EXPRESSION OF ACTIVATION MARKERS AND CHEMOKINE RECEPTORS ON PROLIFERATIVE LYMPHOCYTES FOLLOWING *in vitro* HIV-1 INFECTION

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ABSTRACT

This research emphasized the investigation of expression of activation markers and chemokine receptors on proliferative lymphocytes following *in vitro* HIV-1 infection. Peripheral blood mononuclear cells collected from 10 HIV seronegative healthy Thai donors were stimulated with phytohemagglutinin (PHA) and maintained in interleukin 2 (IL-2) media for 4 days. At day 4, those cells were divided into two portions. The first was infected with HIV-1, and the second was kept as a control. All samples were harvested and determined by flow cytometry at day 4, day 7, and day 10 post infection (pi). Flow cytometric analyses on CD4+ and CD8+ T cells demonstrated that there was a slight decrease but without a statistically significant difference in activation markers (CD25, CD69, CD71, and HLA-DR) and chemokine receptors (CCR5 and CXCR4) between the infected and uninfected cultures. In contrast, a significant decrease in the percentage of CXCR4 on the infected CD4+ T cells was observed at day 10 pi (p=0.049).

More specifically, T cell population was divided into three gates using the proliferating cell sizes (small, intermediate, and large). The results showed that the level of activation markers and chemokine receptors in small T cells were lower than in intermediate size T cells and highest in large T cell population. The results suggested that the expression of activation markers and chemokine receptors might be increased by the level of cell proliferation. Furthermore, this study demonstrated that the percentage of intracellular p24 antigen of small T cells was lower than the large proliferating cells.

The ability of HIV-1 infection was analyzed by flow cytometric assay, using the detection of the percentage of p24 antigen (%p24), which was correlated with the extracellular p24 antigen capture (ELISA) (ng/ml). Moreover, flow cytometry was able to quantify intracellular p24 antigen as of capture (ELISA) technique in proliferative lymphocytes.

This study indicates that activation markers and chemokine receptors might be the markers used for monitoring the progression and prognosis of the disease.

KEY WORDS: ACTIVATION MARKERS/ CHEMOKINE RECEPTORS/ PROLIFERATIVE LYMPHOCYTES/ HIV

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การเปลี่ยนแปลงของ activation markers และ chemokine receptors บนผิวของลิมโฟไซต์ ที่ถูกกระตุ้น เมื่อมีการติดเชื้อ HIV–1 ในหลอดทดลอง (EXPRESSION OF ACTIVATION MARKERS AND CHEMOKINE RECEPTORS ON PROLIFERATIVE LYMPHOCYTES FOLLOWING IN VITRO HIV-1 INFECTION)

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บทคัดย่อ

การวิจัยนี้เป็นการศึกษาการแสดงออก ของ activation markers และ chemokine receptors บนผิวของเซลล์ลิมโฟไซต์ที่ถูกกระตุ้น และมีการติดเชื้อเอชไอวี-1 ในหลอดทดลอง โดยใช้เซลล์โมโนนิวเคลียร์ จากคนไทยปกติที่มีสุขภาพดีจำนวน 10 ราย และนำมากระตุ้นด้วย PHA และเลี้ยงในอาหารเลี้ยงเซลล์ ที่มีสาร IL-2 จนครบ 4 วัน ในวันที่ 4 เซลล์จะถูกแบ่งเป็น 2 ส่วน โดยส่วนแรกถูกทำให้ติดเชื้อเอชไอวี-1 และส่วนที่สองถูกใช้เป็นตัวควบคุม ในวันที่ 4 , 7 และ 10 หลังการถูกทำให้ติดเชื้อ เซลล์จะถูกนำมาวิเคราะห์ด้วยวิธีโฟลไซโตเมทรี ผลการศึกษาพบว่า การวิเคราะห์ activation markers (CD25, CD69, CD71, และ HLA-DR) และ chemokine receptors (CCR5 และ CXCR4) บนผิวของเซลล์ CD4+ และ CD8+ ที่ถูกทำให้ติดเชื้อ HIV-1 มีแนวโน้มลดลง แต่ไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อทดสอบทางสถิติ เมื่อเทียบกับเซลล์ที่ไม่ติดเชื้อ ยกเว้นในวันที่ 10 พบว่า มีการลดลงอย่างมีนัยสำคัญทางสถิติของ CXCR4 บนเซลล์ CD4+ ที่มีการติดเชื้อ (p=0.049)

ได้มีการศึกษาเพิ่มเติมโดยแบ่ง ทีเซลล์ ทั้งหมดเป็น 3 กลุ่ม ตามขนาดของ ทีเซลล์ ที่ถูกกระตุ้นและพบว่า การแสดงออกของ activation markers และ chemokine receptors ในเซลล์ขนาดเล็ก มีปริมาณน้อยกว่าเซลล์ขนาดกลาง และ มีปริมาณสูงสุดในเซลล์ขนาดใหญ่ ผลการศึกษายังแสดงให้เห็นว่า ระดับการแสดงออกของ activation markers และ chemokine receptors อาจเพิ่มขึ้นตามระดับของเซลล์ที่ถูกกระตุ้น นอกจากนี้ในการศึกษาพบว่า ทีเซลล์ขนาดเล็กมีปริมาณร้อยละของแอนติเจน p24 ในเซลล์น้อยกว่าเซลล์ขนาดใหญ่

ความสามารถในการติดเชื้อ HIV-1 ถูกวิเคราะห์ด้วยวิธีโฟลไซโตเมทรี โดยการวัดปริมาณร้อยละของแอนติเจน p24 ภายในเซลล์ ซึ่งมีความสัมพันธ์กับแอนติเจน p24 ภายนอกเซลล์ที่วัดโดยวิธี ELISA นอกจากนั้นวิธีโฟลไซโตเมทรี ยังสามารถวัดปริมาณแอนติเจน p24 ในเซลล์ลิมโฟไซต์ที่ถูกกระตุ้นได้

การศึกษาแสดงให้เห็นว่า activation markers และ chemokine receptors อาจจะนำมาใช้ติดตามผลการดำเนินโรค และการพยากรณ์โรค

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
µg	Microgram
AFRIMS	The Armed Forces Research Institute of Medical Science
AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen Presenting Cells
C	Cysteine
CA	California
CD	Cluster of differentiation
CM253	Chiang Mai 253 (HIV-1 strain)
CO_2	Carbondioxide
CRF	Circulating recombinant form
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
env	Envelope gene
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FBS	Fetal bovine serum
Fe2+	Ferrous
Fe3+	Ferric
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
g	Gram
GM-CFU	Granulocyte-macrophage colony-forming unit
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HAART	Highly active anti-retroviral therapy

LIST OF ABBREVIATIONS (Continued)

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
hr	Hour
HRP	Horse redish peroxidase
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
IU	International Unit
Kb	Kilobase
KDa	Kilo Dalton
LTNP	Long term non progressor
LTR	Long terminal repeat
M	Majority
mAb	Monoclonal antibody
MCP	Macrophage chemoattractant protein
MHC	Major Histocompatibility Complex
Min	Minute
MIP-1 α	Macrophage inflammatory protein-I alpha
MIP-1 β	Macrophage inflammatory protein-I beta
ml	Milliliter
mRNA	Messenger ribonucleic acid
M-tropic	Macrophage-tropic
N	Non-M-non-O
Nef	Negative factor
ng	Nanogram
NK	Natural killer cell
nm	Nanometer
No	Number
NS	No significance

LIST OF ABBREVIATIONS (Continued)

NSI	Non syncytial inducing
NY	New York
O	Outlier
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PE	Phycoerythrin
Per CP	Peridin chlorophyl protein
PFA	Paraformaldehyde
pg	Picogram
PHA	Phytohemagglutinin
pi	Post infection
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
SSC	Side Scatter
$TCID_{50}$	Tissue Culture Infectious Dose 50
TCR	T cell receptor
TMB	Tetramethylbenzidine
T-tropic	T-tropic lymphocyte
UNAIDS	The Joint United Nations Programme on HIV/AIDS
vpr	Viral accessory gene product
α	Alpha
β	Beta
γ	Gamma

CHAPTER I
INTRODUCTION

It has been over twenty-four years since human immunodeficiency virus (HIV), which is the cause of acquired immune deficiency syndrome (AIDS), was first reported (1). It has increased from five cases of *Pneumocystis carinii* pneumonia in homosexual men to become a worldwide pandemic (1). Although the disease was first encountered in homosexual men and injection drug users, the risk groups soon included Haitians (2), transfusion recipients, including those with hemophilia (3, 4), infants (5), female sexual contacts infected men (6, 7), prisoners (8), and Africans (9). According to UNAIDS estimated in 2004, more than three million people have died as a result of AIDS and about five million people are newly infected by HIV. An estimated forty million people worldwide are currently living with HIV, of whom about twenty five million people live in sub-Saharan Africa, where approximately 7% of adults are infected, and more than seven million people live in the South and South-East Asia (10). In addition, sub-Saharan has accounted for around three-quarters of the global death toll (10).

AIDS still remains one of several important problems in Thailand. Although a lot of campaigns occur in Thailand, there are about 30,000 newly infected cases each year. In addition, this infection rate is increasing. Nowadays, there are 700,000 AIDS patients who require medical care and social welfare. More than 90 percent of AIDS patients who die in the age of twenty to forty-four are in the working group (11). This is why doctors and scientists are trying to find new drug treatments and new strategic prevention. Before a right treatment to AIDS is found, mechanisms of immunopathogenesis have to be understood at both molecular and cellular levels. One of several basic knowledge to understand mechanisms of HIV-1 infection is studies of the expression of activation markers and chemokine receptors. *In-vivo* activation of lymphocytes was described in AIDS patients soon after the discovery of HIV and it's

primary cellular receptor CD4 (12, 13). In addition, it was reported that peripheral blood CD4+ cells from AIDS patients proliferate spontaneously at a higher rate than CD4+ cells from controls (14). An increased expression of cell surface activation markers has been found in circulating T cells in HIV-1 infected individuals. These activation markers include HLA-DR (15-18), CD25 (16, 17), CD69 (19), and CD71 (20). The increase in HLA-DR is associated with functional T cell defects (21) and also correlates to disease severity (17). HIV-1 virions activated CD4+ and CD8+ T cells to express CD25 and HLA-DR and preferentially induced apoptosis in CD25+ HLA-DR+ T cells in a CXCR4 dependent manner (22). A significantly lower proportion of CD69 activated T lymphocytes were detected in peripheral blood mononuclear cells (PBMC) from HIV-infected individuals stimulated with phytohemagglutinin (PHA) (23). Moreover, CD71 that is susceptible to short-term effects of zidovudin is surrogate marker candidates for evaluation in anti-HIV treatment (20). Cellular entry of HIV requires a chemokine receptor as its coreceptor (24) and the coreceptor usage can be linked to viral tropism (25) and the disease progression (26-28). Unsurprisingly, the major chemokine receptors (CCR5 and CXCR4) are used as protective gate by using chemokines, chemokine analogues, and monoclonal antibodies.

The purpose of this study was to determine whether the expression of both chemokine receptors (CCR5 and CXCR4) and activation markers (CD25, CD69, CD71, and HLA-DR) on CD4+ T cells and CD8+ T cells can be used as surrogate markers to monitor the progression of HIV-1 infection *in vitro*. In addition to study the relationship between intracellular p24 antigen and level of individual activation markers and chemokine receptors, PHA was added to the culture to stimulate PBMC and maintained in IL-2 media to proliferate cells. The proliferating cells or lymphoblast were divided into three gates according to cell size. The percentage of p24 antigen in each gate was compared with the percentage of each activation markers and chemokine receptors. Therefore, the flow cytometric analysis that was used to detect the change of those cells following *in vitro* HIV-1 infection may be a piece of puzzle to fulfill a right treatment.

CHAPTER II

OBJECTIVES

This study was conducted to investigate the expression of activation markers and chemokine receptors on proliferative lymphocytes following in vitro HIV-1 infection. The aims of this study were:

1. To determine whether the expression of both chemokine receptors (CCR5 and CXCR4) and activation markers (CD25, CD69, CD71, and HLA-DR) on CD4+ T cells and CD8+ T cells separated from Thai normal PBMC and infected with CRF01_AE can be used as surrogate markers to monitor the progression of HIV-1 infection *in vitro*.

2. To study the relationship between intracellular p24 antigen and level of individual activation markers and chemokine receptors on proliferative lymphocytes.

3. To compare the result of p24 antigen from intracellular p24 antigen flow cytometry and p24 antigen capture ELISA.

CHAPTER III
LITERATURE REVIEW

1. Overview of HIV

1.1 HIV classification

Human immunodeficiency viruses (HIV) are divided into at least two types, HIV-1 and HIV-2, of which the former is more widely expand and is also the main cause agent for the AIDS pandemic. These two types show approximately 50 percent similarity at the nucleotide level (29). They cause a similar clinical syndrome, although HIV-1 is clearly more virulent than HIV-2 and associated with a shorter incubation time to AIDS and a higher risk of vertical transmission (30, 31). HIV-1 strains are further classified into three main groups: (i) M (majority), the group of viruses that cause the global pandemic; (ii) O (outlier), a form that is found with a low prevalence in The West of Central Africa and has also been found in Europe (32, 33); and (iii) N (non-M-non-O) (29, 34), a very rare form found in Africa (35). Recently, guidelines for the definition of subtypes and circulating recombinant forms (CRF) have been proposed that take into account the genetic diversity and evolution of the epidemic (36). A new subtype or CRF may be defined by the existence of at least three fully characterized genomes, obtained from epidemiologically unlinked individuals (37). Phylogenetic analyses should group these sequences approximately equidistantly from all other subtypes. The M group of HIV-1 several recognized non-recombinant subtypes and sub-subtypes, and at least 14 CRF (38). HIV-1 group M is subdivided into several subtypes or clades that are designated by letters A-D, F-H, J and K (29). Further analysis of complete genome sequences from viruses initially described is belonging to subtype E (now classified as CRF01_AE) and subtype I has revealed these viruses to be recombinant strains, and not distinct subtypes as initially proposed (39, 40). The subtypes are distributed throughout the world. For example, subtype A

and D predominate in the sub-Saharan Africa; subtype B is mostly found in the Americas, Japan, Australia, the Caribbean and Europe; subtype C dominates in South Africa and India; and subtype E is discovered in Thailand, the Central African Republic and other countries of Southeast Asia. In addition, More than 90 percent of HIV-1 infected persons in Thailand have HIV-1 subtype E (41). Subtype F (Brazil and Romania), G and H (Russia and Central Africa), and I (Cyprus) have a low prevalence. Most subtypes occur in Africa, although subtype B is less prevalent there.

1.2 HIV-1 structure

Human immunodeficiency virus (HIV), which is the cause of acquired immune deficiency syndrome (AIDS), is an RNA virus that belongs to the family of retroviruses. In addition, the virion is composed of two identical strands of RNA genome, which each strand is about 9.2 kb long (42). The viral genome and associated enzymes, including reverse transcriptase, integrase, and protease are packaged in nucleocapsid proteins (core proteins or p24 capsid proteins) (Figure 1). Nucleocapsid proteins are contained in p17 protein matrix, which are surrounded by phospholipid bilayer envelope derived from host cell membrane. The viral envelope is bound by glycoproteins (gp120 and gp41). Glycoprotein 120 (gp120) plays an important role in binding to CD4 and coreceptor. GP120 is always bound to glycoprotein 41 (gp41); in addition, gp120 and gp41 are also envelope proteins of HIV-1 and are always in trimeric form (43). GP120 is the first contact between HIV-1 and CD4 cell (44), following coreceptor binding to open and enter the cell. That is why several strategies block this step to prevent HIV-1 from entering the cell. HIV genomic organization and the encoded proteins are shown in Figure 2.

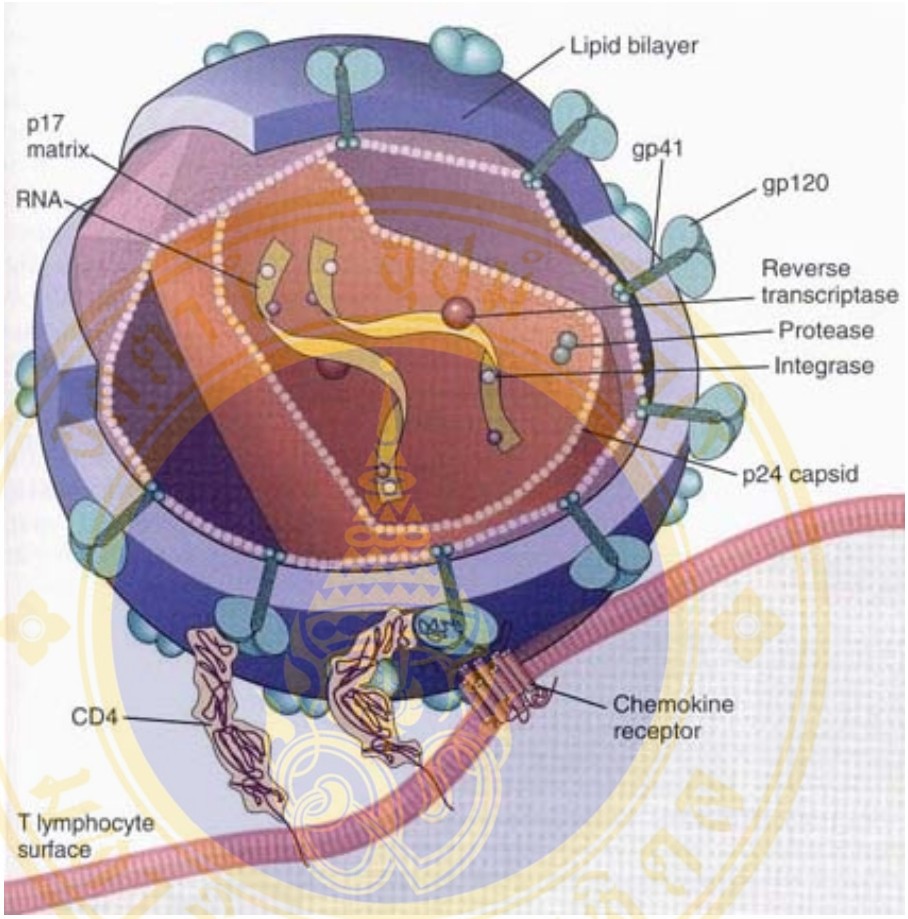


Figure 1. Structure of human immunodeficiency virus type 1 (HIV-1) (45).

An HIV-1 particle is shown next to a T lymphocyte surface. HIV-1 comprises two identical plus stranded RNA molecules of approximately 9.2 kb long. The viral genome is embedded in a nucleocapsid protein (core protein or p24 protein) together with certain viral enzymes, including reverse transcriptase, integrase, and protease. The nucleocapsid protein is surrounded by a matrix layer (p17 protein) that all are enclosed by a phospholipid membrane envelope derived from the host cell. Virally encoded membrane proteins (gp41 and gp120) are bound to the envelope. CD4 and chemokine receptor on the host cell surface function as HIV-1 receptor and HIV-1 coreceptor protein, respectively.

