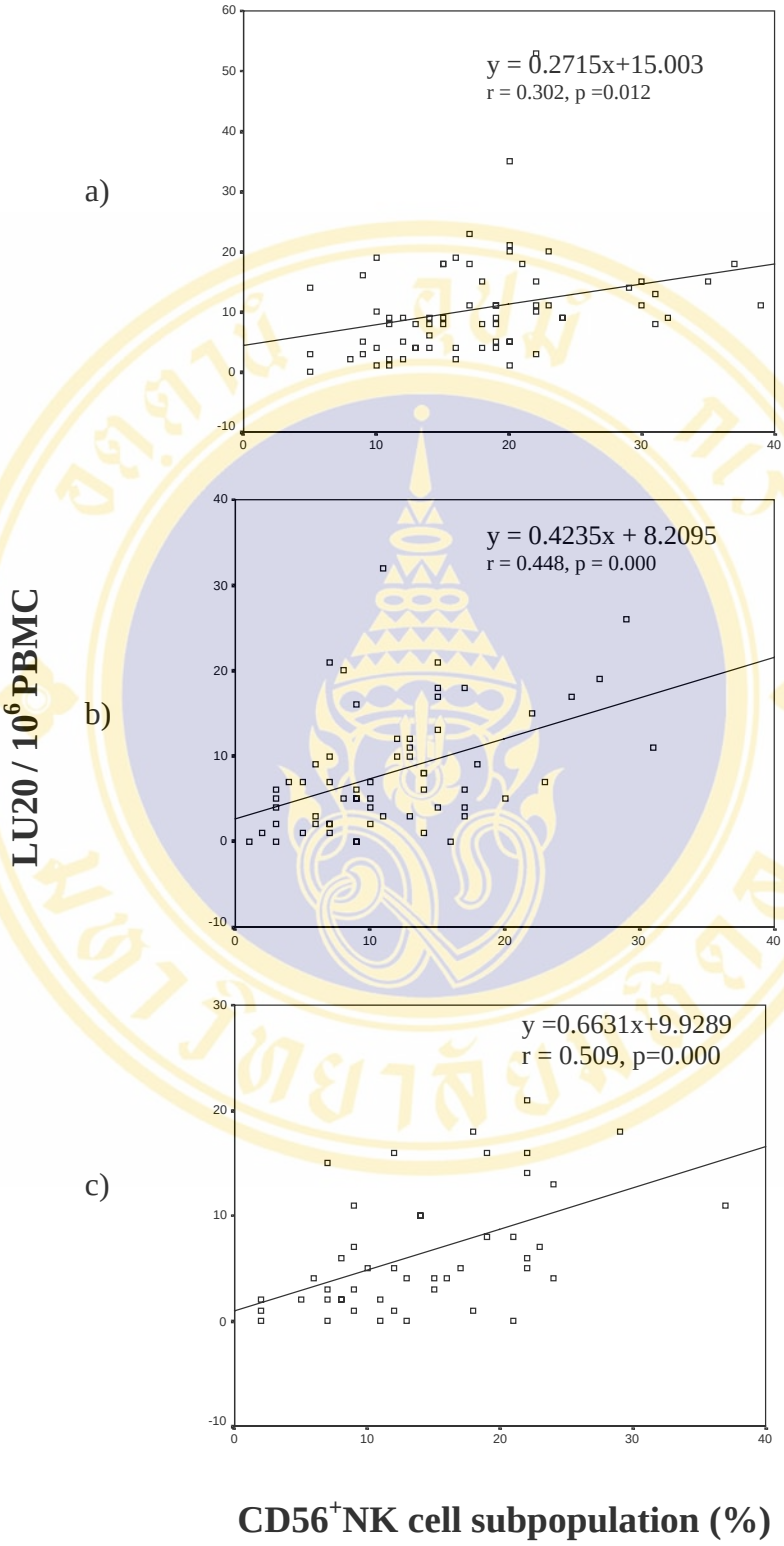

Figure 19. Correlation between LU20 / 10^6 PBMC and %$CD56^+$NK cell subpopulation in; a) HIV seronegative subjects; b) HIV seropositive subjects with CD4≥14% and; c) HIV seropositive subjects with CD4<14%

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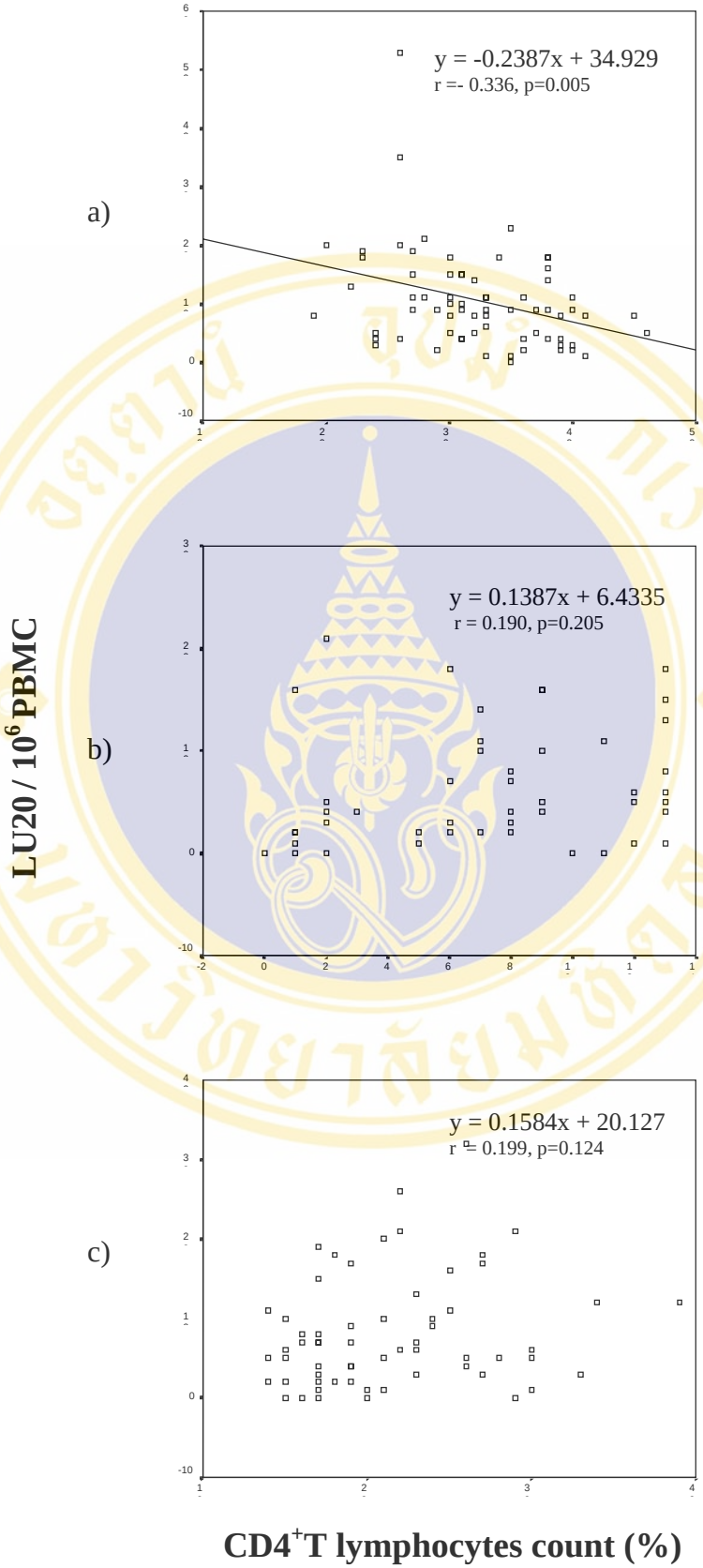

Figure 20. Correlation between LU20 /10^6 PBMC and %$CD4^+$T cell count in; a) HIV seronegative subjects; b) HIV seropositive subjects with CD4<14% and ; c) HIV seropositive subjects with CD4≥14%

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CHAPTER VI
DISCUSSION

Immune mechanisms are mediated by multiple white blood cell types: granulocytes, monocytes and lymphocytes. A functional classification of lymphocytes can further distinguish B, T and NK cells, utilizing the expression of specific cell surface glycoproteins and/ or the specific mechanism by which the cell protects the host from foreign antigens. The number and functional capacity of lymphocyte subsets reflect the overall state of immune competence of an individual. Enumeration of lymphocyte subsets by flow cytometric method has made possible the precise identification of lymphocyte immunophenotypes. In a variety of clinical situation, number of lymphocyte subset have become important in the disease diagnosis and monitoring the immunorestoration treatments. For example, the relative proportion of lymphocyte subsets may be altered in certain disease states, such as depletion of $CD4^{+}$T lymphocytes during the course of HIV infection (2).