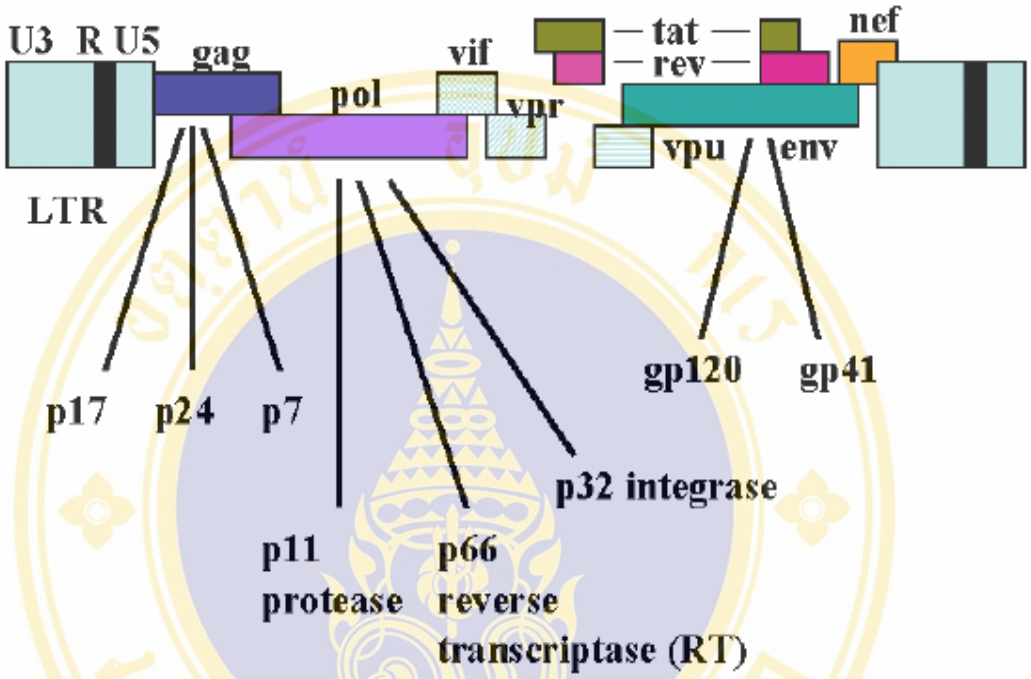


Figure 2. Genomic organization and feature of the HIV genome. (http://www.hivmedicine.com)

1.3 Genetic variation of HIV-1

HIV-1 has a high degree of its genetic variability as a result of no proofreading activity for reverse transcription (46-49), point mutation, recombination events during replication (50), the high rate of virus production (51, 52), the high number of replication cycles (53), and host selective immune pressures (54). Moreover, an estimated one mutation is generated per replication cycle. Approximately 10 billion (10^{10}) virus particles are produced each day (51). The average of viral generation time has been estimated at 2.6 days (51). Therefore, HIV-1 replication has a rate of 140 generations per year, for ten or more years (53). The mutation rate of HIV-1 is about $3x10^{-5}$ nucleotides per replication cycle, while the size of HIV-1 genome is only 9,200 nucleotides. As mentioned above, if 10 billion (10^{10}) virions are generated each day, it means mutation can occur at every nucleotide in the viral genome very frequently in a day.

2. CD4 as a primary receptor for HIV-1

It has been known for 2 decades that the primary receptor HIV-1 is the CD4 molecule, expressed on surface of the mature T-helper lymphocytes in peripheral blood and lymph node, monocytes, macrophages, and also dendritic cells (55, 56). The extracellular domain of CD4 on T cells is composed of four regions D1-D4, which have been characterized that represent immunoglobulin-like domains. The V2 of CD4 has an important role for the binding of gp120 to CD4. In addition, this region overlaps the part of the CD4 where its natural ligands, HLA class II molecules, bind specifically. The identification of the gp120-binding site on the CD4 receptor of activated CD4+ T cells attempts to use soluble CD4 (sCD4) to neutralize the circulating virus in patients. The goal of this experiment aims to inhibit viral spread. However, it had evidence that even though laboratory viral isolates were easily neutralized by sCD4, a neutralization of primary patient-derived isolates had not been succeeded. Inversely, sCD4 was able to induce conformational changes within the viral envelope glycoprotein, which promoted the infection of target cells (57). CD4 engages to the T-cell receptor complex (TCR) on CD4+ T cells and attaches to the HLA class II molecules on antigen presenting cells (APCs), including dendritic cells, macrophages, B-lymphocytes and Langerhans cells. The binding of gp120 to CD4 is not only an important step for viral entry, but also interferes with intracellular signal transduction pathways and promotes apoptosis in CD4+ T cells (58). Although HIV-1 uses CD4 as its primary receptor, CD4 receptor alone is not enough for entering cell of HIV-1. In addition, experiments using non-human cell lines transfected with human CD4 showed that experiment of human CD4 on the cell surface of a non-human cell line was not sufficient to allow entry of HIV. Thus, the existence of additional human co-receptors necessary for viral entry was postulated (45).

3. The course of HIV-1 infection

The course of HIV-1 infection can be divided into three stages (59), including primary infection (acute HIV disease), clinical latency (chronic phase), and AIDS stage (clinically apparent disease). Primary infection stage that occurs a few weeks after the initial infection is associated with a high level of viremia and a sharp fall in peripheral blood CD4+ T cell counts (60-66). The level of CD4+ T cells may rebound toward normal as the patient enters the clinically latent stage of disease (Figure 3); however, they have never returned to pre-infection level. The clinical features during acute phase of HIV disease are fever, headache, sore throat with pharyngitis, generalized lymphadenopathy, and rashes. The acute phase of HIV disease is followed by a clinical latency stage that may last from few to more than ten years. This stage is distinguished by low but persistent level of viral replication, predominantly in lymph nodes, and steady decline of CD4+ T cells in which HIV-1 is replicating (59, 67). The majority of patients remain relatively healthy during the period; however, sometimes nonspecific symptoms such as diarrhea, fever, night sweat, and weight loss may be seen. The hallmark of AIDS stage is an accelerated loss of CD4+ T cells, increment of viral replication, and exhaustion of immune system. As a result of the weakening immune system, opportunistic infections appear such as protozoa (*Pneumocystis carinii, Cryptosporidium*), bacteria (*Toxoplasma, Mycobacterium avium, Norcadia, Salmonella*), fungi (*Candida, Cryptococcus neoformans, Coccidioides immitis, Histoplasma capsulatum*), and viruses (Cytomegalovirus, Herpes simplex, Varicella-zoster). In addition, tumors such as lymphomas (including EBV-associated B cell lymphomas), Kaposi's sarcoma, and cervical carcinoma may be seen. Without anti-retroviral therapy, the patient usually dies within a few years after onset of AIDS.

Depending on survival time, HIV-infected individuals can be classified into three groups. Natural history cohorts have demonstrated that the mean time from initial infection to development of AIDS is approximately eight to twelve years (68, 69). However, 10-15 percent of HIV-infected patients who are called as rapid progressors develop AIDS within two to three years following initial infection (59). Inversely, about 5 percent of patients who are called as long-term non-progressors (LTNP) show very slow disease progression during a period of 10-15 years after primary infection (70).

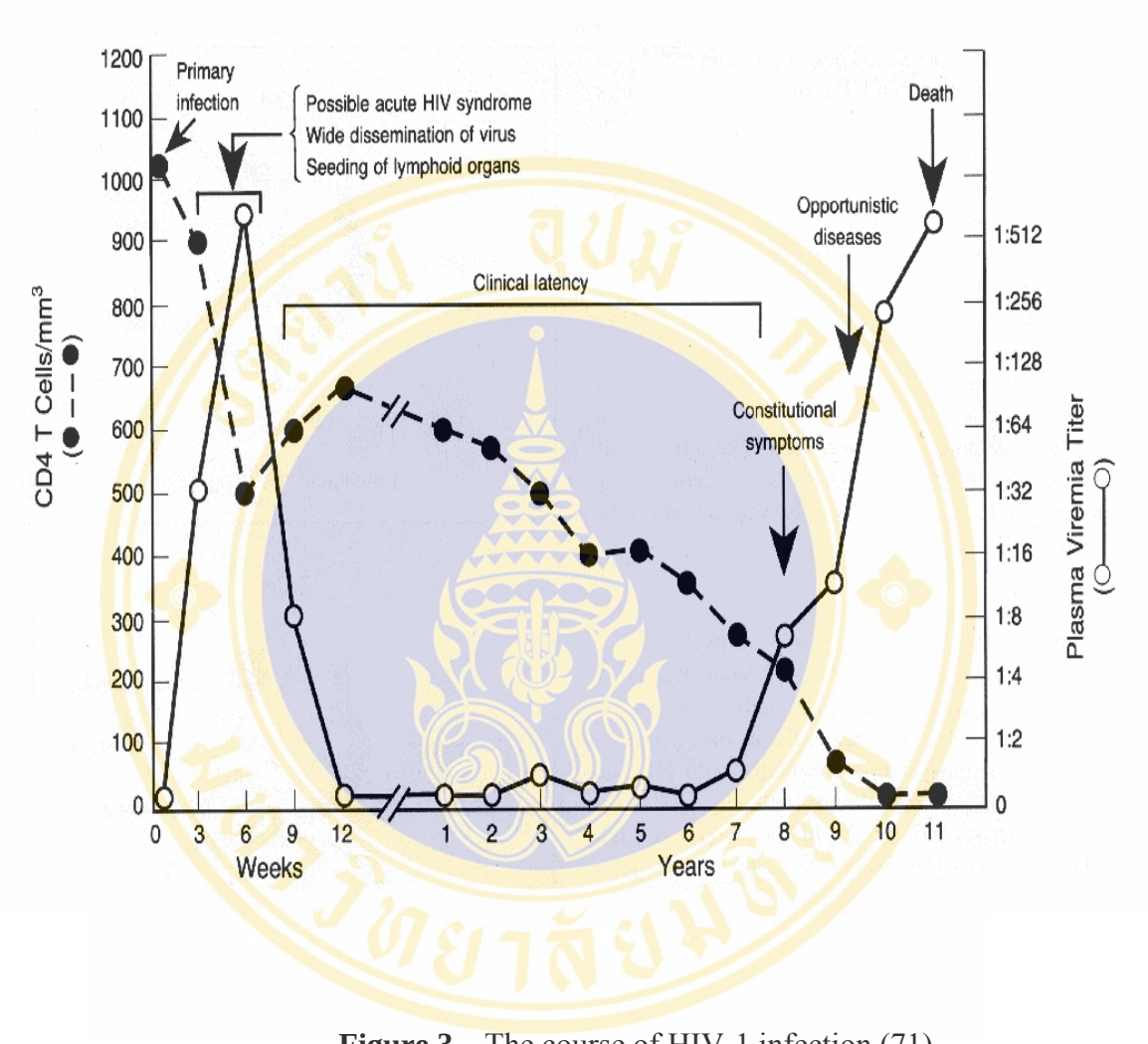


Figure 3. The course of HIV-1 infection (71).

Schematic representation of the decline in CD4+ T lymphocytes and the kinetics of viremia during the various courses of HIV-1 infection are observed in typical progressors.

4. The HIV-1 life cycle

The main target for HIV-1 is cell expressing the CD4 antigen, although other cell-surface molecules have also been exhibited to function as in distinct cell types (72, 73). The CD4 host cells that have an affinity for HIV-1 are CD4+T-cells and monocytes. The viral life cycle starts when HIV-1 attaches to the host cell (Figure 4). The entry of HIV-1 into the cell has several steps that are mediated by the trimeric form of the viral envelope protein. After binding of HIV-1 gp120 and CD4 receptor, conformational changes are initiated in the gp120 that leads to display of a previously hidden coreceptor-binding site. In addition, the coreceptors are usually the chemokine receptors CCR5 or CXCR4. Coreceptor binding that induces sequential conformational changes in the transmembrane part gp41 promote fusion of the HIV-1 and host cell membrane. The viral core is delivered into the cytoplasm where the nucleocapsid of the virion is then uncoated and released. The process of reverse transcription is begun in the cytoplasm. In addition, HIV-1 reverse transcriptase (RT) is a multifunctional enzyme, which has three distinct activities: RNA-dependent and DNA- dependent DNA polymerase activity and RNase H activity that degrades the RNA-DNA intermediates formed during proviral synthesis. The RNA is converted through the action of RT into double-stranded DNA. The double stranded DNA copy is translocated into the nucleus and randomly integrated in the host genome by using the activity of HIV-1 integrase enzyme. Successfully integrated DNA is called a provirus may become silent and latent period until the host cell is stimulated (74). The proviral DNA is used as a template to produce the yield of viral messenger RNA (mRNA) that is translated into viral proteins. The viral RNA and viral proteins are contained into intracellular immature particles. These bud from the cell membrane into a new virus. After budding step, protease enzyme is requested to process other HIV proteins into their functional forms. Thereby, the maturation of the new virion is complete and can infect another target cell.

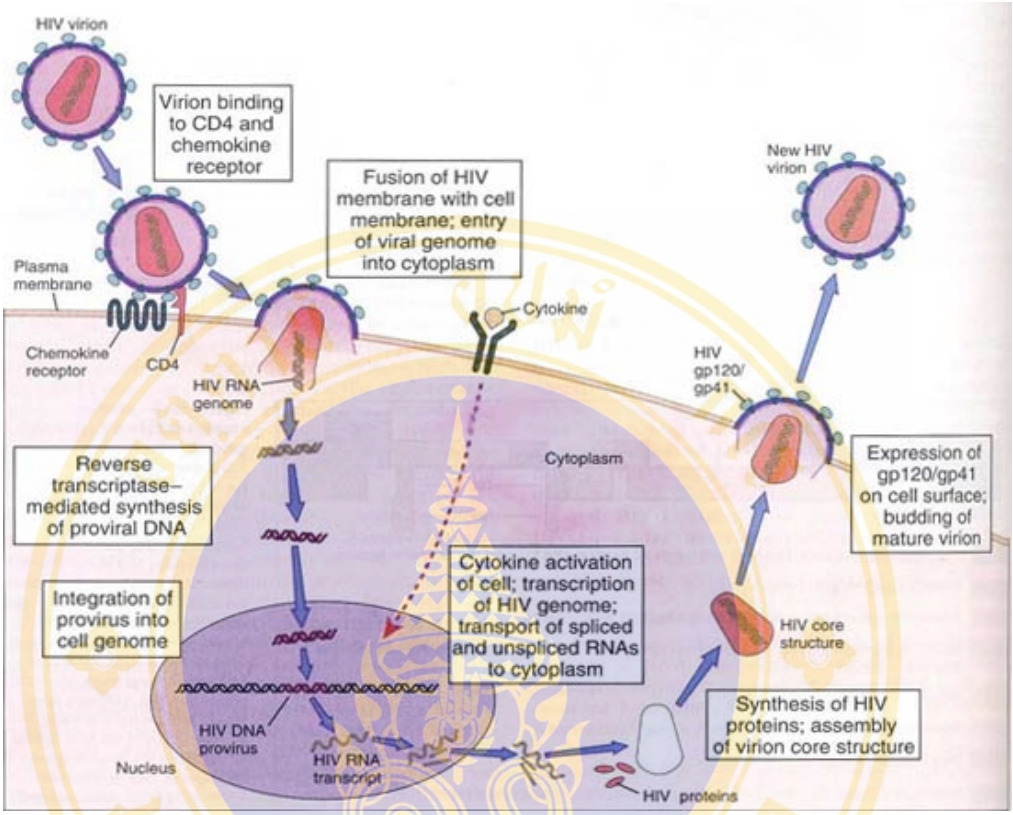


Figure 4. The HIV-1 life cycle (45).

The HIV-1 life cycle begins with the high affinity binding of its gp120 to CD4 as its receptor on the host cell surface. Following binding, fusion with the host cell membrane occurs between gp41 and chemokine receptor as its coreceptor, and the HIV-1 genomic RNA is un-coated and internalized. The RNA is converted into double-stranded DNA by the activity of reverse transcriptase enzyme. The double-stranded DNA, provirus, is randomly inserted into the host genome by using the activity of the integrase enzyme. After cellular activation, the provirus is transcribed and translated into viral mRNA and viral protein, respectively. Budding of the progeny virion occurs through the host cell membrane.

5. Chemokine receptors as coreceptor for HIV-1 entry

The HIV-1 coreceptors belong to the chemokine receptor family. Chemokine receptors play an important role in host defense and they have been used by several pathogens for entering cell. These proteins are seven-transmembrane G-protein coupled receptors that usually bind to soluble ligands called chemokines. The chemokines are a large family of chemotactic cytokines with molecular weights in the range of 8-12 kDa, they directly stimulate leukocytes to migrate to the sites of inflammation (75). The chemokines have been subdivided into four subfamilies: C, CC (or β), CXC (or α), and CXXXC, according to the position of conserved cysteine residues (C) that lie close to the N-terminus region of the protein (76-78). The β-chemokines (79-81) "regulated by activation, normal T-cell expressed and secreted" (RANTES), macrophage inflammatory protein (MIP)-lα, MIP-1β, macrophage-derived chemokine (MDC) and the α-chemokine (82, 83) stromal derived factor (SDF)-l are *in vitro* potential HIV-1 inhibitors as shown in Table 1. Other chemokines have potential to inhibit HIV such as eotaxin (84, 85), monocyte chemotactic protein (MCP)-2 and –3 (86, 87). Cellular entry of HIV-1 requires CD4 as its major receptor and chemokine receptor as its coreceptor (88-91). Moreover, the major HIV-1 coreceptors are CCR5 and CXCR4 (92). Before the discovery of the chemokine receptors which act as coreceptors in HIV-1 infection, three overlapping classification systems were used to describe the phenotype of HIV-1 isolates. The first system was based on virus growth kinetics in cell culture. The viruses were defined as slow/low (SL) or rapid/high (RH) depending on their growth in peripheral blood mononuclear cells (PBMC) (26). A second system classified viruses as syncytium inducing (SI) or non-syncytium-inducing (NSI) on the basis of whether they induced syncytia in MT-2 cells which express the chemokine receptor CXCR4 but not CCR5 (88). However, NSI viruses also form syncytia with CCR5-expressing cells. In a third system primary isolates were defined as Macrophage (M)-tropic or T-cell-line (T)-tropic. This system was not optimal because activated primary CD4+ T-lymphocytes are replicated by all primary isolates and T-tropic viruses sometimes infect macrophages. After the discovery of HIV-1 coreceptors, Berger and coworkers suggested a new classification system for HIV-1 in 1998 (93). In this system the virus isolates are categorized

according to their coreceptor usage. Moreover, CCR5-using viruses, CXCR4-using viruses, and both receptor-using viruses are designated as R5, X4, and R5X4, respectively (91, 94). These two coreceptors can be linked to viral tropism. CCR5-using HIV-1 variants (R5, M-tropic virus) predominate in the early infection and asymptomatic phase whereas CXCR4-using HIV-1 variants (X4, T-tropic virus) relate to symptomatic phase and AIDS (26-28).

Table 1. Biological properties of β and α chemokines and chemokine receptors (95-97).

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

6. Activation markers

After cell stimulation, activation markers that are symbolic of activated cells express on the surface membrane, including CD25, CD69, CD71 and HLA-DR (Table 2). CD25 is the α chain of IL-2 receptor (IL-2R) that is produced and secreted, together with IL-2 by stimulated T lymphocytes. IL-2_{α}R binding to IL-$2_{\beta\gamma}$R forms the high affinity receptor to IL-2 (98, 99). CD25 symbolizes activated T lymphocytes and it is expressed by lymphocytes within 24-48 hr after mitogenic stimulation (100-103). The rapid expression of this activation antigen and its easy measurement by flow cytometry suggest that expression of CD25 molecule may be useful to evaluate functionality of lymphocytes. Moreover, immune response in HIV-1 infected individuals was gradually impaired. Imunomodulation with IL-2 is one of the complementary treatments used in combined anti HIV-1 therapy. Administration of IL-2 is expected to improve immune responses and to activate latently infected cells; however, IL-2 may increase the pool of susceptible cells, by triggering, not only proliferation, but also expression of chemokine receptors in CD4 cells. Modern medicine used IL-2 with HAART in HIV-1 infected individuals.