Phenotyping for reference ranges of lymphocyte subsets can be influenced by time of blood collection, duration and temperature of specimen storage before staining, flow cytometric analysis, and also by methodologies (62-65). Previous multi-center study reported the effects of time lapse from phlebotomy to staining and fixing, and also time lapse from staining and fixing to analysis by flow cytometry (64). In addition, the Japanese study suggested circadian variation in circulating T cell subsets as evaluated in whole blood by flow cytometry technique. Rhythmicity with high values of lymphocyte numbers was shown during night time and low values during daytime (62).

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In order to control the variations from the above technical practices, we performed phleblotomy during 9.00-11.00 a.m., followed by staining and fixing within 3 hours; then, flow cytometrically analyzed within 24 hours after staining and fixing. We also kept blood specimens in air-conditioned room (25^0 C) until stained.

Previous investigators demonstrated that changes in absolute number of lymphocyte subset were not always consistent with changes in percentages values (55). It should be kept in mind that absolute number was derived from three variable values: white blood cell count, total lymphocyte count and percentages of lymphocyte subset. Therefore, the value presented by percentages may not correlated with that of the absolute number (Tables 9 and 10). In addition, with respect to the difference in the values of WBC count by sex (Table 8), we suggested that statistical analyses of the datasets presented in term of percentage values should be more accurate, because the result obtained is independent from other variable confounding factors.

The result of our study was compared to previous works done in Thai blood donors as reported by 1) Webster *et al.* (10) in the multi-site study in Bangkok, i.e., Siriraj Hospital, Chulalongkorn Hospital and Armed Forces Research Institute of Medical Sciences (AFRIMS) in 1996, and 2) Vithayasai *et al*. (58) in the Chiang Mai study. The different studies presented their data in different ways and parameters. Collectively, some points of similarity and some points of discrepancy were found. In overall, all two studies demonstrated that Thai people had relative low $CD4^+$T cell count. The $CD4^+$T cell reference values of the present study and of that from Chulalongkorn Hospital were comparable *viz,* mean = 32.1 *v.s.* 32.8 % or 663.8 *v.s.* 730 cells/µl for both sexes, but both values were relatively lower than those pooled data generated by Siriraj and AFRIMS Laboratories *viz*,37.4% or 840 cells/µl, and lower than those reported by Chiang Mai Laboratory *viz,* 36.1% or 910 cells/µl . The mean CD4/CD8 ratio in our study was about the same as that demonstrated by Webster *et al*. (10) *viz* 1.3 *v.s.* 1.35, while it was 1.5 by Vithayasai *et al*. (58). Both Webster *et al*. (10) and the present study revealed high NK cell counts especially in male subjects.

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Gathering from various reports, the most important finding was variation of lymphocyte reference ranges according to ethnic groups (Table 1). Mongoloids had lower $CD4^+$ T cell count, but higher number of NK cells (1, 10, 56). The values of $CD4^+$T cells in Mongoloid people from different countries were similar, i.e., 32% in Thai people, 32% in Chineses living in Hong Kong (56), 34.8% in Chineses living in Malaysia and 32.6% in native Malay people (1). It was noteworthy that Indian people in Malaysia had higher $CD4^+$T cells count; Indians are classified as Caucasian ethnic group. Mongoloid ethnic with relatively high $CD4^+$T cell count was observed in Japanese (72). Nevertheless, the count was still lower than those reported in Caucasians. Reports for Caucasians from Western countries demonstrated higher $CD4^+$ T cell counts varying from 42 to 48% (53-55, 63). Our study found that 26.4% of Thai people had $CD4^+$T cell count <500 cells/ µl, and 13.6% had $CD4^+$T cell count < 400 cells/ µl. The subject who had lowest amount of $CD4^+$T cell count of 281 cells/µl possessed normal values of WBC and $CD4^+$T cell count; however, her lymphocyte count was only 15%. This subject was apparently healthy during the week of blood collection, and had no obvious medical history. Similarly, 2.9% of Chinese living in Hongkong had $CD4^+$T cell count <300 cells/ µl; and 15.9% had $CD4^+$T cell count < 500 cells/ µl (56).

Refer to guidelines from US. and CDC, $CD4^+$T cell levels are used as criteria for AIDS case definitions, for initiation of anti-retroviral drugs and for pre-exposure prophylaxis of opportunistic infections (69-71). Treatment of Mongoloid people infected with HIV by basing on level of $CD4^+$T cell count recommended for Caucasians should be taken into careful reconsideration.

The compensation for Mongoloids for their low $CD4^+$T count is their high NK cell level. We also studied the changes in lymphocyte subset in HIV infected cases by determining the change in number of $CD8^+$T cells, $CD19^+$B cell and NK cells in correlation with the level of $CD4^+$T cell count (Table 13 and Figures 6-8). This study revealed that level of $CD4^+$T cell count had inversed correlation with the $CD8^+$T and NK lymphocyte count. Nevertheless, we have not seen any significant correlation between $CD4^+$T and $CD19^+$B lymphocyte counts. This study indicated that during the progression

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of HIV infection, a quantitative defect in $CD4^+$T cell count had resulted in an increase in expansion of $CD8^+$T and NK lymphocyte level.

Activation of NK cells by immunological modulators or drugs may be a supplementary treatment for cancer and viral, especially HIV infections. It has been suggested that NK cells may be a factor protecting the health of HIV seropositive individuals with very low CD4 cell counts(83). A low level of NK cells in HIV infected cases was associated with rapid disease progression, compared to those who progressed more slowly (17). The Los Angeles Center of the Multicenter AIDS Cohort Study demonstrated that percentages of NK cells severely decreased in the presence of HIV infection (81). NK cells are relatively resistant to HIV infection. Whether, the HIV can replicate in NK cells or not is still inconclusive (185-187). NK cells can mediate killing activity which is not antigen specific. Therefore, NK cells can potentially overcome the problem of HIV antigenic diversity, while this is an important factor limiting the roles of specific cytotoxic T lymphocytes and neutralizing antibody in HIV infection. Previous work done by de Souza *et al.* (84) reported that at comparable number, NK cells of Thais had higher cytotoxicity function than North Americans. The role of NK cells in HIV infected Thais and other Mongoloid people deserves further investigation.

The present study looked into those NK cells which expressed CD16 and/or CD56 molecules. CD16 molecules function as Fc receptors that bind IgG (Fcγ receptor III), thus, these receptors can link NK cells to the target cells coated with IgG and lead to target cell destruction *via* the mechanism so called antibody-dependent cell-mediated cytotoxicity (ADCC) (146, 188). $CD56^+$ NK cells play an important role in the production of several kinds of cytokines following an induction by monokines (146, 188). It was previously reported that activated NK cells may release RANTES, MIP-1α and MIP-1β, the cytokines capable of binding HIV coreceptor, and thus, blocking HIV replication (19). NK cells also release tumor necrosis factor and IFNγ which enhance immunological response (189). Our study demonstrated that 86% of NK cells express both $CD16^+$ and $CD56^+$ molecules on the same cell; and both subpopulations have cytotoxic function. NK cells also recognize and directly kill target cells through killer activating receptors. These killing activity is usually overriden by killer-inhibitory

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receptors on regcognition of MHC class I molecule under normal situation. Unfortunately, immunophenotypes of these molecules is not known (188).

NK cell is a critical component of innate immune response and thought to be important during the early stage of HIV infection (148, 190, 191). Although cytokine expressing $CD8^+$T cells have been extensively documented, there have been few studies on cytokine production from NK cells.

Previous group of investigators had found that NK cells are as potent as $CD8^+$T cells in the mediation of suppression of HIV replication, predominantly by secretion of CC-chemokines (21). In addition, Fehniger *et al.* (191) demonstrated that supernatant from NK cells can inhibit the replication not only of M-tropic HIV-1, but also of T-tropic HIV-1 strain. Moreover, Scharton *et al.* (192) demonstrated that NK cell could produce IFN-γ during the first week of infection, and occured more rapidly than might be expected for conventional primary T cell response. Oliva *et al*. (19) found the level of RANTES, MIP-1α and MIP-1β synthesized by NK cells was comparable to those produced by the stimulated $CD8^+$T cells and the study done by Vitale *et al.* (20) reported a higher percentages of IFN-γ producing NK cells as compared to number of IFN-γ producing T cells from both the HIV seronegative and HIV seropositive subjects.

Concerning the methods used for determining cytokine and chemokine production such as ELISA, RT-PCR, T cell cloning and ELISpot, all of these methods are time consuming and labor intensive. Moreover, these techniques require long activation time and cannot determine the frequencies and the phenotypes of the cytokine-producing cells at the single cell level (93, 117, 193-196). The flow cytometric method has been designed to simply and rapidly assess intracellular cytokine production in different leukocyte subsets resided in PBMCs or whole blood samples. These procedures determine the percentages of activated cells and the identification of leukocyte subsets capable of expressing various cytokines and cell surface antigens simultaneously. The ability to assess the key intracellular functional markers by multiparametric flow cytometry offer some unique advantages in a number of clinical applications.