Table 2. Activation markers and cellular expression (100, 103).

CD designation	Common synonym	Main cellular expression
CD25	IL–2 Receptor	Activated T and B cells, activated macrophages
CD69	Early activation antigen	Activated leukocytes, including T cells, B cells, NK cells, neutrophils, Eosinophils, Platelets, Langerhans cells
CD71	Transferrin receptor	Activated T and B cells, macrophages, proliferating cells
HLA–DR	MHC class II molecule	Monocytes, Macrophages, B lymphocytes, Activated T lymphocytes

CD69 is one of the earliest activation antigen expressed on activated leukocytes (104), including T lymphocytes, B lymphocytes, NK cells, neutrophils, eosinophils platelets and Langerhans cells. This activation marker is hardly detectable on resting PBMC but it is rapidly expressed on stimulated cells. Krowka and coworkers found that HIV-1 infected donors had significantly lower frequencies of CD4+ and CD8+ lymphocytes expressing CD69 after stimulation with PHA in comparison to uninfected donors (23). This activation marker may be used to test the impairment of the function of CD4+ T cells to express CD69 antigen.

All living organisms, from the smallest to the largest ones (microorganisms, plants and animals) need iron as essential growth mineral and have elaborate mechanisms for its acquisition, transport and storage. Iron exists as two active forms, ferric (Fe3+) and ferrous (Fe2+), which contribute to its flexibility. Indeed, it has the ability to accept and donate electrons readily. Iron is a useful catalytic center for many cellular reactions such as energy metabolism, respiration (105) and DNA synthesis (106). For HIV-1 replication, the virus requires the presence of an iron to the upregulation of proviral transcription (107). Furthermore, HIV -infected cells use the metal to produce viral particles (108). A direct link can then be established between iron overload and the stimulation of HIV replication (109). The iron carrier proteins used by vertebrates to transport iron in the plasma and cells are transferrin. They are monomeric glycoproteins and have a molecular weight of 80 kDa. Transferrin binds two atoms of Fe3+ with high affinity and is a means of transporting iron through the plasma to and among the receptor on bone marrow cells, the liver the reticulum endothelial system (RES), the spleen and muscle cells. CD71 is known as the transferrin receptor that plays an important role in iron metabolism and cell growth. CD71 is also expressed in activated T and B cells, macrophages, and other proliferating cells.

HLA-DR is a member of human HLA class II. It is expressed on activated T lymphocytes, B lymphocytes, monocytes, and macrophages. The surface expression of HLA-DR is significantly increased and predicts the progression to AIDS in adults (21, 110). Resino and coworkers found positive correlation between viral load and

HLA-DR expression on CD4+ T cells (111). Therefore, HLA-DR may be a sensitive prognostic marker for HIV-1 infected individuals.

There are many kinds of anti-retroviral drugs, including reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors, which can function after HIV-1 entering the cell. Therefore, the strategies to inhibit HIV-1 binding and form fusion are more benefit. Unfortunately, the knowledge of these steps is just like a puzzle, especially more studies on the level of the activation markers after HIV-1 infection is required. As mentioned above, several studies support that the ligands of CCR5 (MIP-1α, MIP-1β, and RANTES) (80), the ligand of CXCR4 (SDF-1α) (82), chemokine analogues (112), and antibodies (113) can protect or at least reduce the rate of HIV-1 infection. However, the blocking step should be used carefully. Because the different cell tropism of HIV-1 uses the different chemokine receptor as its coreceptor. Therefore, the use of chemokines, chemokine analogues and antibodies to block HIV-1 coreceptor may be the selective pressure for changing to use other kinds of chemokine receptors. In addition, this phenomenon may make the patient in danger, if HIV-1 that used CCR5 is turned to use CXCR4, which is more virulent. That is why this study focuses on the expression of chemokine receptors and activation markers on proliferative lymphocytes following in vitro HIV-1 infection. The results of study may help us to understand the alteration of chemokine receptors and activation markers on the population of CD4 cells and CD8 cells after HIV-1 infection.

CHAPTER IV
MATERIALS AND METHODS

1. HIV-1 strain

The 9667 (CM235, CRF01_AE) was NSI primary isolate of Thai patients in the north {kindly provided by Dr. John Mascola, the Armed Forces Research Institute of Medical Science (AFRIMS)}.

2. Blood donors/subjects

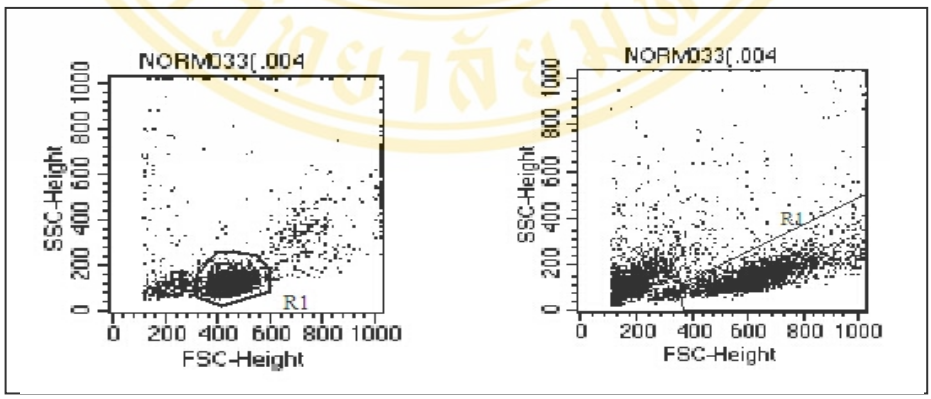
Peripheral blood was drawn by venipuncture from 10 HIV-seronegative healthy Thai donors of the Blood Bank at the Faculty of Medicine, Siriraj Hospital.

3. Peripheral blood mononuclear cells (PBMC) separation

PBMC were separated by gradient centrifugation on ficoll hypaque (Histopaque 1077; Sigma, St. Louis, MO). After the centrifugation step, PBMC were harvested and washed three times in phosphate-buffered saline (PBS; GIBCO, Grand Island, NY). The pellet was resuspended in complete RPMI {cRPMI: RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 20 mM L-glutamine (all reagents from GIBCO, Grand Island, NY)} plus 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) at 20 x 10^6 cells/ml. Finally, cell suspension was aliquoted (1 ml/cryogenic tube) and frozen in –20°C for 0.5-1 hour (hr) then vials containing frozen cells were transferred to –70°C deep freezer for 24 hr and in liquid nitrogen on the next day.

4. Phytohemagglutinin (PHA) stimulation

Frozen PBMC were thawed rapidly in water bath at 37°C and were washed to remove DMSO. After thawing step, PBMC were centrifuged and resuspended at 1-2 x 10^6 in cRPMI containing 1 μg/ml PHA (DIFCO, Detroit, MI). On day0, PHA-stimulated cells were incubated in T-25 culture flask upright overnight. After incubating with PHA, cells were centrifuged and cultured in IL-2 medium {cRPMI supplemented with 10% (10 IU/ml) recombinant interleukin-2 (rIL-2; Boehringer-Mannheim, Indianapolis, IN)} at 37°C and 5% CO_2 for subsequent experiments. After activation step, cells were determined by staining 0.5 x 10^6 cells with 5 μl each of fluorescein isothiocyanate (FITC)-conjugated anti-CD25, CD71, and CCR5 monoclonal antibody, phycoerythrin (PE)-conjugated anti-CD69, HLA-DR, and CXCR4 monoclonal antibody, and peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 and CD8 monoclonal antibody. The percentage of each marker on CD4+ T cells and CD8+ T cells in the PBMC was analyzed by using flow cytometry. The cell size and quantity of PBMC were increased after PHA activation (Figure 5). In addition, the increment of the expression of activation markers and chemokine receptors was also observed on PHA-stimulated PBMC.



Unstimulation **PHA stimulation**

Figure 5. Flow cytometry gate of unstimulated and PHA-stimulated PBMC.

Schematic representation of flow cytometry gate compared between unstimulated (left) and PHA-stimulated (right) PBMC. After PHA stimulation, the cells were increased in size and quantity.

5. Virus stock preparation

Virus stock preparation was kindly provided by Dr. Ronnarit Haponsaph. Primary isolate of CRF01_AE strain was propagated to produce high titered stock. Five million PHA stimulated PBMC of three donors were resuspended in 1 ml of viral supernatant and incubated for 1 hr at 37°C. After infection, cells were washed using cRPMI to remove excess unbound virus and cultured in IL-2 medium for 8-12 days. At peak of p24 antigen levels determined by p24 antigen capture (Coulter, Miami, FL), virus stocks were frozen in 1-ml aliquots and stored at -70°C for subsequent use. The normal donor PBMC was used for each virus titration. Primary isolate was not passaged through neoplastic cell lines and the virus used throughout was uncloned.

6. Virus stock titration

Virus stock titration was kindly done by Dr. Ronnarit Haponsaph. Viral stock was titered in five-fold dilutions using five replicate wells per virus dilution. 100 µl of diluted (1:6.7) normal human plasma (NHP), 1 x 10^6 PHA-PBMC in 100 µ1 of IL-2 medium and 100 µ1 of viral stock (prepared at three times the desired final dilution) were mixed in 15-ml tubes (one tube per virus dilution). The final virus dilutions were from 5^{-2} to 5^{-7}; cells were infected overnight (16-18 hr). The next morning, the cells were washed two times in 10 ml of wash medium (cRPMI with 2% FBS). After the second wash, cells were resuspended in 1.0 ml of IL-2 medium and 200 µl were plated in five replicate wells of a 96-well U-bottom culture plate. At day4, 100 µl of supernatant were removed and 100 µ1 of fresh IL-2 medium were added. At day8, the supernatant was removed for p24 antigen capture. The dilution of virus required to infect 50% of replicate wells ($TCID_{50}$) was calculated by the Spearman-Karber method (114).

7. Growth kinetics quantitated by HIV-1 p24 antigen capture (ELISA) and Intracellular p24 detection (flow cytometry)

Depending on the used virus, pretitered virus stock (2265$TCID_{50}$) was diluted appropriately to 500$TCID_{50}$ during the infection period. Experiments including CRF01_AE-infected and uninfected groups were set up in 15 ml tube during infection period by counting cells and adjust to 1 x 10^6 cells in 100 µ1. 100 and 300 µ1 of IL-2 media was added to 15 ml tube of infected and uninfected groups, respectively. A 200-µl aliquot of 500$TCID_{50}$ virus was incubated with 100 µ1 of PHA-stimulated cells in infected tube overnight. Uninfected PHA-PBMC were then added at 1 x 10^6 cells per tube or well in 100 µ1 of IL-2 medium (final volume of 400 µ1 per tube on day0 pi). After overnight infection, cells were washed with 10 ml wash medium. 1.5 ml of IL-2 medium was added to each tube and contents were transferred to 24-well plate in triplicate. At days4, 7, and 10, 450 µl supernatant plus 50 µl lyse buffer (Coulter, Miami, FL) were collected for p24 antigen capture by ELISA. Cells were subcultured 1:2 (0.5 ml), counted and resuspended at 0.5 x 10^6 cells/tube for intracellular p24 detection by flow cytometry. 1 ml of fresh IL-2 medium was added to each well and final volume was 1.5 ml.

8. P24 antigen capture ELISA

HIV-1 p24 antigen in samples was detected using p24 antigen capture ELISA technique (Organon Teknika, Boxtel, Netherlands). According to the manufacturer's instructions, this test system used a murine monoclonal antibody to HIV-1 p24 antigen coated onto wells. A specimen of our culture (100 µl) and disruption buffer (25 µl) was added to the coated well and incubated for 1 hr at 37°C. In addition, disruption buffer destroyed virus particles in the specimen and monoclonal antibody on the well bound to HIV-1 p24 antigen. After incubation, each well was washed with diluted phosphate buffer for 4 times. Subsequently, 100 µl of anti HIV-1 (human) conjugate label with horseradish peroxidase (HRP) was added for 1 hr at 37°C. Moreover, the labeled antibody binds to the solid phase antibody/antigen complexes previously formed. Following a wash and incubation with 100 µl of tetramethylbenzidine (TMB) substrate for 30 min at room temperature (RT), a blue color was produced that turned yellow when the reaction was stopped with 100 µl of 1 N sulfuric acid (H_2SO_4). Color absorbance was measured using a dual-wavelength spectrophotometer at 450 and 620-700 nm. The intensity of the color developed was directly proportional to the amount of HIV-1 p24 antigen containing within the sample. If the sample contained no HIV-1 p24 antigen, the labeled antibody could not be bound specifically and only a low background color developed. P24 standard curve was prepared in RPMI1640 to range in concentration from 5 to 160 pg/ml including negative well. The p24 content was determined by comparison with standard curve. An internal control was included in all experiments.

9. Intracellular P24 antigen by flow cytometry

Flow cytometric determination of intracellular HIV-1 p24 antigen was performed using the method described by Darden et al (115). Briefly, cells were counted and 0.5 x 10^6 cells were aliquoted to 12 x 75-mm polystyrene tubes. Cells were washed one time by adding 3 ml PBS with 2% heat-inactivated FCS (PBS-FCS) and centrifuging. After washing, supernatant was decanted and cells were mixed gently. Surface staining was performed first using an isotype-matched control or CD4-PE as well as CD8-PerCP. Cells were incubated at 4^0C for 30 minutes (min) in the dark, washed once, and resuspended in 100 µl of fixation medium (Fix and Perm kit, reagent A, Caltag Laboratories, Burlingame, CA) for 15 min at RT in the dark. After fixation, PBMC were washed once and resuspended in 100 µl of permeabilization medium (reagent B, Caltag Laboratories, Burlingame, CA). At this step, anti-p24-FITC (KC57; Coulter, Miami, FL) was added. Cells were stained for 20 min at RT in the dark, washed, and resuspended in 500 µl of 1% buffered paraformaldehyde. Samples were stored at least half an hour or overnight at 4^0C and then analyzed by flow cytometry for total p24+ HIV-1-infected cells. FACSort and CellQuest software (Becton Dickinson Biosciences, San Jose, CA) was used and instrument calibration was setup using Ca1iBRITE beads and FACSCOMP software (Becton Dickinson Biosciences, San Jose, CA). Dead cells and debris were excluded from lymphocyte gate and fifty thousand cells were collected and analyzed by CellQuest software for each sample. The percent of CD4+/CD8+, CD4+/p24+, CD8+/p24+, CD4-/p24+ and total p24+ cells in the lymphocyte gate were determined from gated two-color dot plots of p24-FITC versus CD4-PE, p24-FITC versus CD8-PerCP and CD4-PE versus CD8-PerCP. Uninfected cells were stained for isotype-matched controls. Compensation controls (CD3-FITC alone-treated tube and CD4-PE alone-treated tube) were used to set the appropriate quadrant markers (less than 0.5% of the cells will be p24+ in the uninfected cultures). To specify the percentage of p24 in proliferative lymphocytes, PBMC were divided into three gates by the size of forward scatter, including small (360-600 or gate1), medium (600-800 or gate2), and large (800-1,000 or gate3) group (Figure 6).

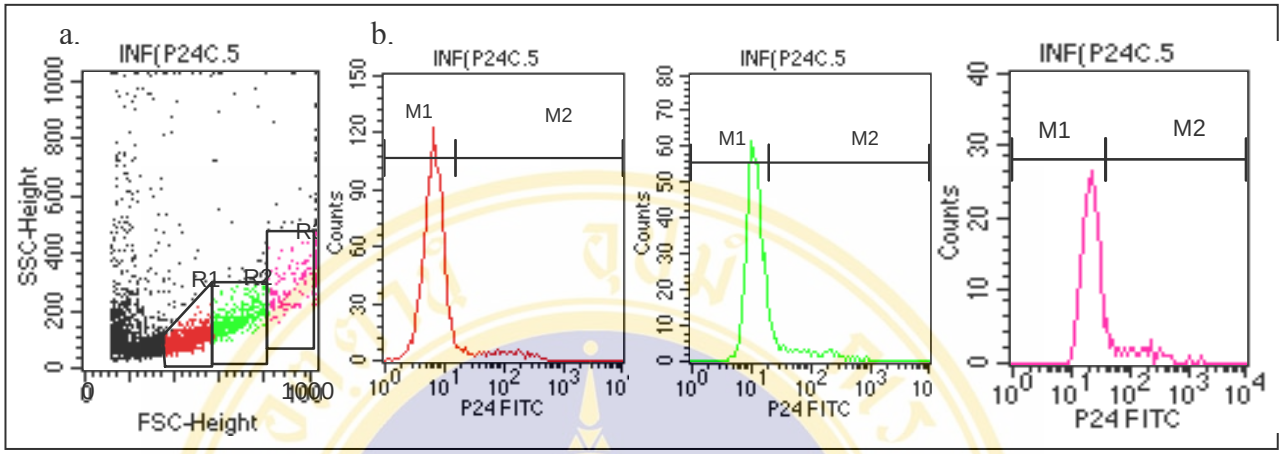


Figure 6. Flow cytometry gate of percent intracellular p24+ cells in lymphocyte gate.

The scatter plot of flow cytometry gate of percent intracellular p24+ cells (a) was divided into three gates (b), including gate1 (R1), gate2 (R2), and gate3 (R3) according to cell size.

10. Surface staining

Chemokine receptors and activation markers were stained on day0 PHA, day4 PHA, day4, day7, and day10 post infection. Cells were counted and 0.3-0.5x10^6 cells were aliquoted to 12x75-mm polystyrene tubes. Cells were washed one time and centrifuging. After washing step, cells in tube1 were stained by 5 µl each of anti-CCR5-FITC, CXCR4-PE, and CD4-PerCP monoclonal antibody (mAb). Similar to tubes 1, 2, 3, 4, 5, and 6 were stained by 5 µl each of anti-CCR5-FITC, CXCR4-PE, and CD8-PerCP mAbs; anti-CD25-FITC, HLA-DR-PE, and CD4-PerCP mAbs; anti-CD25-FITC, HLA-DR-PE, and CD8-PerCP mAbs; anti-CD71-FITC, CD69-PE, and CD4-PerCP mAbs; anti-CD71-FITC, CD69-PE, and CD4-PerCP mAbs, respectively. Cells were incubated at RT for 15 min in dark and were washed once with PBS. One percent paraformaldehyde was added to all tubes and they were then analyzed by flow cytometer. Uninfected cells were stained for isotype-matched controls. Compensation controls which used to set appropriate quadrant markers were stained by single monoclonal antibody, i.e. anti-CD25-FITC alone-treated tube and anti-HLA-DR-PE alone-treated tube. Twenty thousands to fifty thousands PBMC were counted and analyzed. Chemokine receptors (CCR5 and CXCR4) and activation markers (CD25, CD69, CD71, and HLA-DR) were gated on both CD4+ T cells and CD8+ T cells. To specify the percentage of activation markers and chemokine receptors in proliferative lymphocytes, PBMC were divided into three gates by the size of forward scatter, including small (360-600), medium (600-800), and large (800-1,000) group (Figure 7).