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The present study was a further effort to reveal the production of various types of intracellular cytokines in NK and $CD8^+$T cells. It was shown that NK and $CD8^+$T cells were the expandable sources of cytokine and chemokine production in healthy subjects and HIV-1 infected ones. Few percentages of cytokine or chemokine producing $CD8^+$T, and NK cells were observed in unstimulated PBMC samples (Figures 13-15 and Table 14). Our study showed that NK cells and $CD8^+$T cells from HIV infected cases were functionally active with regards to the production of cytokine and chemokine in response to PMA and ionomycin as compared to HIV seronegative subjects. Both NK and $CD8^+$T cells from HIV seronegative and HIV seropositive subjects were capable of producing high level of IFN-γ and MIP-1 β; whereas, the production of RANTES and MIP-1α was mainly produced from $CD8^+$T cells (Table 15). Thus, HIV infection of any disease stage may not affect the ability of production of cytokine and chemokine. In contrast, Azzoni *et al.* (119) found that NK cells from HIV-infected cases were impaired in their ability to produce IFN-γ as compared to the HIV seronegative donors. Moreover, Peruzzi *et al.* (197) suggested that the gp120 mediated decrease in IFN-γ production by NK cells may contribute to the cytokine imbalance observed in HIV infection.

NK cell number and function are altered in many diseases, including HIV, certain cancers, viral infection, and autoimmune disease (16, 17). Rapid disease progression after HIV infection also has been correlated with a decrease in NK cell cytotoxic activity (180). Decrease in amount and cytotoxic function of NK cells by HIV infection was also found in several studies (81, 83, 84, 185).

This study also measured NK cell cytotoxic activity by ^{51}Cr release assay. The significant correlation between the cytotoxic activity and level of NK cells or their subpopulation could be observed. Correlation between the NK cytotoxic activity and level of $CD4^+$T cell count was also determined; and an inverse correlation was found only in HIV seronegative subjects. In contrast, there was no any correlation between $CD4^+$T cell count and NK cell cytotoxic activity as observed in HIV infected cases. Our subjects included some asymptomatic HIV infected cases with low $CD4^+$T cell count who had high NK cell cytotoxic activity (data not shown) which was similarly reported by Ironson *et al*. (83). NK cytotoxic activity may, therefore, be a factor protecting the health of these

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people. The host may maintain a degree of protection from HIV-related complications by compensating for the loss of specific T-cell immunity with non-specific immune defense.

Regarding the sex influence on NK cell cytotoxic activity in HIV seronegative subjects, a statistically significant difference was not observed. As compared to HIV seronegative subjects, a decline in NK cell activity was observed in all HIV seropositive subjects with $CD4^+$T count <14 and ≥14 %. There was no significant difference in percentage of NK cells as compared the HIV-1 seronegative subjects with the HIV seropositive subjects who had $CD4^+$T count less than 14 % (Table 17). This might be due to the compensation of number of NK cells for the decrease in number of $CD4^+$T cells.

Our study was different from what had been reported by de Souza (84) who demonstrated that NK cell number and function of the Thai HIV seronegative and seropositive subjects were not significantly different. In contrast, as compared between the HIV seronegative and seropositive North American subjects, they demonstrated the profound impairment of NK cell number and function (80, 84). They also demonstrated that the number and percentage of NK cells in HIV seronegative Thais and North Americans were similar, but the cytotoxic activity was significantly greater in the Thais. Meanwhile, Douglas *et al*. (80) reported that there was no significant difference in NK cytotoxic activity by race.

Various mechanisms have been proposed to explain why NK cell function is impaired in HIV infection. Bonavida *et al.* (179) demonstrated that defective NK cell activity can be the result from; a) a decreased number of effector cells; b) inability of the NK cells to recognize and interact with NK-sensitive targets; c) failure of NK target cells to trigger and activate the NK cells to release NK cytotoxic factor; and d) failure of the NK cell to synthesize NK cytotoxic factor.

Peruzzi *et al.* (197) demonstrated a direct effect of envelope gp120 which can inhibit NK cytotoxic activity. The addition of gp120 to the cell culture was associated with a significant inhibitory effect on NK lysis and was clearly dose dependent (198). Moreover, Cauda *et al.* (199) showed that synthetic peptides corresponding to amino acid sequences 735-752 and 846-860 of the HIV transmembrane gp41, conjugated to protein

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carriers, significantly inhibited the NK cell activity of normal PBMCs against the K562 cell line.

Since IFN-γ can be synthesized by $CD4^+$T cells and play an important role in stimulating in innate cell-mediated immunity through NK cell cytotoxic activity (104). The decline of $CD4^+$T cells in the progression of HIV infection may affect the level of IFN-γ production and NK cell cytotoxicity activity. IFN-γ and IL-2, the T_H1 cytokines which potently stimulate the NK activity, decrease markedly as the T_H1-T_H2 shift progress during the course of HIV infection (98). In addition, the products of T_H2 cytokine e.g., IL-10 and IL-12 have been found to dramatically inhibit NK cell activity (200). Nevertheless, our study demonstrated that the expression of IFN-γ by NK and $CD8^+$T cells in HIV infected cases had no defect.

Some previous studies reported the mechanism responsible for the impairment of NK cell function during HIV infection such that NK cell served as a potential reservoir for HIV-1 or can be infected by HIV-1. Nevertheless, results obtained from different groups of investigators were controversial (185-187, 201, 202).

A second set of NK cells sharing characteristics with NK and T cell has been defined as NKT cells (157-160). NKT cells could be infected by HIV as they express both CD4 and CCR5 molecules. Therefore, resulted in NKT cell death. This event may be the other reason why NK cell cytotoxicity was impaired (165). Unutmas *et al*. (166) found that $CD4^+$/$CCR5^+$NKT cells were more susceptible to R5-tropic, but less susceptible to X-4 tropic virus *in vitro*. Recent findings have shown that number of NKT cells in PBMCs of HIV-1 infected individual are dramatically reduced as compared with healthy donors; and a positive correlation between high viral load and lower $CD4^+$NKT numbers was established (164, 166).

Our study aimed to investigate the role of NK cells in the protection against HIV disease progression based on the advantages that a) Thai people had higher frequency of NK cells as compared to the Caucasians: b) NK cells could retain their activity on chemokine/cytokine production, even though, their cytotoxic activity may decrease: c) NK cell function is not antigen specific; therefore, they can overcome the problem of

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HIV escape from humoral and cell-mediated immune response. Therefore, if NK cell activity can be sustained by any immunostimulator, it should provide an alternate approach for HIV treatment.

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CHAPTER VII
CONCLUSION

The present study comprised three major parts : 1) flow cytometric analyses to establish the lymphocyte immunophenotype reference ranges for Thai people together with the additional investigation in HIV infected cases on lymphocyte immunophenotype changes according to level of $CD4^+$ T cell count; 2) intracellular cytokine/chemokine production in NK cells and $CD8^+$T cells of HIV seronegative and HIV seropositive subjects; 3) NK cell cytotoxic activity assayed in HIV seronegative and HIV seropositive subjects

Lymphocyte subpopulation , i.e., T, B and NK cells including NK cell subsets were enumerated by two color-flow cytometry in a total of 125 HIV seronegative Thai adults. The study demonstrated relatively low $CD4^+$T cell count in these subjects, i.e., 26.3% of them had a $CD4^+$T cell count of less than 500 cells/ μl. In contrast, their NK cell counts were relatively high. Collective data from several studies including the present one suggested that high NK cell counts may be a compensation for $CD4^+$T cell count in Mongoloid people. It was roughly estimated that about 86% of NK cells harbored CD16 and CD56 molecules. Statistical analyses of the percentage values showed that females had significantly higher CD3 (total $CD3^+$T cell), but lower NK cell counts as compared to males ($p<0.05$). Regarding age variation, an increase of 1.1% of $CD4^+$T cell count per decade was seen. The change of $CD4^+$T cell count had an effect on level of $CD8^+$T cell and NK cell in term of inverse correlation, i.e., HIV infected cases with low level of $CD4^+$T cell count had higher number of $CD8^+$T cells and NK cells. In contrast, change in level of $CD4^+$T cell count had no effect on B cell level.