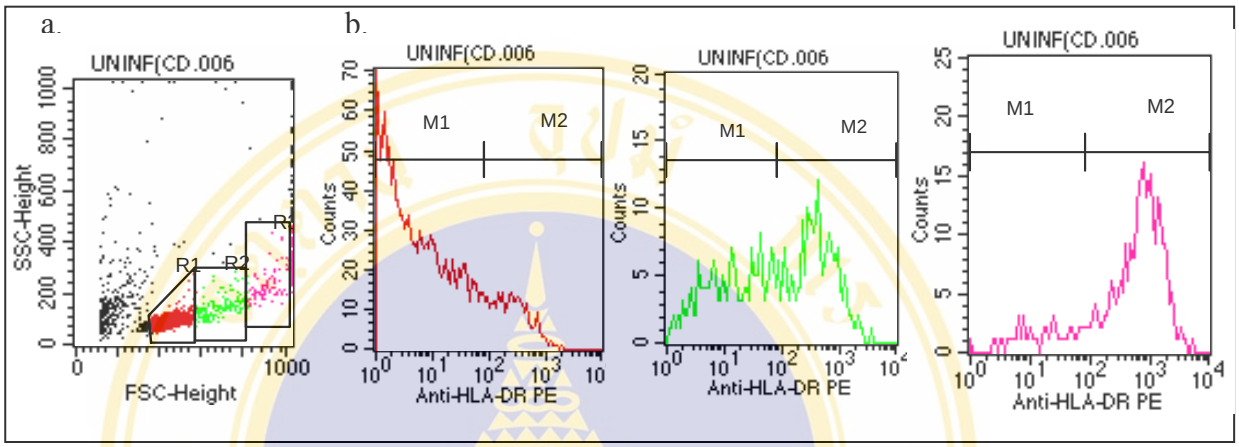


Figure 7. Flow cytometry gate of percent HLA-DR in lymphocyte gate

The scatter plot of flow cytometry gate of percent intracellular p24+ cells (a) was divided into three gates (b), including gate1 (R1), gate2 (R2), and gate3 (R3) according to cell size.

11. Statistical Analysis

Results of individual chemokine receptors (CCR5 and CXCR4) and individual activation markers (CD25, CD69, CD71, and HLA-DR) were observed and compared between 1) PHA-unstimulated CD4+ T cells (day0 PHA) and PHA-stimulated CD4+ T cells (day4 PHA or day0 pi), 2) PHA-un-stimulated cells (day0 PHA) and PHA-stimulated CD8 cells (day4 PHA or day0 pi), 3) uninfected CD4+ T cells and infected CD4+ T cells, 4) uninfected CD8+ T cells and infected CD8+ T cells. In addition, uninfected and infected cells were observed on days4, 7, and 10 post infection and were compared by Wilcoxon Signed Ranks Test. While Friedman one-way analysis of variance was used to compare the percentage of each parameter between each day. The significant differences of the results were then further analyzed by Wilcoxon Signed Ranks Test. For all statistical tests, a p value < 0.05 was significant.

Data obtained from the intracellular p24 flow cytometry and p24 antigen capture ELISA were analyzed by using Pearson correlation method. Individual results of the both were observed and compared with the results of individual activation markers and individual chemokine receptors.

CHAPTER V
RESULTS

1. Determination of chemokine receptors and activation markers on the unstimulated and PHA-stimulated PBMC

PBMC from ten healthy normal blood donors were thawed, stimulated, and cultured by incubating in flask with PHA at the concentration of 1μg/ml to proliferate the cells for 24 hrs. PBMC were mixed and transferred to tube, washed to remove PHA. Then the cells were counted and adjusted to culture in IL-2 medium for three days. For investigation of chemokine receptors, unstimulated and PHA-stimulated PBMC were stained with two panels of three-color fluorescent conjugated monoclonal antibodies: 1) anti-CCR5-FITC, anti-CXCR4-PE, and anti-CD4-PerCP; 2) anti-CCR5-FITC, anti-CXCR4-PE, and anti-CD8-PerCP. However, the expression of activation markers was performed by staining with four other panels of three-color fluorescent conjugated monoclonal antibodies: 1) anti-CD25-FITC, anti-HLA-DR-PE, and anti-CD4-PerCP; 2) anti-CD25-FITC, anti-HLA-DR-PE, and anti-CD8-PerCP; 3) anti-CD71-FITC, anti-CD69-PE, and anti-CD4-PerCP; 4) anti-CD71-FITC, anti-CD69-PE, and anti-CD8-PerCP. Finally, all samples were analyzed by flow cytometry and representative data are shown in Figures 8-9, respectively.

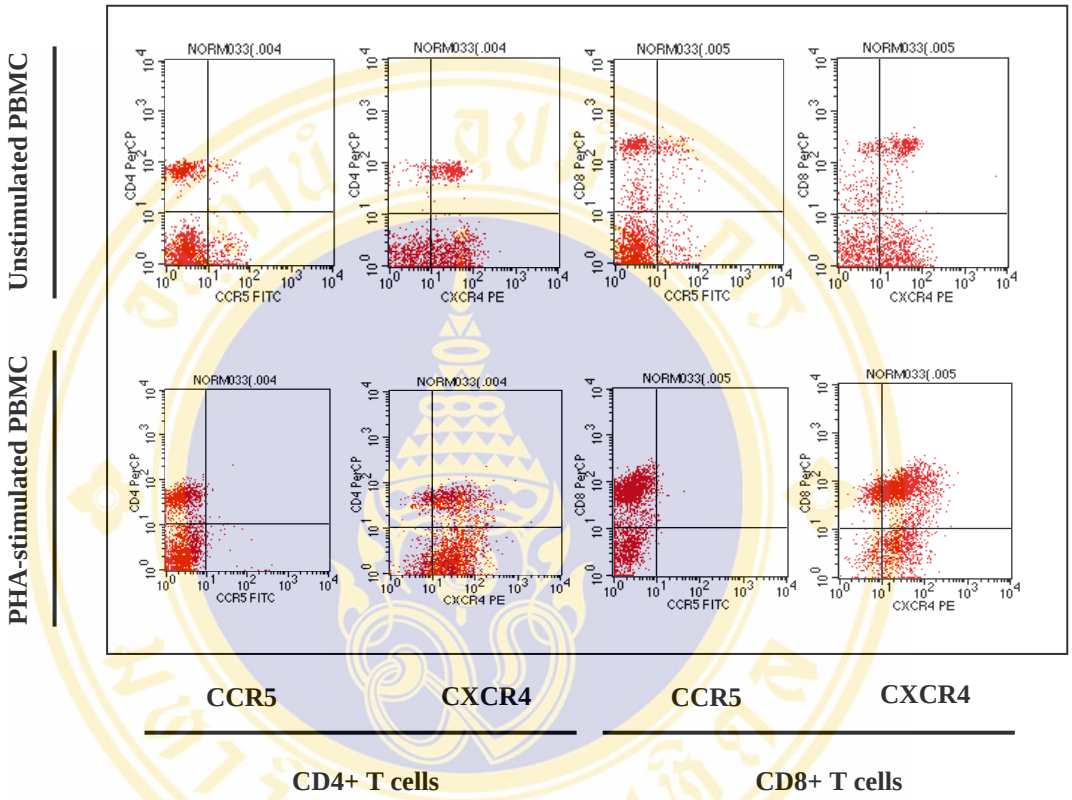


Figure 8. Representative flow cytometric two-color dot plots of CCR5 and CXCR4 expression on both unstimulated (upper panel) and PHA-stimulated (lower panel) CD4+ and CD8+ T cells.

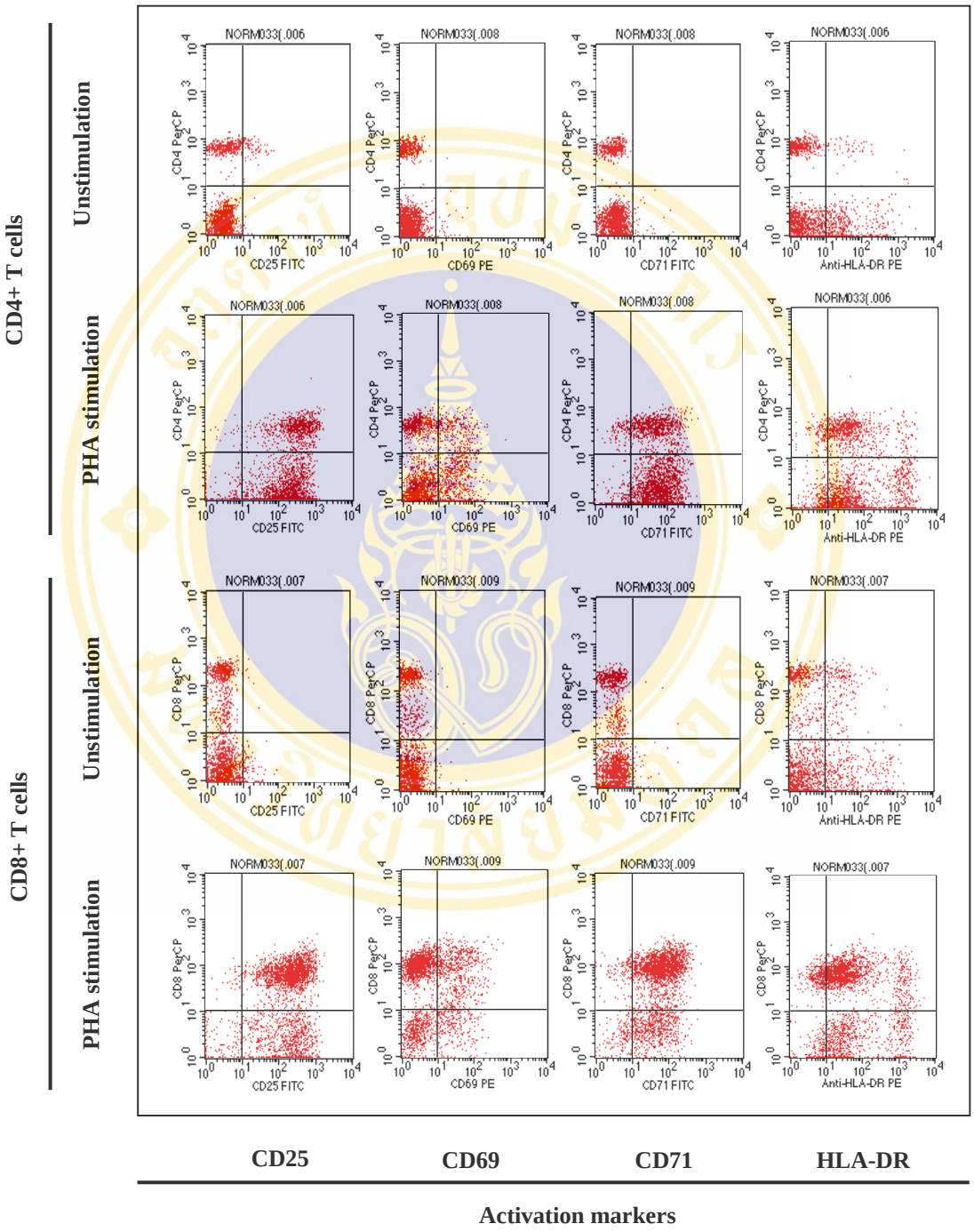


Figure 9. Representative flow cytometric two-color dot plots of activation markers (CD25, CD69, CD71, and HLA-DR) expressed on both unstimulated and PHA-stimulated CD4+ and CD8+ T cells.

The expression of CCR5 and CXCR4 on both CD4+ and CD8+ T cells are summarized in Table 3A and Figures 10-11. CCR5 expression on CD8+ T cells of PHA-stimulated PBMC was significantly decreased when compared with unstimulated cells. In contrast, CXCR4 expression of PHA-stimulated CD4+ and CD8+ T cells was significantly higher than unstimulated PBMC.

Table 3. Changes on expression level of chemokine receptors on CD4+ and CD8+ T cells* after PHA stimulation (A) and HIV-1 infection (B).

A. PHA stimulation of uninfected PBMC

Day	Chemokine receptors	PBMC		*p* value
		Unstimulation (%)	PHA stimulation** (%)	
Day0	**CD4+ T cells**			
	CCR5	3.55 ± 1.12	2.18 ± 2.14	NS
	CXCR4	27.33 ± 10.36	34.98 ± 11.58	0.009
	CD8+ T cells			
	CCR5	8.09 ± 3.12	1.86 ± 1.65	0.005
	CXCR4	18.60 ± 5.52	30.88 ± 12.02	0.005

B. HIV-1 infection in PHA-activated PBMC

Day	Chemokine receptors	PBMC		*p* value
		Uninfection (%)	Infection (%)	
Day4 pi	**CD4+ T cells**			
	CCR5	3.21 ± 4.22	2.62 ± 3.02	NS
	CXCR4	32.58 ± 12.73	30.80 ± 12.26	NS
	CD8+ T cells			
	CCR5	5.41 ± 8.25	5.17 ± 7.62	NS
	CXCR4	37.62 ± 19.23	38.01 ± 20.27	NS
Day7 pi	**CD4+ T cells**			
	CCR5	6.86 ± 4.49	4.49 ± 3.14	NS
	CXCR4	36.45 ± 11.96	28.04 ± 12.20	NS
	CD8+ T cells			
	CCR5	10.95 ± 8.67	10.50 ± 6.80	NS
	CXCR4	43.94 ± 15.28	49.69 ±16.93	NS
Day10 pi	**CD4+ T cells**			
	CCR5	8.44 ± 6.55	6.06 ± 5.58	NS
	CXCR4	31.64 ± 13.33	18.48 ± 11.27	0.049
	CD8+ T cells			
	CCR5	10.92 ± 5.51	11.60 ± 6.28	NS
	CXCR4	39.96 ± 12.43	46.73 ± 13.47	NS

* Mean±SD, ** Day4 PHA or Day0 pi, pi = post infection, NS = no significance

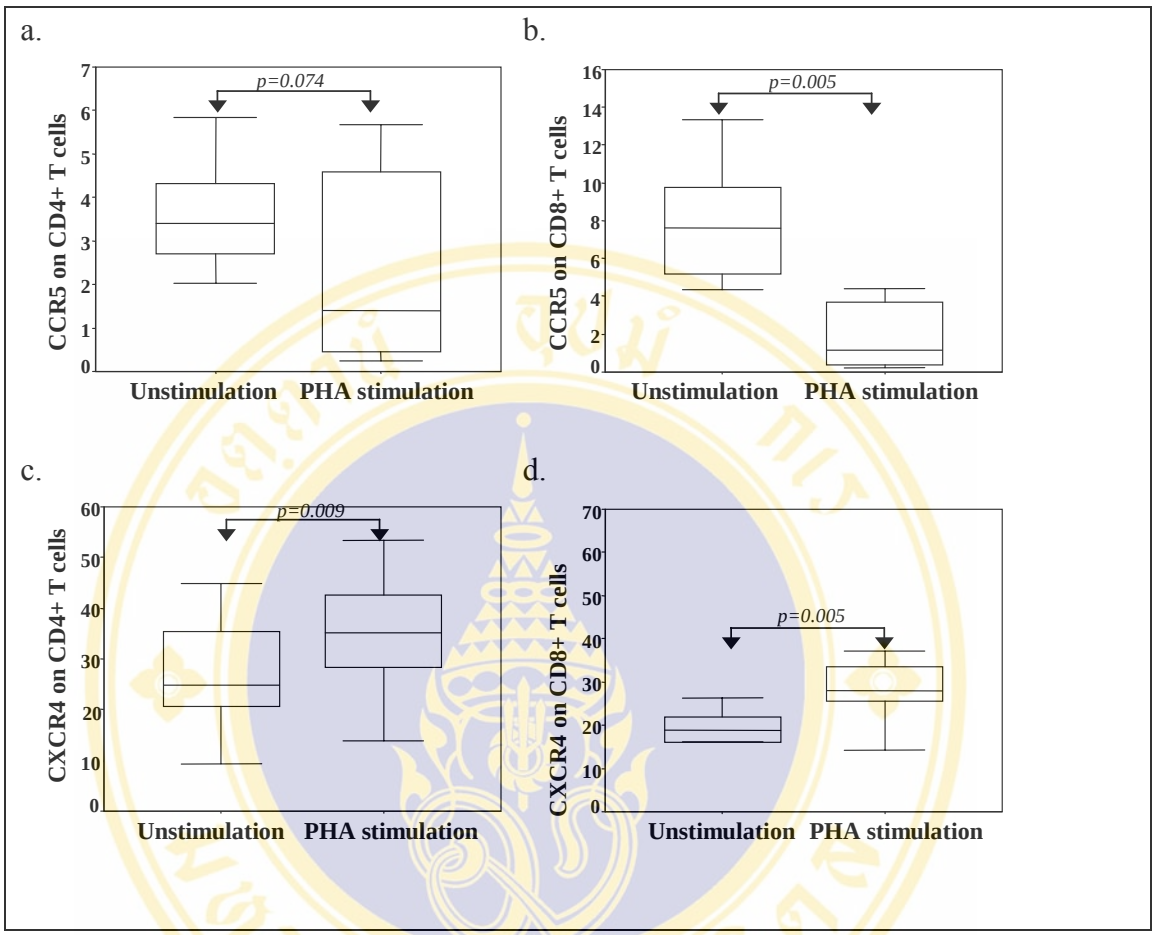


Figure 10. Comparison of chemokine receptors expressed on CD4+ T cells (a,c) and CD8+ T cells (b,d) between unstimulated and PHA-stimulated PBMC. a) CCR5 on CD4+ T cells, b) CCR5 on CD8+ T cells, c) CXCR4 on CD4+ T cells, and d) CXCR4 on CD8+ T cells.

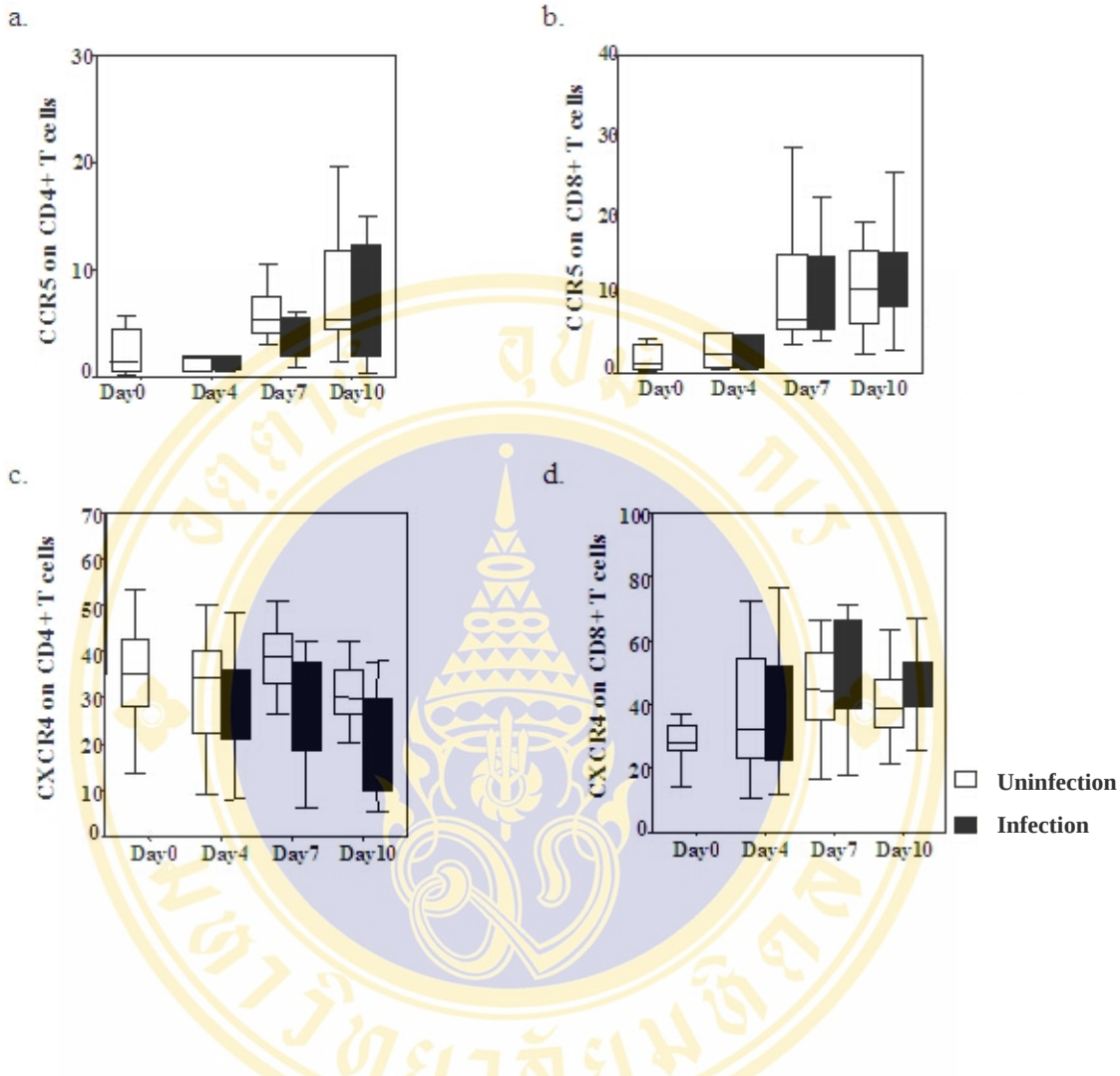


Figure 11. Comparison of chemokine receptors expressed on CD4+ T cells (a,c) and CD8+ T cells (b,d) between uninfected and HIV-1 infected PBMC. a) CCR5 on CD4+ T cells, b) CCR5 on CD8+ T cells, c) CXCR4 on CD4+ T cells, and d) CXCR4 on CD8+ T cells.

The expression of all activation markers (CD25, CD69, CD71, and HLA-DR) on unstimulated and PHA-stimulated PBMC is demonstrated in Table 4A and Figures 12-15. All activation markers on CD4+ and CD8+ T cells of PHA-stimulated PBMC were significantly higher than unstimulated PBMC.

Table 4. Changes on expression level of activation markers on CD4+ and CD8+ T cells* after PHA stimulation (A) and HIV-1 infection (B).

A. PHA stimulation of uninfected PBMC

Day	Activation markers	PBMC		*p* value
		Unstimulation (%)	PHA stimulation** (%)	
Day0	**CD4+ T cells**			
	CD25	4.16 ± 1.31	42.90 ± 15.68	0.008
	CD69	1.21 ± 2.99	11.69 ± 4.89	0.008
	CD71	0.77 ± 1.14	41.59 ± 12.57	0.008
	HLA-DR	6.36 ± 6.83	38.58 ± 14.49	0.011
	CD8+ T cells			
	CD25	0.15 ± 0.06	43.78 ± 18.16	0.008
	CD69	0.46 ± 0.45	18.22 ± 17.69	0.008
	CD71	0.17 ± 0.06	42.37 ± 18.34	0.008
	HLA-DR	11.41 ± 3.30	37.99 ± 12.81	0.008

B. HIV-1 infection in PHA-activated PBMC

Day	Activation markers	PBMC		*p* value
		Uninfection (%)	Infection (%)	
Day4 pi	**CD4+ T cells**			
	CD25	35.81 ± 15.80	33.87 ± 14.42	NS
	CD69	3.98 ± 4.06	3.01 ± 3.02	NS
	CD71	25.05 ± 15.41	23.35 ± 14.12	NS
	HLA-DR	16.69 ± 10.99	16.17 ± 11.21	NS
	CD8+ T cells			
	CD25	19.74 ± 9.03	20.83 ± 9.57	NS
	CD69	6.49 ± 7.52	5.90 ± 6.37	NS
	CD71	24.23 ± 7.84	24.48 ± 8.03	NS
	HLA-DR	20.42 ± 5.64	21.41 ± 6.04	NS
Day7 pi	**CD4+ T cells**			
	CD25	26.83 ± 11.91	21.23 ± 11.10	NS
	CD69	6.56 ± 4.45	3.94 ± 3.24	NS
	CD71	17.46 ± 11.68	14.19 ± 10.21	NS
	HLA-DR	22.42 ± 13.72	18.12 ± 12.73	NS
	CD8+ T cells			
	CD25	12.26 ± 5.27	13.64 ± 5.60	NS
	CD69	9.31 ± 7.54	11.03 ± 10.81	NS
	CD71	17.23 ± 7.49	19.31 ± 6.94	NS
	HLA-DR	30.35 ± 6.76	33.94 ± 10.36	NS
Day10 pi	**CD4+ T cells**			
	CD25	17.34 ± 10.91	10.91 ± 8.13	NS
	CD69	8.69 ± 6.74	5.07 ± 4.95	NS
	CD71	12.85 ± 11.28	9.26 ± 9.02	NS
	HLA-DR	23.41 ± 15.55	15.49 ± 12.71	NS
	CD8+ T cells			
	CD25	10.79 ± 6.16	11.20 ± 5.15	NS
	CD69	13.66 ± 11.28	14.75 ± 11.54	NS
	CD71	13.89 ± 7.85	16.25 ± 8.40	NS
	HLA-DR	35.95 ± 9.00	40.11 ± 17.34	NS

* Mean±SD, ** Day4 PHA or Day0 pi, pi = post infection, NS = no significance

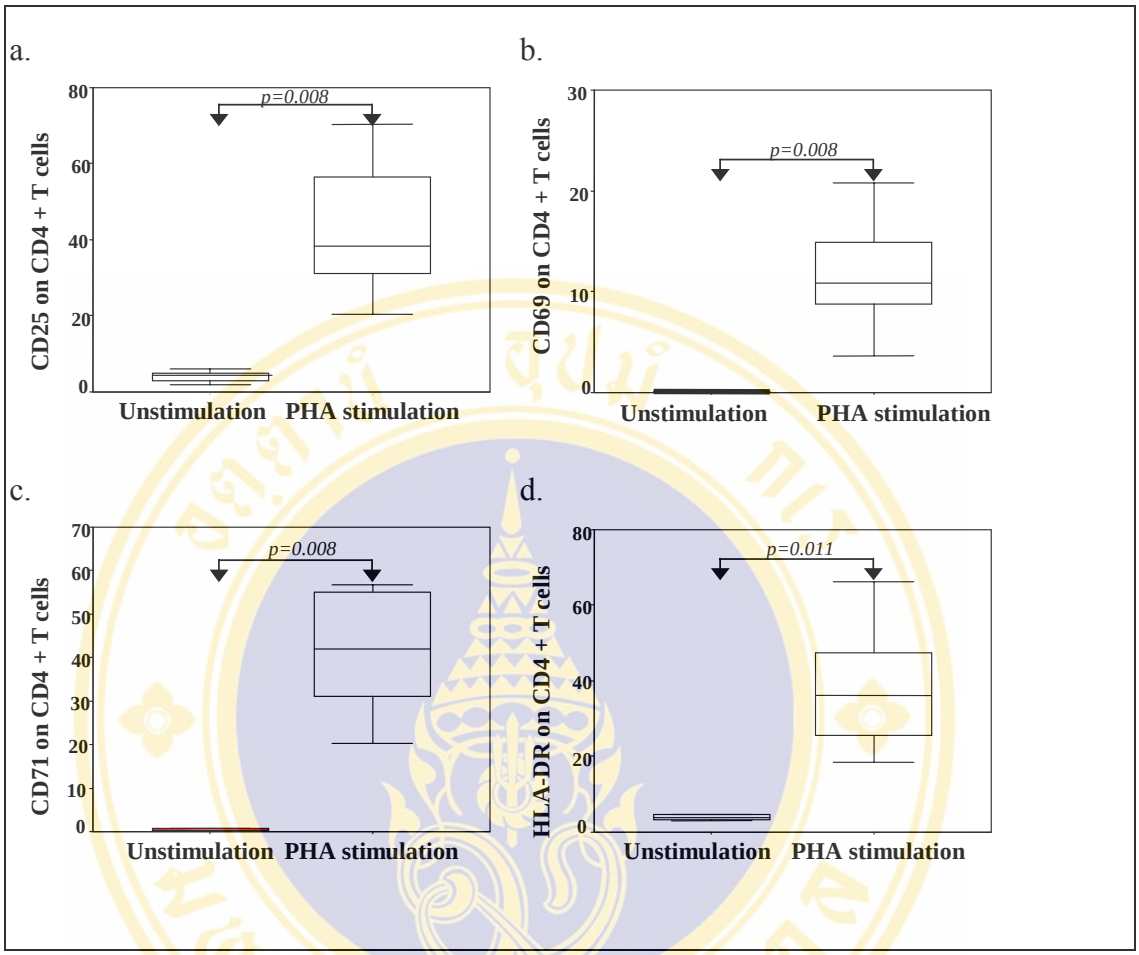


Figure 12. Comparison of activation markers expressed on CD4+ T cells between unstimulated and PHA-stimulated PBMC. a) CD25 on CD4+ T cells, b) CD69 on CD4+ T cells, c) CD71 on CD4+ T cells, and d) HLA-DR on CD4+ T cells.

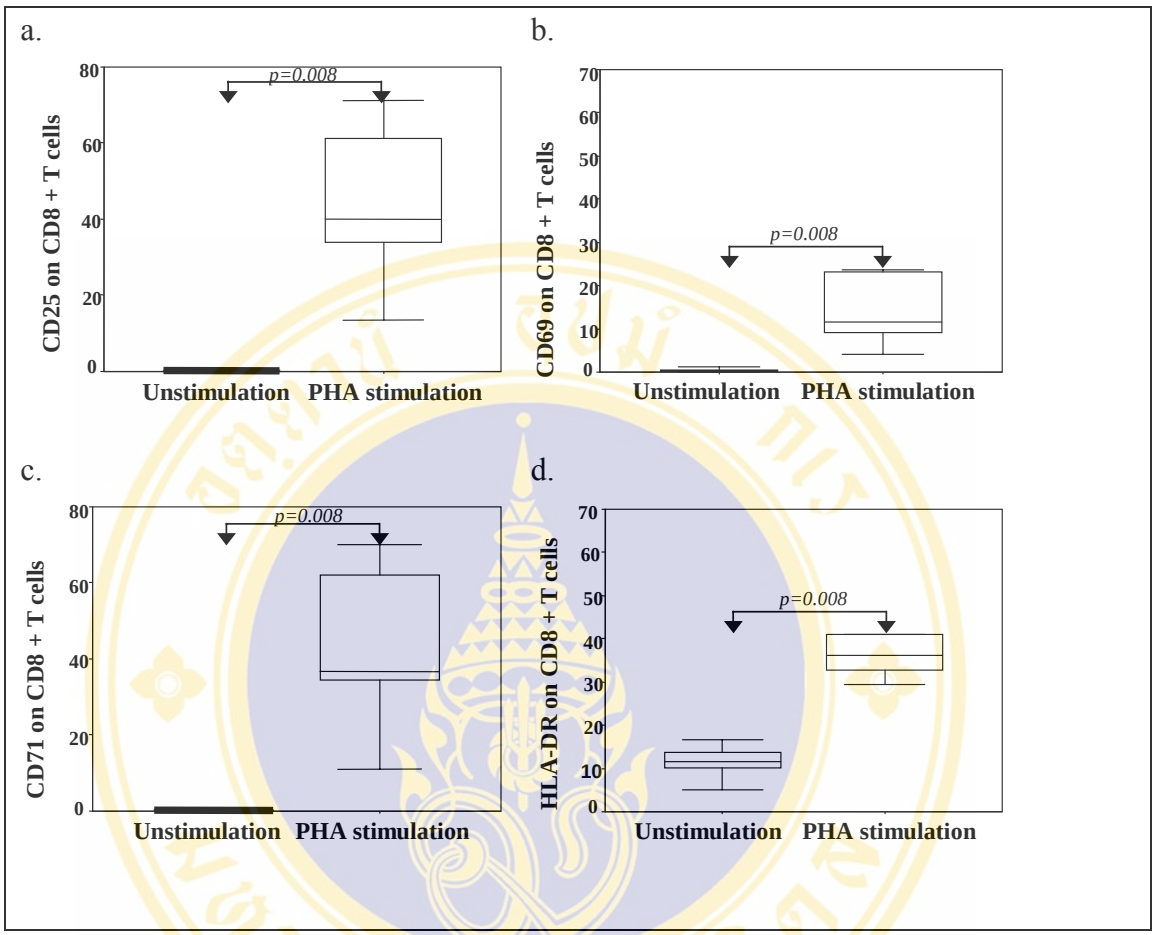


Figure 13. Comparison of activation markers expressed on CD8+ T cells between unstimulated and PHA-stimulated PBMC. a) CD25 on CD8+ T cells, c) CD69 on CD8+ T cells, c) CD71 on CD8+ T cells, and d) HLA-DR on CD8+ T cells.

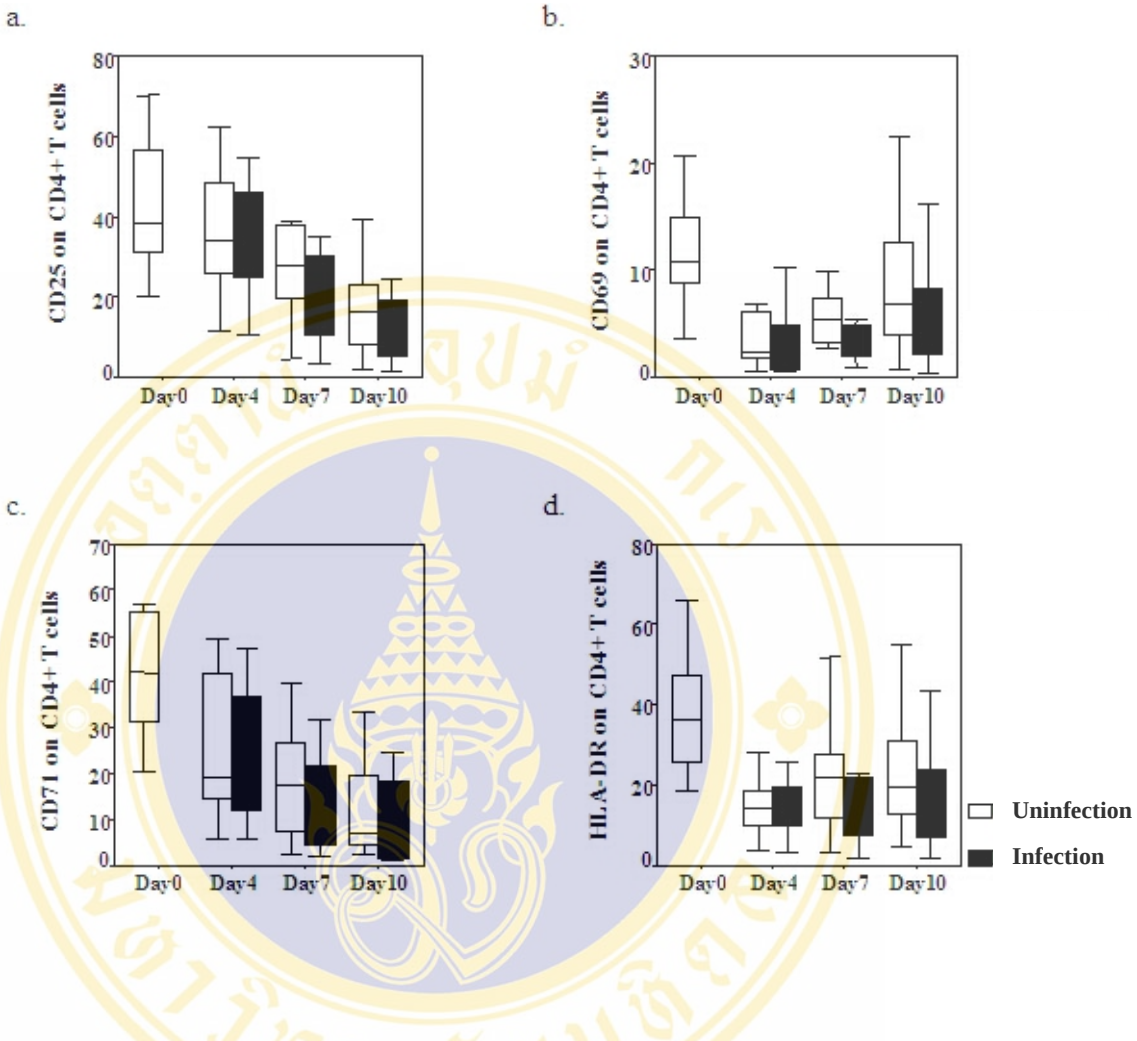


Figure 14. Comparison of activation markers expressed on CD4+ T cells between uninfected and HIV-1 infected PBMC. a) CD25 on CD4+ T cells, b) CD69 on CD4+ T cells, c) CD71 on CD4+ T cells, and d) HLA-DR on CD4+ T cells.

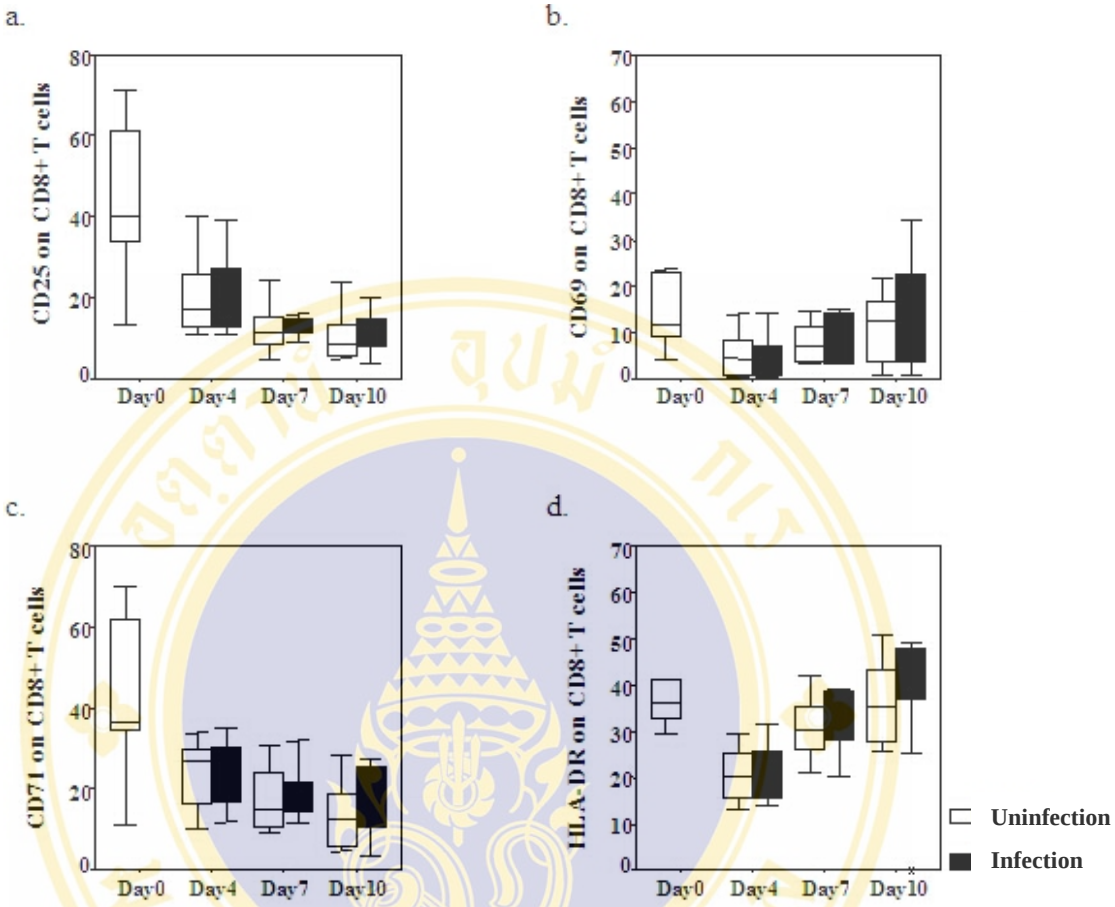


Figure 15. Comparison of activation markers expressed on CD8+ T cells between uninfected and HIV-1 infected PBMC. a) CD25 on CD8+ T cells, c) CD69 on CD8+ T cells, c) CD71 on CD8+ T cells, and d) HLA-DR on CD8+ T cells.