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Three color flow cytometric analysis was conducted to determine intracellular cytokine/chemokine production in $CD3^+$T, $CD8^+$T and NK cells. The study included a total of 10 HIV seronegative subjects and 29 HIV seropositive subjects. The HIV seropositive subjects were further devided into group of CD4<14% (n=9) and group of CD4≥14% (n=19). The study demonstrated that spontaneous synthesis of IFN γ, RANTES, MIP-Iα and MIP-Iβ was clearly observed in $CD8^+$T cells at low frequency, but not in NK cells. Moreover, it has been shown upon stimulation with PMA and ionomycin that $CD8^+$T cells and NK cells were important sources of IFN γ and MIP-Iβ; whereas RANTES and MIP-Iα were mainly produced from $CD8^+$T cells. The finding hold true in both in HIV seronegative and HIV seropositive subjects. This study suggested that HIV-1 infection has less effect on the ability of NK and $CD8^+$T cell in the cytokine/chemokine production.

NK cytotoxic activity in 69 HIV seronegative and 107 HIV seropositive subjects were measured by a standard ^{51}Cr release assay. It was shown that NK cell cytotoxic activity had no statistically significant difference by sex as observed in HIV seronegative subjects. Furthermore, NK cytotoxic activity of HIV seropositive subjects were significantly lower than that of HIV seronegative subjects.

NK cell possess several advantages in the control of HIV disease progression such as: 1) Thai people had high NK cell number; 2) NK cell recognition is antigen non specific so that it can overcome the problem of as escape of HIV mutant from cytotoxic T lymphocytes and neutralizing antibodies which both of them are antigen specific in function. This study, thus, propose NK cell as a novel approach on HIV disease treatment.

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APPENDIX

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APPENDIX

A. Reagents for cell cultivation

A.1 Growth media for K-562 erythroleukemic cell line

10% fetal calf serum (FCS) (GIBCO BRL®, Grand Island, New York) in RPMI 1640 (GIBCO, USA) plus100 units/ml penicillin and 100 µg/ml streptomycin.

A.1.1 5X RPMI 1640

Dissolve RPMI 1640 powder (GIBCO) with deionized tri-distilled water to make 5X solution; and sterile through filtration with 0.22 µm millipore membrane. The 5X media is frozen at –20^{o}C until used.

A.1.2 1X RPMI 1640 (500 ml)

5X RPMI 1640	100	ml
1M N-2 hydroxyethylpiperazine-2-ethane sulfonic acid (HEPES)	1	ml
Penicillin (40,000 units/ml) (GIBCO)	1.25	ml
Streptomycin (40,000 units/ml) (GIBCO)	1.25	ml

Adjust to pH 7.2-7.4 with 5% $NaHCO_3$ and add deionized, tri-distilled water to 500 ml

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A.1.3 10% FBS in 1X RPMI 1640 (growth media)

1X RPMI 1640	450	ml
Fetal bovine serum (Biochrom KG, Berlin, Germany)	50	ml

A.2 Cell freezing media (10% dimethylsulfoxide in 20% FBS RPMI 1640)

40% FBS in 1X RPMI 1640

1X RPMI 1640	6	ml
FBS	4	ml

20% DMSO in 1X RPMI 1640

1X RPMI 1640	8	ml
DMSO (Sigma, St. Louis, Mo, USA)	2	ml

Working protocol

Suspend cells in cold 40% FBS RPMI 1640.

Add 20% DMSO in RPMI 1640 dropwise at an equal volume to achieve the final concentration of 10% DMSO and 20% FBS in RPMI media

A.3 Phosphate buffer saline Ca^{2+} and Mg^{2+} free (1X), pH 7.5 for cell washing, flow cytometry

NaCl	8	g
KCl	0.20	g
KH_2PO_4 (anhydrous)	0.12	g
Na_2HPO_4 (anhydrous)	0.91	g
Deionized, tri-distilled water	1,000	ml

Adjust to pH 7.5 by 1N NaOH. Sterilize by autoclaving at 121^{o}C under pressure of 15 lbs/square inch for 15 minutes, then, store at room temperature

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A.4 1M HEPES buffer

HEPES	253.3	g
Deionized, distilled water	1000	ml

A.4.1 Dissolve 253.3 g HEPES with deionized, distilled water to 1000 ml

A.4.2 Adjust to pH 7.4 with 1N NaOH

A.4.3 Sterilize through filtration with 0.22 μm millipore membrane

A.5 0.4% Trypan blue

This reagent is used for dead cell staining in the process of cell counting by light microscope

Trypan blue dye (Sigma)	0.4	g
1X PBS	100	ml

A.5.1 Dissolve thoroughly 0.4 g trypan blue dye with 100 ml of 1X PBS

A.5.2 Filter the solution through Whatman filter paper and keep at room temperature

B. Reagents for flow cytometry

B.1 1% Paraformaldehyde

Paraformaldehyde (sigma)	1	g
1X PBS	100	ml

Filter through 0.1 μm filtered paper, store in the dark bottle at 4 ^{o}C.