2. Determination of chemokine receptors and activation markers on PHA-stimulated PBMC during the period of HIV–1 infection

On day4, the proliferative PHA-stimulated PBMC was divided into two groups. The first group had only PHA-stimulated PBMC and the second group of PHA-stimulated PBMC was infected with HIV-1 at 500 $TDIC_{50}$. These two groups had been cultured for 10 days. Then, they were stained with specific three-color fluorescent conjugated monoclonal antibodies as a previous process on days 4, 7, and 10 post infection (pi). Examples of chemokine receptors and activation markers on CD4+ and CD8+ T cells are shown in Figures 16-21. The mean percentage of CD4+ and CD8+ expressed on chemokine receptors and activation markers is summarized in Tables 3B and 4B, respectively.

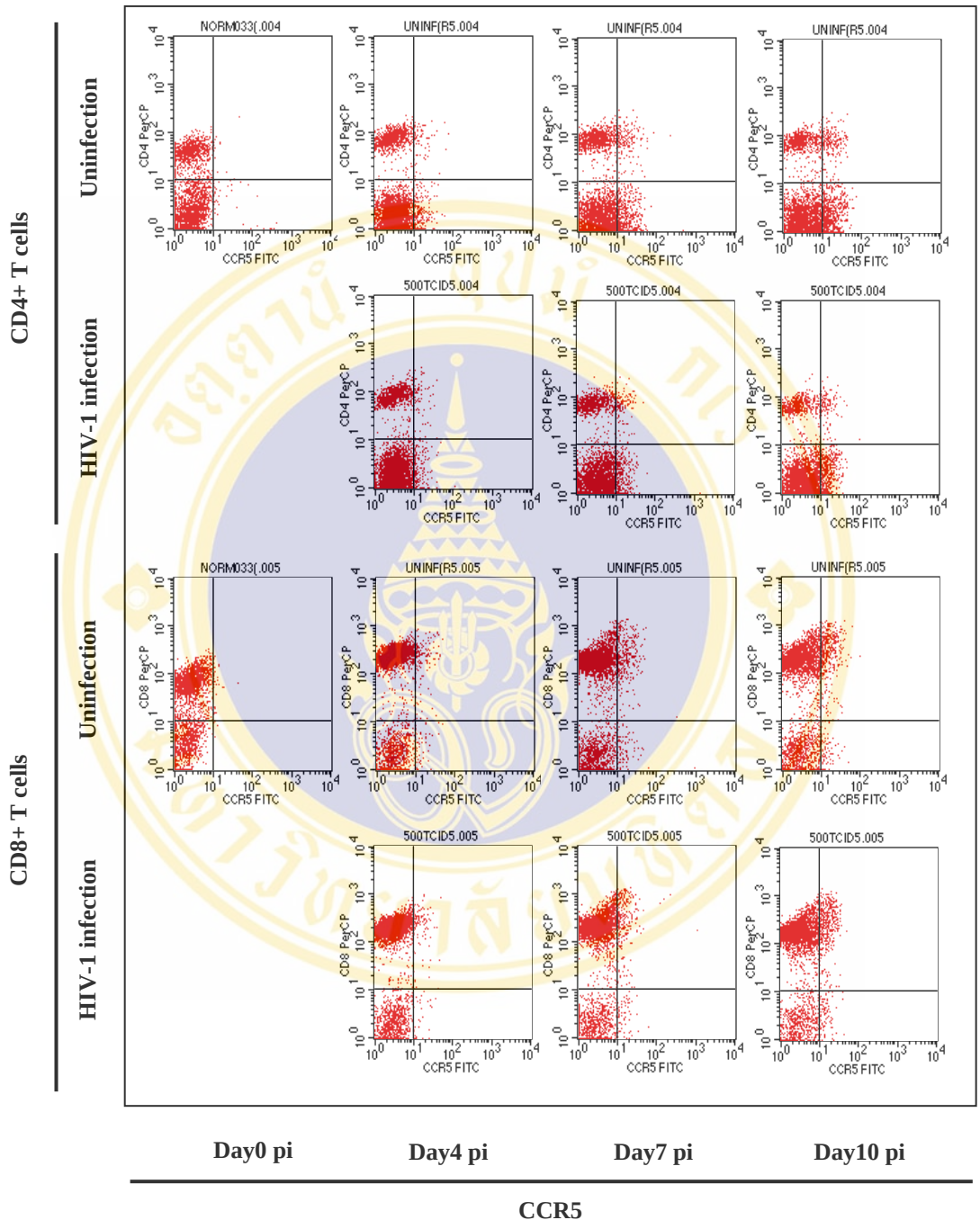


Figure 16. Flow cytometry analysis of CCR5 expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

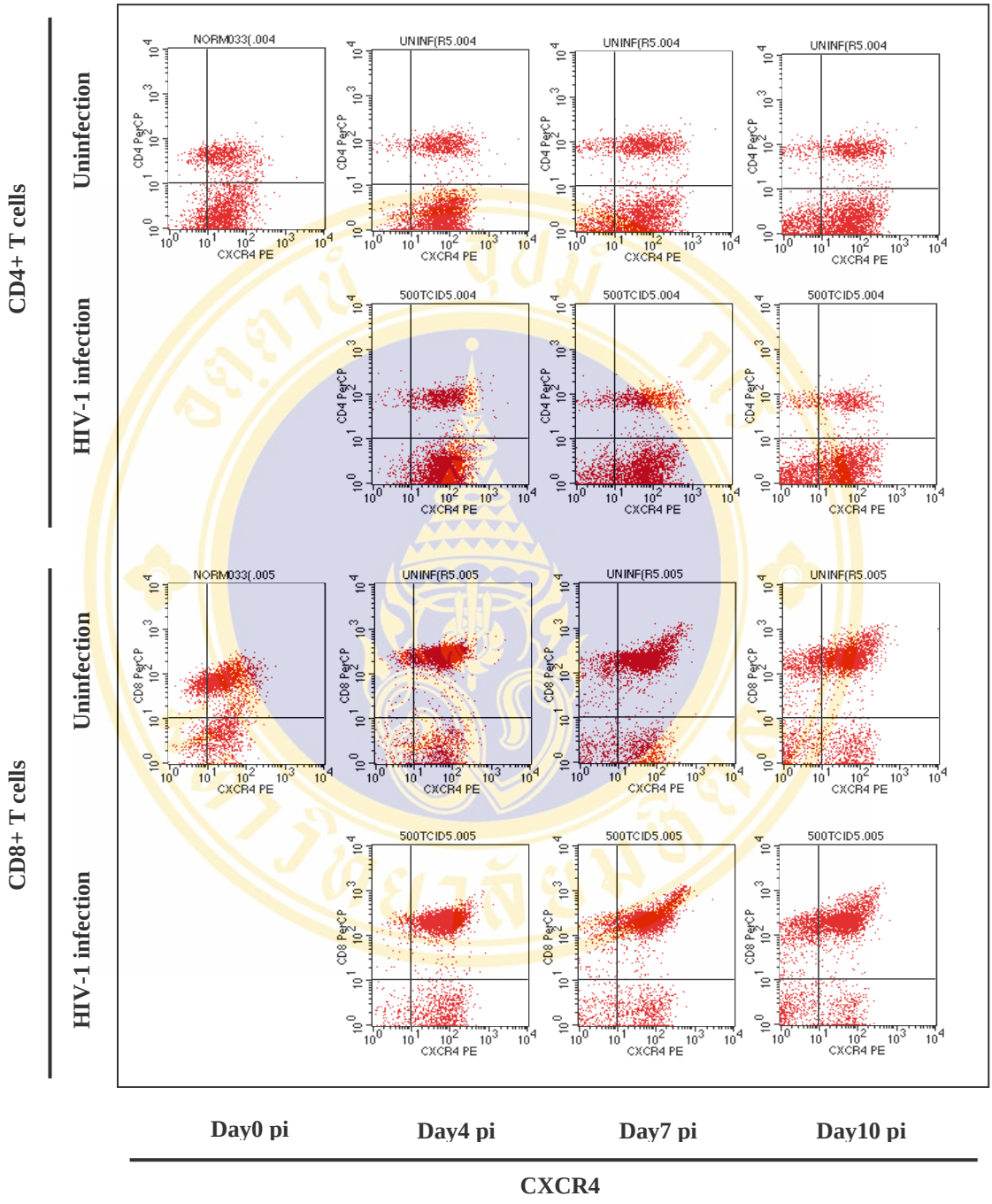


Figure 17. Flow cytometry analysis of CXCR4 expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

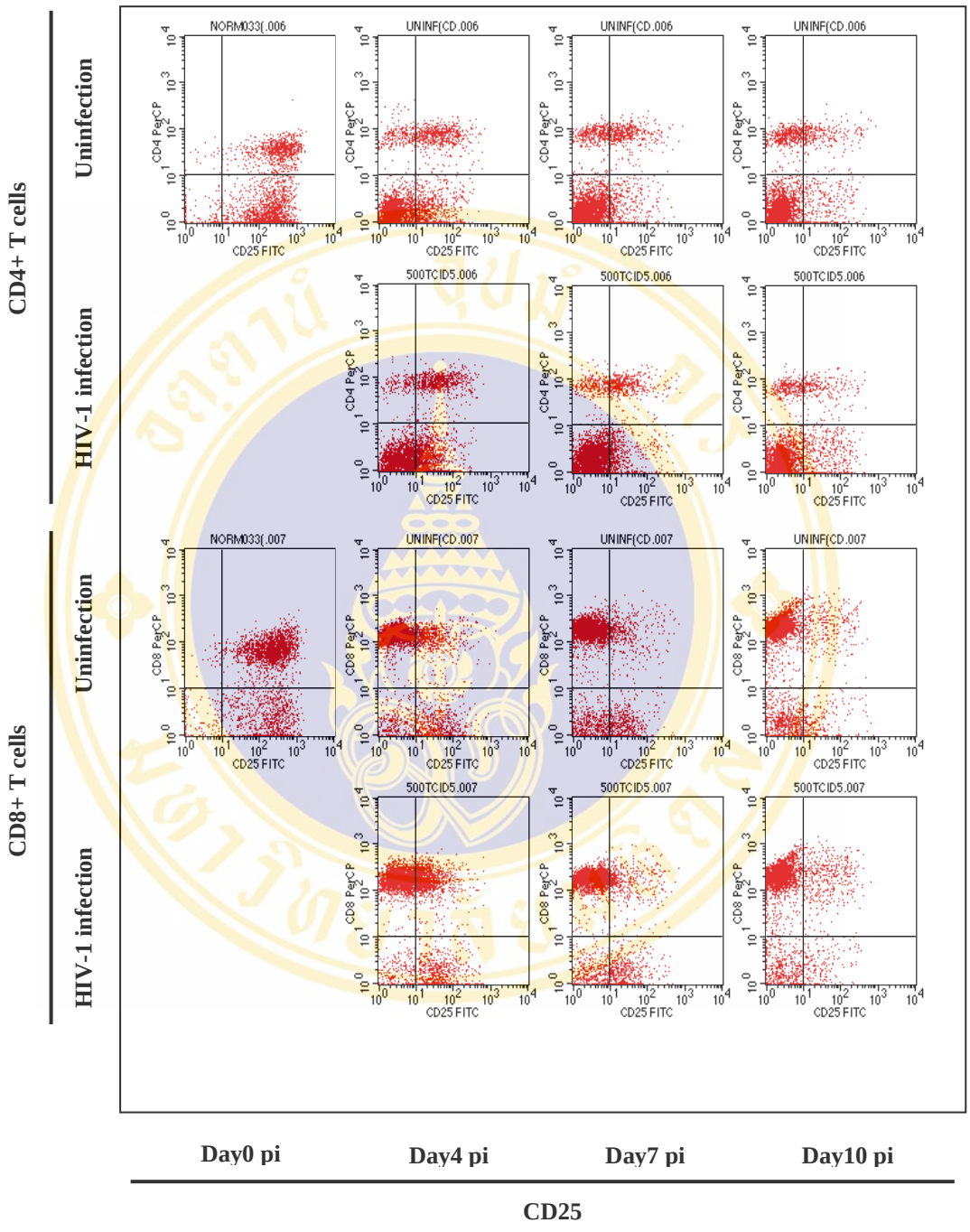


Figure 18. Flow cytometry analysis of CD25 expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

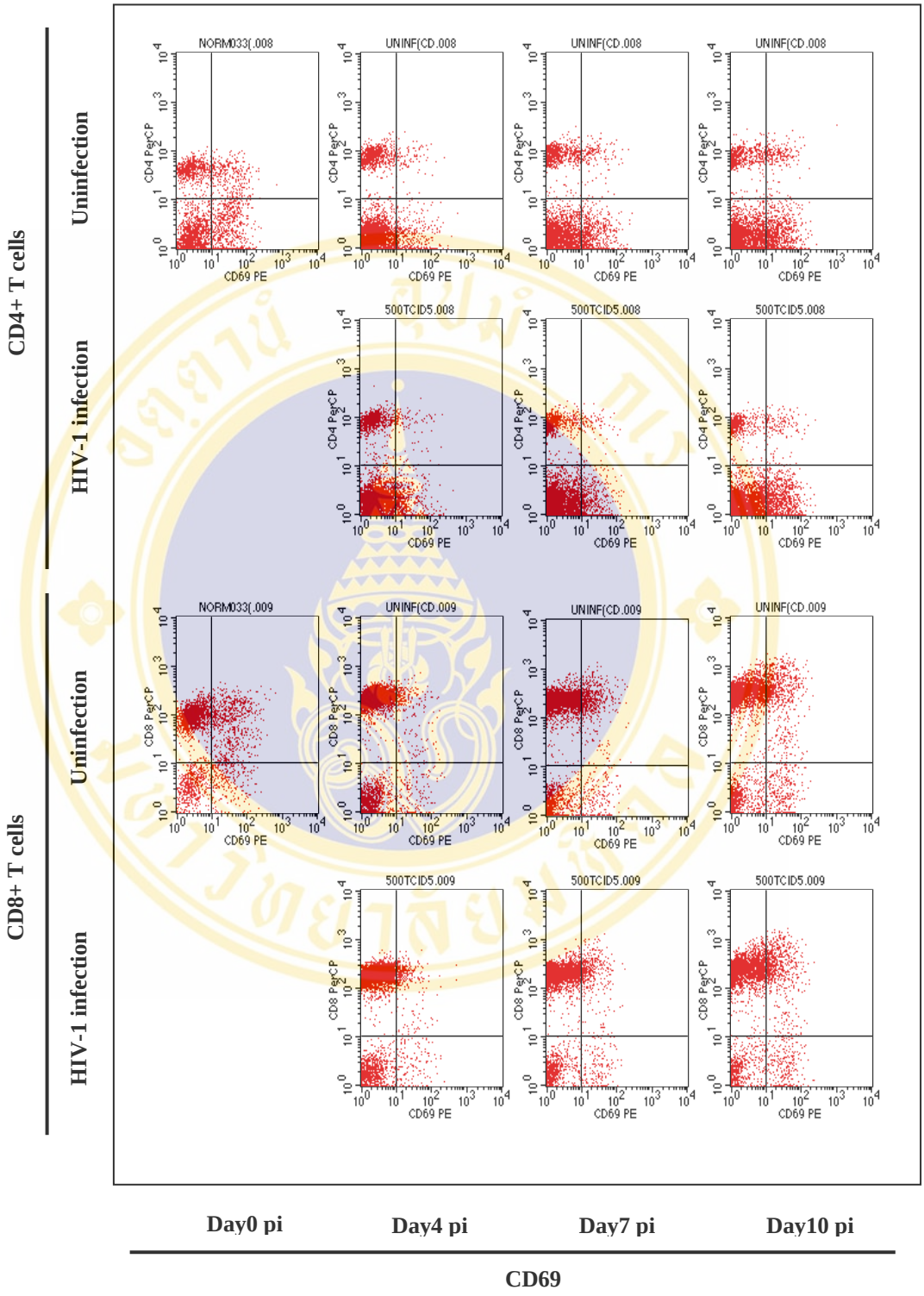


Figure 19. Flow cytometry analysis of CD69 expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

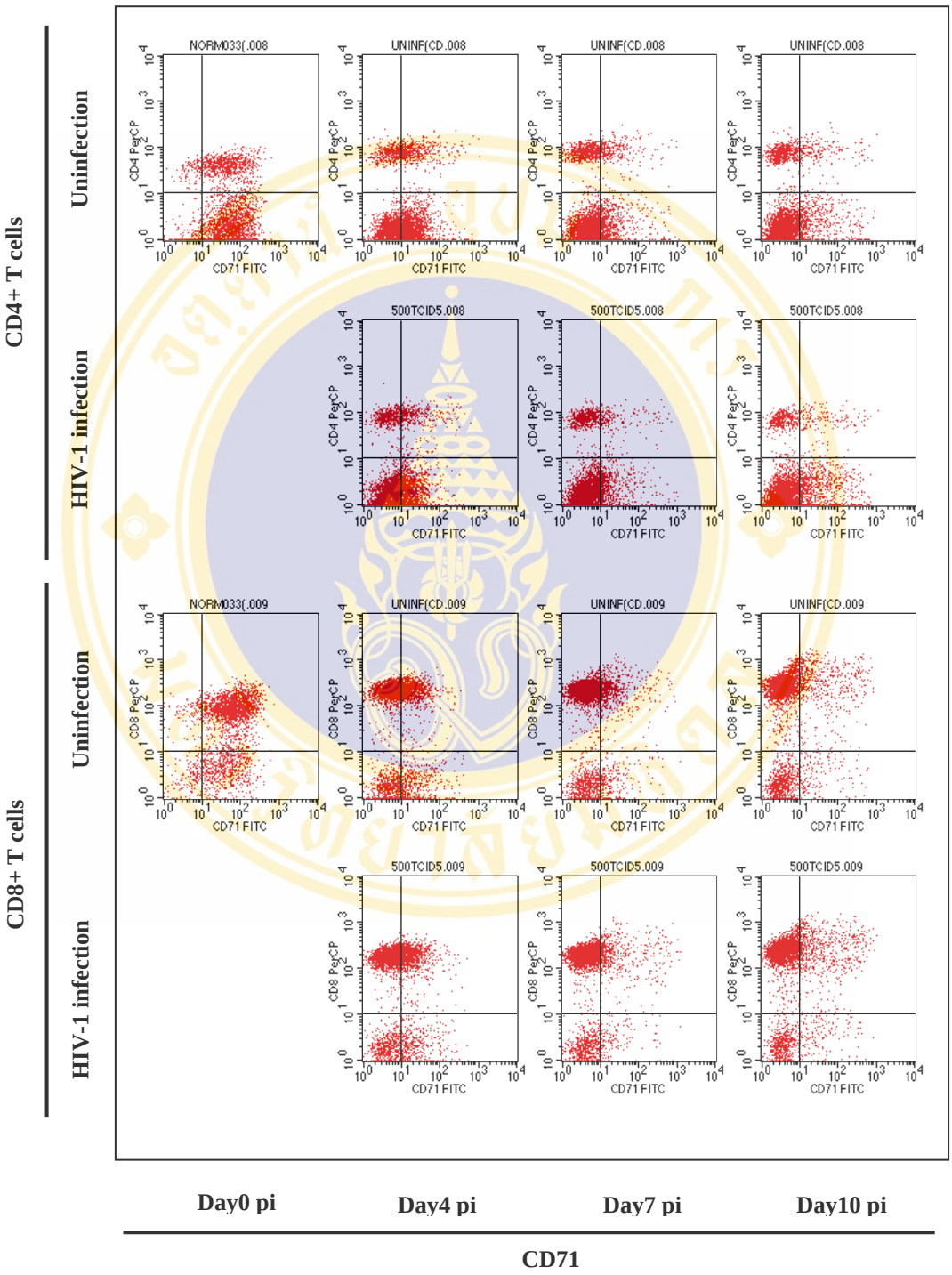


Figure 20. Flow cytometry analysis of CD71 expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

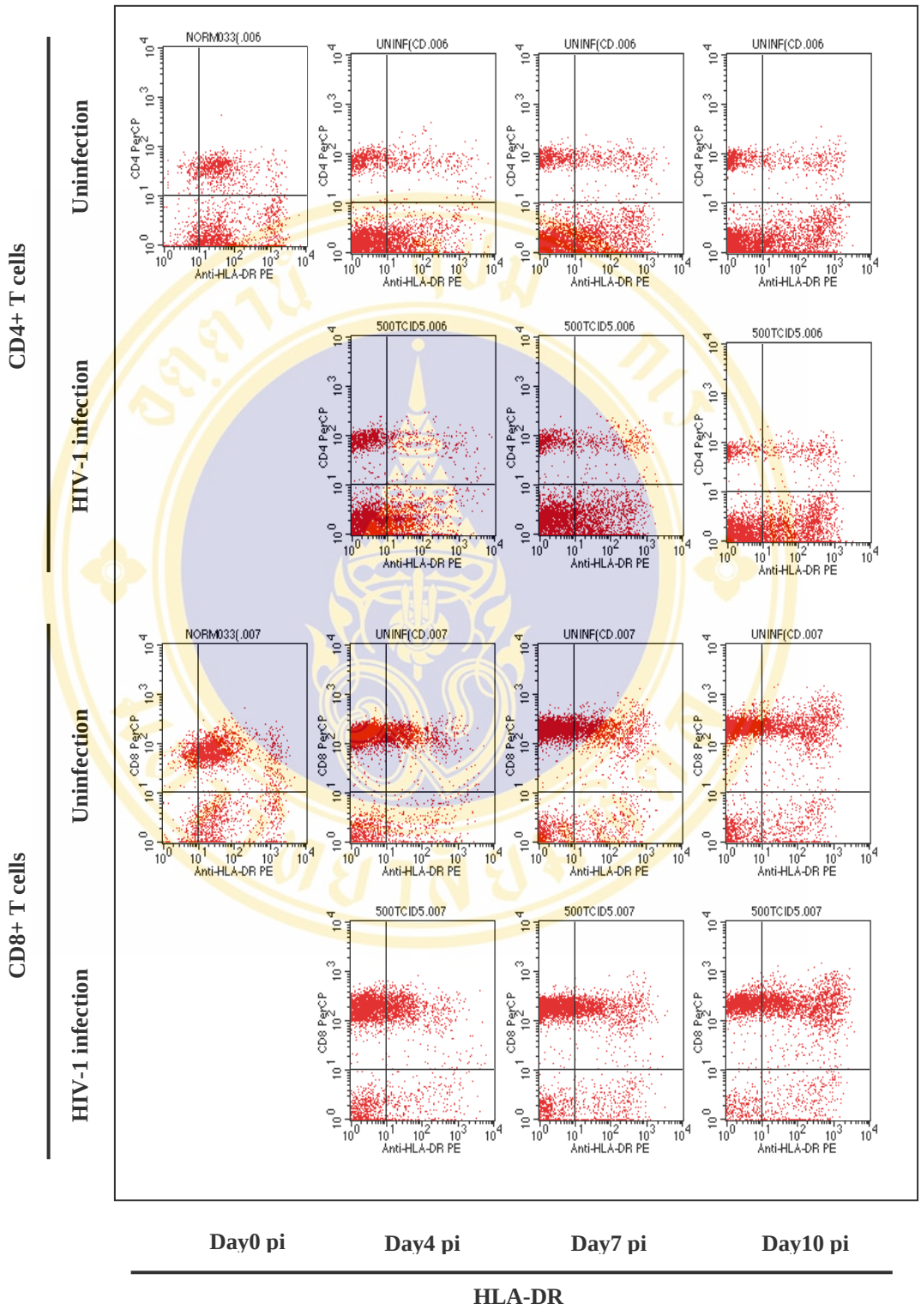


Figure 21. Flow cytometry analysis of HLA-DR expression on both uninfected and HIV-1 infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

As shown in Table 5 and Figure 22, CCR5 and CXCR4 expression on CD4+ and CD8+ T cells were slightly declined in both groups but there were no significant differences between uninfected and HIV–1 infected cells on day4 and day7. However, on day10 CXCR4 expression on CD4+ T cells between both groups was significantly different (p=0.049) but the expression of other chemokine receptor was not significant difference. The mean percentage of activation markers that expressed on CD4+ and CD8+ T cells is summarized in Tables 4B, 6 and Figure 23. No significant difference was observed in the percentage of all activation markers.

Table 5. Statistical comparison of chemokine receptors CCR5 (A), and CXCR4 (B) on CD4+ and CD8+ T cells during the period of HIV-1 infection.

A. CCR5

	Day	Statistical comparison					
		Uninfected PBMC			HIV-1 infected PBMC		
		Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
CD4+ T cells							
Uninfection	Day0	NS	0.005	0.022			
	Day4		NS	0.028	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	0.047
	Day7 pi						NS
CD8+ T cells							
Uninfection	Day0	NS	0.005	0.007			
	Day4		0.037	0.047	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	0.047
	Day7 pi						NS

B. CXCR4

	Day	Statistical comparison					
		Uninfected PBMC			HIV-1 infected PBMC		
		Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
CD4+ T cells							
Uninfection	Day0	NS	NS	NS			
	Day4		NS	NS	NS		
	Day7			0.047		NS	
	Day10						0.049
HIV-1 infection	Day4 pi					NS	0.013
	Day7 pi						0.005
CD8+ T cells							
Uninfection	Day0	NS	0.005	0.005			
	Day4		0.017	NS	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					0.009	0.047
	Day7 pi						NS

n=10; NS = no significance

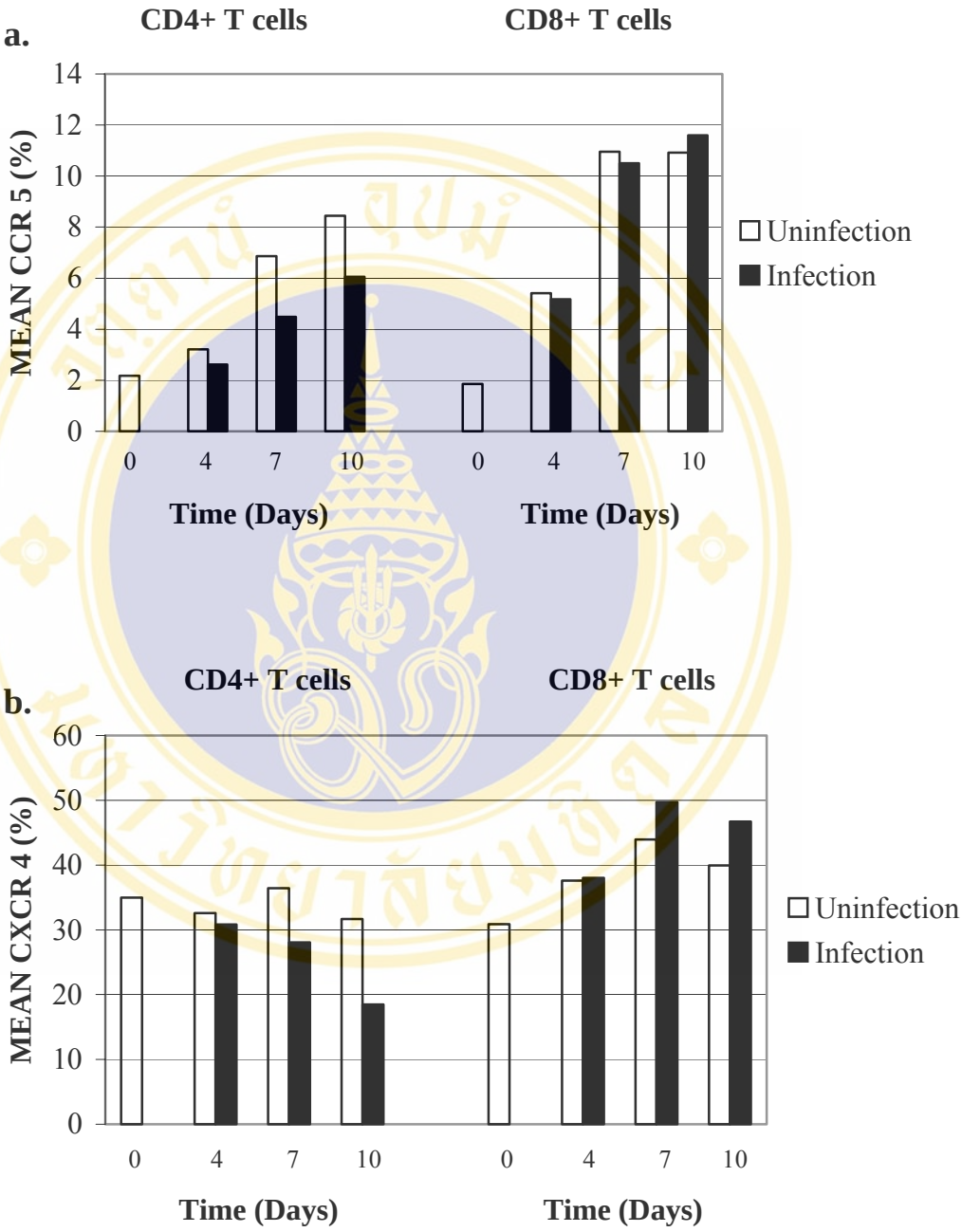


Figure 22. Comparison of chemokine receptors CCR5 (a) and CXCR4 (b) expressed on both uninfected and infected T lymphocytes during the course of HIV-1 infection.

Table 6. Statistical comparison of activation markers CD25 (A), CD69 (B), CD71(C), and HLA-DR (D) on CD4+ and CD8+ T cells during the period of HIV-1 infection.

A. CD25

		Statistical comparison					
	Day	**Uninfected PBMC**			**HIV-1 infected PBMC**		
		Day4	**Day7**	**Day10**	**Day4 pi**	**Day7 pi**	**Day10 pi**
CD4+ T cells							
Uninfection	Day0	NS	0.022	0.005			
	Day4		0.007	0.005	NS		
	Day7			0.007		NS	
	Day10						NS
HIV-1 infection	Day4 pi					0.008	0.005
	Day7 pi						0.005
CD8+ T cells							
Uninfection	Day0	0.005	0.005	0.005			
	Day4		0.007	0.017	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	0.017
	Day7 pi						NS

B. CD69

		Statistical comparison					
	Day	**Uninfected PBMC**			**HIV-1 infected PBMC**		
		Day4	**Day7**	**Day10**	**Day4 pi**	**Day7 pi**	**Day10 pi**
CD4+ T cells							
Uninfection	Day0	0.005	0.037	NS			
	Day4		0.017	0.005	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	NS
	Day7 pi						NS
CD8+ T cells							
Uninfection	Day0	0.007	0.022	NS			
	Day4		0.037	0.005	NS		
	Day7			0.028		NS	
	Day10						NS
HIV-1 infection	Day4 pi					0.007	0.005
	Day7 pi						NS

C. CD71

	Day	Statistical comparison					
		Uninfected PBMC			HIV-1 infected PBMC		
		Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
CD4+ T cells							
Uninfection	Day0	0.028	0.007	0.005			
	Day4		0.028	0.017	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					0.007	0.007
	Day7 pi						0.007
CD8+ T cells							
Uninfection	Day0	0.013	0.007	0.007			
	Day4		NS	0.028	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	0.047
	Day7 pi						NS

D. HLA-DR

	Day	Statistical comparison					
		Uninfected PBMC			HIV-1 infected PBMC		
		Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
CD4+ T cells							
Uninfection	Day0	0.007	0.037	0.047			
	Day4		0.007	0.028	NS		
	Day7			NS		NS	
	Day10						NS
HIV-1 infection	Day4 pi					NS	NS
	Day7 pi						NS
CD8+ T cells							
Uninfection	Day0	0.005	NS	NS			
	Day4		0.005	0.005	NS		
	Day7			0.017		NS	
	Day10						NS
HIV-1 infection	Day4 pi					0.005	0.037
	Day7 pi						NS

n=9; NS = no significance

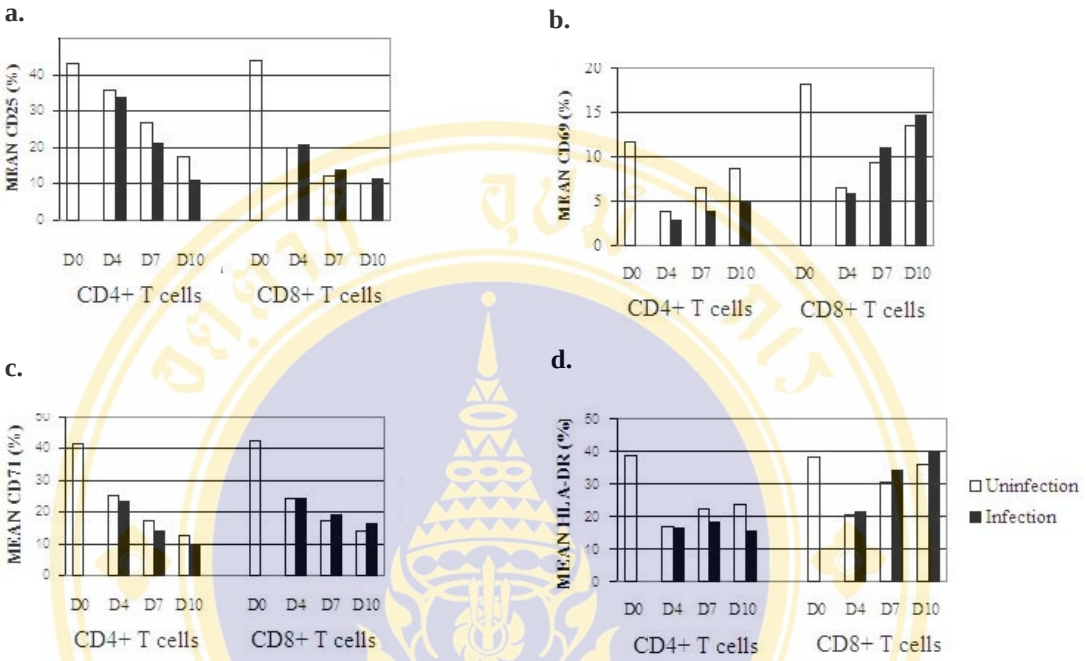


Figure 23. Comparison of activation markers CD25 (a), CD69 (b), CD71 (c), and HLA-DR (d) expressed on both uninfected and infected CD4+ and CD8+ T cells during the period of HIV-1 infection.

3. Comparison of chemokine receptors on PHA-stimulated PBMC during the period of HIV–1 infection

The data of chemokine receptors (Figures 16-17) from flow cytometric assay were analyzed and compared according to the period of HIV-1 infection. The uninfected PBMC were compared among the result on day4 after PHA stimulation (day4 PHA or day0 pi), day4 post HIV-1 infection (day4 pi), day7 pi, and day10 pi, while the result on HIV-1 infected PBMC were compared among days 4 pi, 7 pi, and 10 pi. For uninfected CD4+ T cells, although not significant, a slight but consistent increment in the expression of CCR5 molecules was observed on day4 versus day7 (p=0.074) (Tables 3B and 5A). While significant increment was observed on day0 versus day7 (p=0.005), day0 versus day10 (p=0.022) (Tables 3A, 3B and 5A), and day4 versus day10 (p=0.028). The percentage of CCR5 on HIV-1 infected CD4+ T cells significantly increased on day4 pi versus day10 pi (p=0.047). For uninfected CD8+ T cells, significant increment was observed on day0 versus day7 (p=0.005), day0 versus day10 (p=0.007), day4 versus day7 (p=0.037), and day4 pi versus day10 pi (p=0.047). Moreover, significant increment of CCR5 on HIV-1 infected CD8+ T cells was found on day4 pi versus day10 pi (p=0.047).

The percentage of CXCR4 on uninfected CD4+ T cells significantly decreased on day7 versus day10 (p=0.047) (Tables 3B and 5B). Similarly, the period of HIV-1 culture induced a decline in the percentage of CXCR4 on infected CD4+ T cells on day4 pi versus day10 pi (p=0.013), and day7 pi versus day10 pi (p=0.005). Inversely, a period of HIV-1 infection induced significant increment of the percentage of CXCR4 on both uninfected CD8+ T cells {day0 versus day7 (p=0.005), day0 versus day10 (p=0.005), and day4 versus day10 (p=0.017)} and HIV-1 infected CD8+ T cells {day4 pi versus day7 pi (p=0.009), and day4pi versus day10 pi (p=0.047)}.