B.2 FACS lysing solution

This reagent is used for lysing the red blood cells following direct immunofluorescence staining of human peripheral blood cells with monoclonal antibodies prior to flow cytometric analysis.

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Working 1X FACS lysing solution

10X FACS lysing solution (Becton Dickson Immunocytometry system, San Jose, CA, USA)	1	ml
Deionized,distilled water	9	ml

The prepared solution is stable for up to month when stored at room temperature.

B.3 FACS permeabilizing solution

This reagent is used for permeabilization of lymphocyte membrane prior to intracellular immunofluorescence staining with monoclonal antibodies.

Working FACS permeabilizing solution (1X)

10X concentrated FACS permeabilizing solution (Becton Dickinson)	1	ml
Deionized, distilled water	9	ml

Dilute concentrated FACS permeabilizing solution to 1:10 with deionized, distilled water. The prepared solution is stable for up to one month when stored at room temperature. Do not use this reagent if discoloration or precipitation is seen .

B.4 Phorbol 12-myristate 13-acetate (PMA)

This reagent is used to stimulate the cells to produce cytokine.

PMA stock solution (0.1 mg/ml)

PMA powder (Sigma)	1	mg
DMSO	10	ml

Store the stock PMA solution in small aliquots (e.g.,20 µl) at –20 ^{o}C ; do not repeat freeze-thaw cycle

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PMA working solution (1 ng/ml)

Stock PMA solution	2	µl
Steriled PBS	198	µl

PMA working solution is used at concentration of 25 µl / 1ml whole blood or 12.5 µl of PMA/ 500 µl of whole blood to obtain the final concentration of 25 ng/ml

B.5 Ionomycin

This reagent is used to stimulate the cells to produce cytokine.

Ionomycin stock solution (0.5 mg/ml)

Ionomycin powder (Sigma)	1	mg
Ethanol (gold shield ethyl alcohol, 200 proof)	2	ml

Store the stock solution in small aliquots (e.g.,20µl) at –20 ^{o}C.

Ionomycin working solution (0.05 mg/ml)

Stock ionomycin solution	10	µl
Steriled PBS	90	µl

Ionomycin working is used at concentration of 20 µl/ ml of whole blood or 10 µl of ionomycin/ 500µl of whole blood to obtain the final concentration of 1µg/ ml

B.6 Brefeldin A (Sigma B-7651)

This reagent is used for blocking an intracellular protein release out of the cells during in vitro cell activation process.

Brefeldin A stock solution (5 mg/ml)

Brefeldin A powder (γ,4-Dihydroxy-2- [6-hydroxy-1-heptenyl] - 4 - cyclopentane-crotonic acid –λ lactone) (Sigma)	5	mg
DMSO	1	ml

Store the stock solution in small aliquots (e.g.,20 µl) at –20 ^{o}C ; do not repeat freeze-thaw cycle.

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Brefeldin A working solution (0.5 mg/ml)

Stock Brefeldin A solution	20	µl
Sterile PBS	180	µl

BFA working solution is used at concentration of 0.5 µg/ µl of whole blood (5 µl BFA plus 500 µl whole blood) to achieve the final concentration of 10µg/ ml

C. Reagents for PBMC preparation

C.1 Standard Ficoll-hypaque (Robbins Scientific, Sunnyvale, CA, Norway)

This reagent is used for PBMCs preparation from the EDTA blood. The bottle should be wrapped with aluminium foil and stored in a refrigerator.

D. Reagents for NK cytotoxicity assay

D.1 Sodium chromate [$Na_2\ ^{51}CrO_4$ (^{51}Cr)]

Just before use, dilute $Na_2\ ^{51}CrO_4$ to the concentration of 1 mCi/ml of steriled PBS. The amount of 200 µCi of ^{51}Cr per $1x10^6$ K562cells is required. The volume of ^{51}Cr is to be adjusted in each experiment according to the decay of the radioisotope.

D.2 10% Sodium dodecyl sulfate (SDS)

Purchase ready-made and sterile SDS from GIBCO (Gaithersburg, MD, USA). SDS has a long shelf life at room temperature.

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