4. Comparison of activation markers on PHA-stimulated PBMC during the period of HIV–1 infection

The data of activation markers (Figures 18-21) from flow cytometric assay were analyzed and compared according to the period of HIV-1 infection. The uninfected PBMC was compared among the result on days 0, 4, 7, and 10, while the result on HIV-1 infected PBMC was compared among day4 pi, day7 pi, and day10 pi. The percentage of CD25 (Tables 4A, 4B, and 6A) on uninfected CD4+ T cells significantly decreased on day0 versus day7 (p=0.022), day0 versus day10 (p=0.005), day4 versus day7 (p=0.007), day4 versus day10 (p=0.005), and day7 versus day10 (p=0.007). Similarly, the significant decline of CD25 on HIV-1 infected CD4+ T cells was observed on day4 pi versus day7 pi (p=0.008), day4 pi versus day10 pi (p=0.005), and day7 pi versus day10 pi (p=0.005). Similar to CD25 on CD4+ T cells, the percentage of CD25 on uninfected CD8+ T cells significantly decreased on day0 versus day4 (p=0.005), day0 versus day7 (p=0.005), day0 versus day10 (p=0.005), day4 versus day7 (p=0.007), and day4 versus day10 (p=0.017). While the significant decline of CD25 on HIV-1 infected CD8+ T cells was observed on day4 pi versus day10 pi (p=0.017).

The percentage of CD69 (Tables 4A, 4B, and 6B) on uninfected CD4+ T cells significantly decreased on day0 versus day4 (p=0.005), and day0 versus day7 (p=0.037) but significant increment of CD69 molecules was observed on day4 versus day7 (p=0.017), and day4 versus day10 (p=0.005). However, no significant difference of the percentage of CD69 was observed on HIV-1 infected CD4+ T cells. Similarly, the significant decline of CD69 on HIV-1 uninfected CD8+ T cells was observed on day0 versus day4 (p=0.007) and day0 versus day7 (p=0.022) whereas significant increment of CD69 was observed on day4 versus day7 (0.037), day4 versus day10 (0.005), and day7 versus day10 (0.037). While the percentage of CD69 on infected CD8+ T cells significantly increased on day4 pi versus day10 pi (0.007), and day4 pi versus day10 pi (0.005).

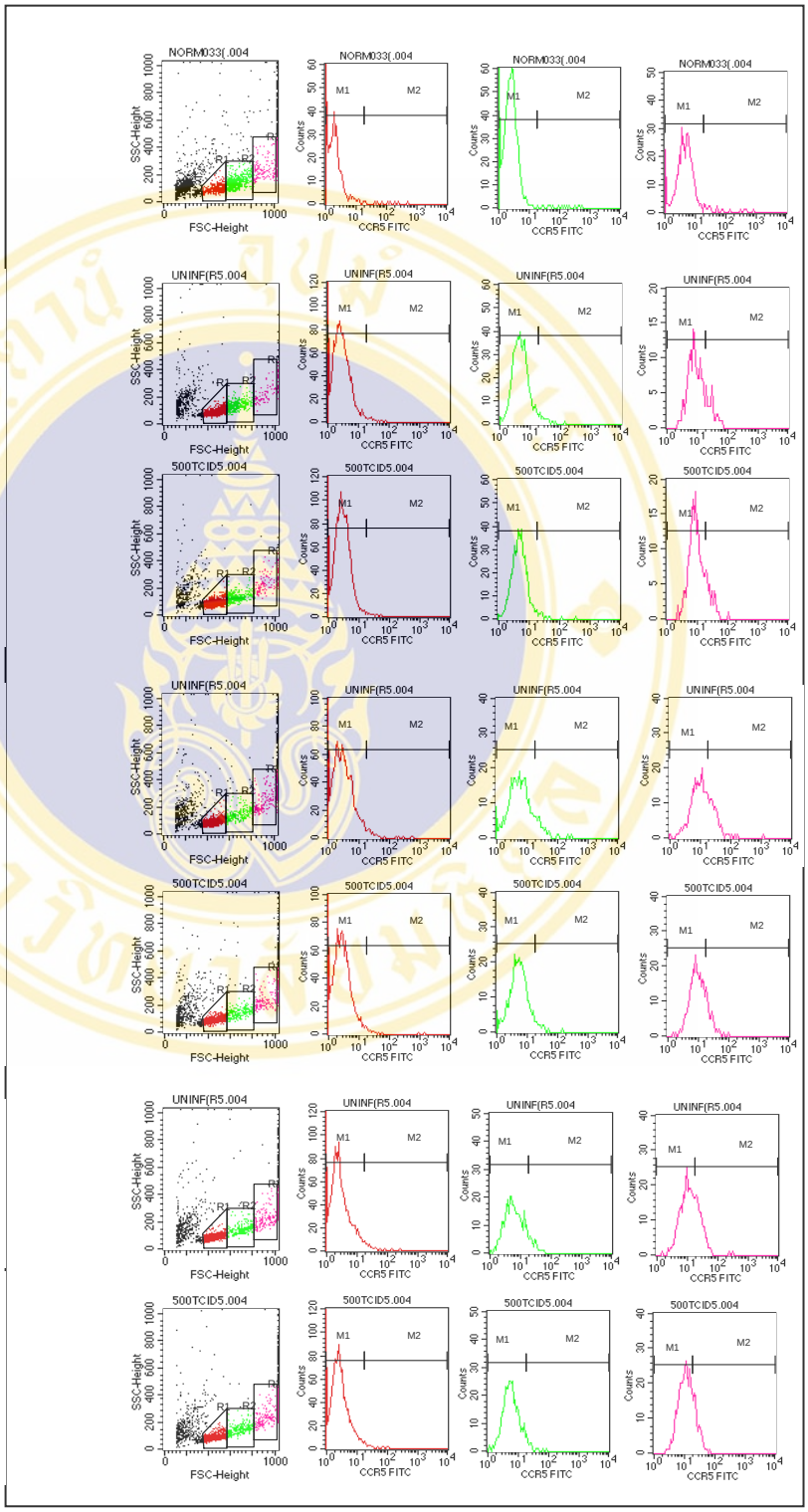
The percentage of CD71 (Tables 4A, 4B, and 6C) on uninfected CD4+ T cells significantly decreased on day0 versus day4 (p=0.028), day0 versus day7 (p=0.007), day0 versus day10 (p=0.005), day4 versus day7 (p=0.028), and day4 versus day10 (p=0.017). Similarly, the significant decline of CD71 on HIV-1 infected CD4+ T cells was observed on day4 pi versus day7 pi (p=0.007), day4 pi versus day10 pi (p=0.007), and day7 pi versus day10 pi (p=0.007). Similar to CD71 on CD4+ T cells, the percentage of CD71 on uninfected CD8+ T cells significantly decreased on day0 versus day4 (p=0.013), day0 versus day4 (p=0.007), day0 versus day10 (p=0.007), and day4 versus day10 (p=0.028). While the significant decline of CD71 on HIV-1 infected CD8+ T cells was observed on day4 pi versus day10 pi (p=0.047).

The percentage of HLA-DR (Tables 4A, 4B, and 6D) on uninfected CD4+ T cells significantly decreased on day0 versus day4 (p=0.007), and day0 pi versus day7 (p=0.037), and day0 versus day10 (p=0.047) but significant increment of HLA-DR molecules was observed on day4 versus day7 (p=0.007), and day4 versus day10 (p=0.028). However, no significant difference of the percentage of HLA-DR was observed on HIV-1 infected CD4+ T cells. Similar to uninfected CD4+ T cells, the percentage of HLA-DR on uninfected CD8+ T cells significantly decreased on day0 versus day4 (p=0.005) but significant increment of HLA-DR molecules was observed on day4 versus day7 (p=0.005), and day4 versus day10 (p=0.005), and day7 pi versus day10 pi (p=0.017). Moreover, the significant increment of HLA-DR on HIV-1 infected CD8+ T cells was observed on day4 pi versus day7 pi (p=0.005), and day4 pi versus day10 pi (p=0.037).

5. Determination of chemokine receptor and activation marker changes on PBMC in each gate during the period of HIV-1 infection

The data from flow cytometry was divided into three gates by the size of proliferative lymphocytes (Figures 24, 25). The percentage of chemokine receptors and activation markers of each gate was analyzed and compared during the period of HIV-1 culture. In addition, the result of chemokine receptors and activation markers on uninfected PBMC was compared among the result on days0 pi, 4 pi, 7 pi, and 10 pi, while the result on HIV-1 infected PBMC was compared among days4 pi, 7 pi, and 10 pi.

a.



Gate1 **Gate2** **Gate3**

CCR5

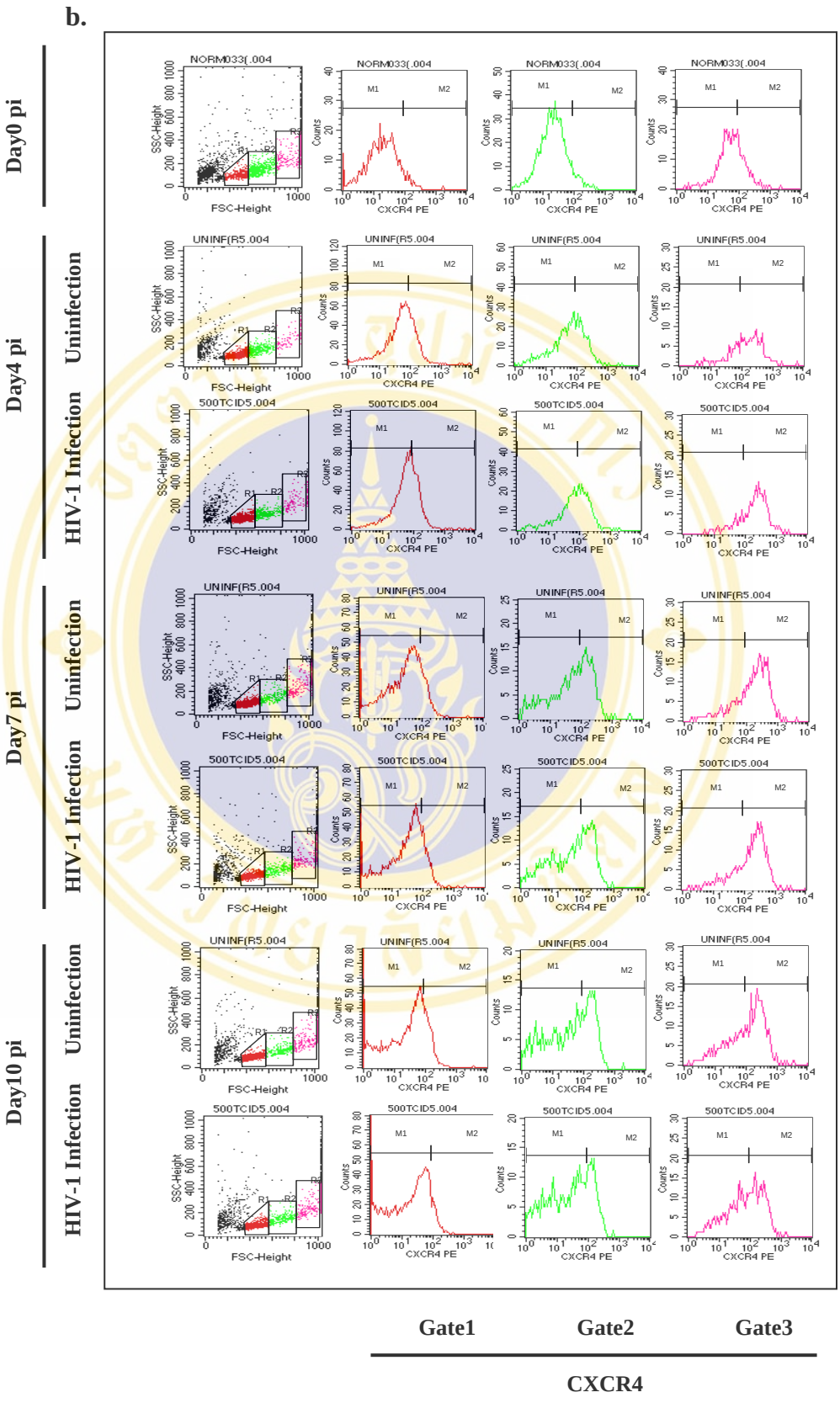
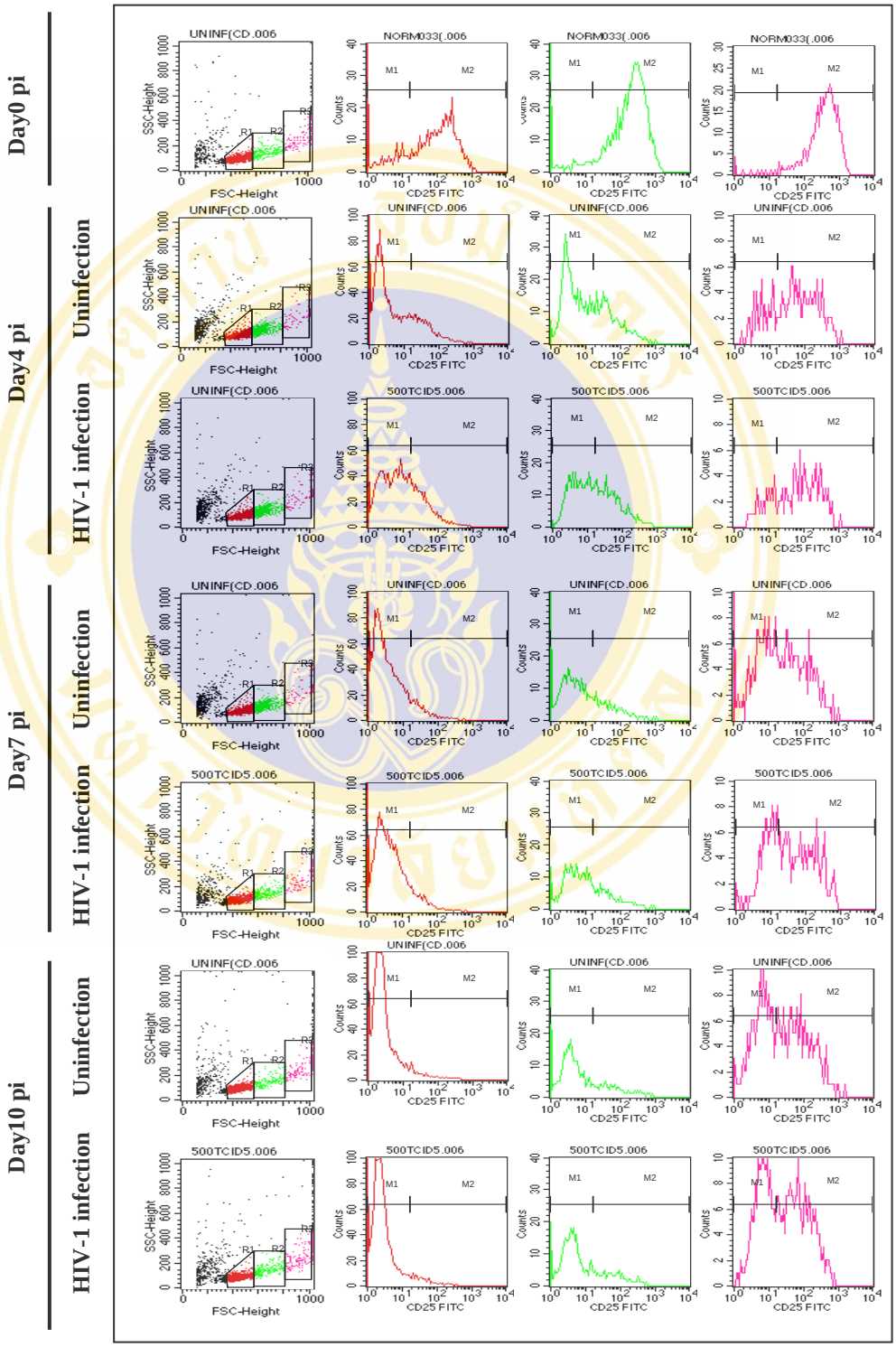
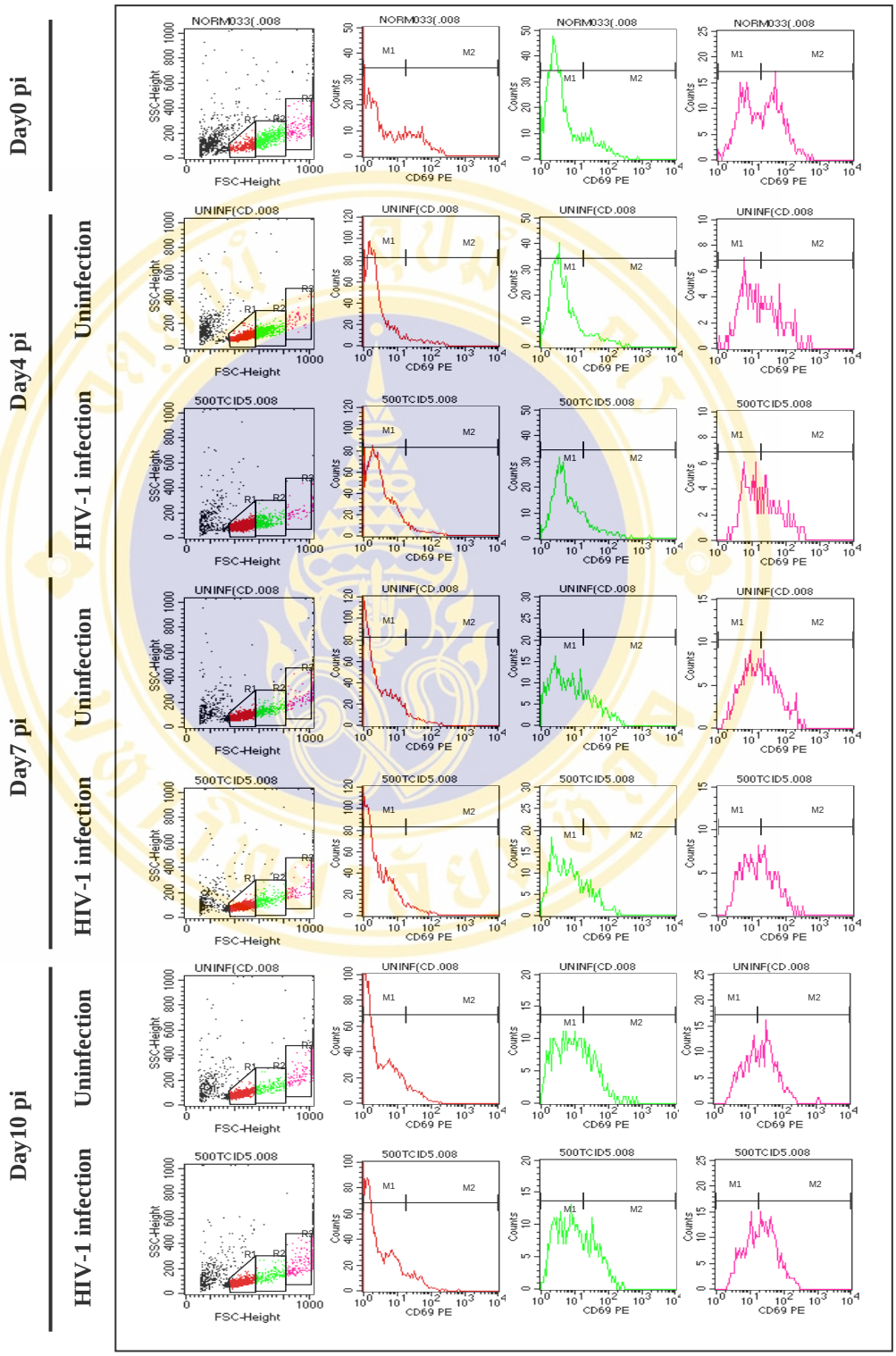


Figure 24. Flow cytometry analysis of a) CCR5 and b) CXCR4 expression in lymphocyte gate on both uninfected and HIV-1 infected PBMC during the period of HIV-1 infection.

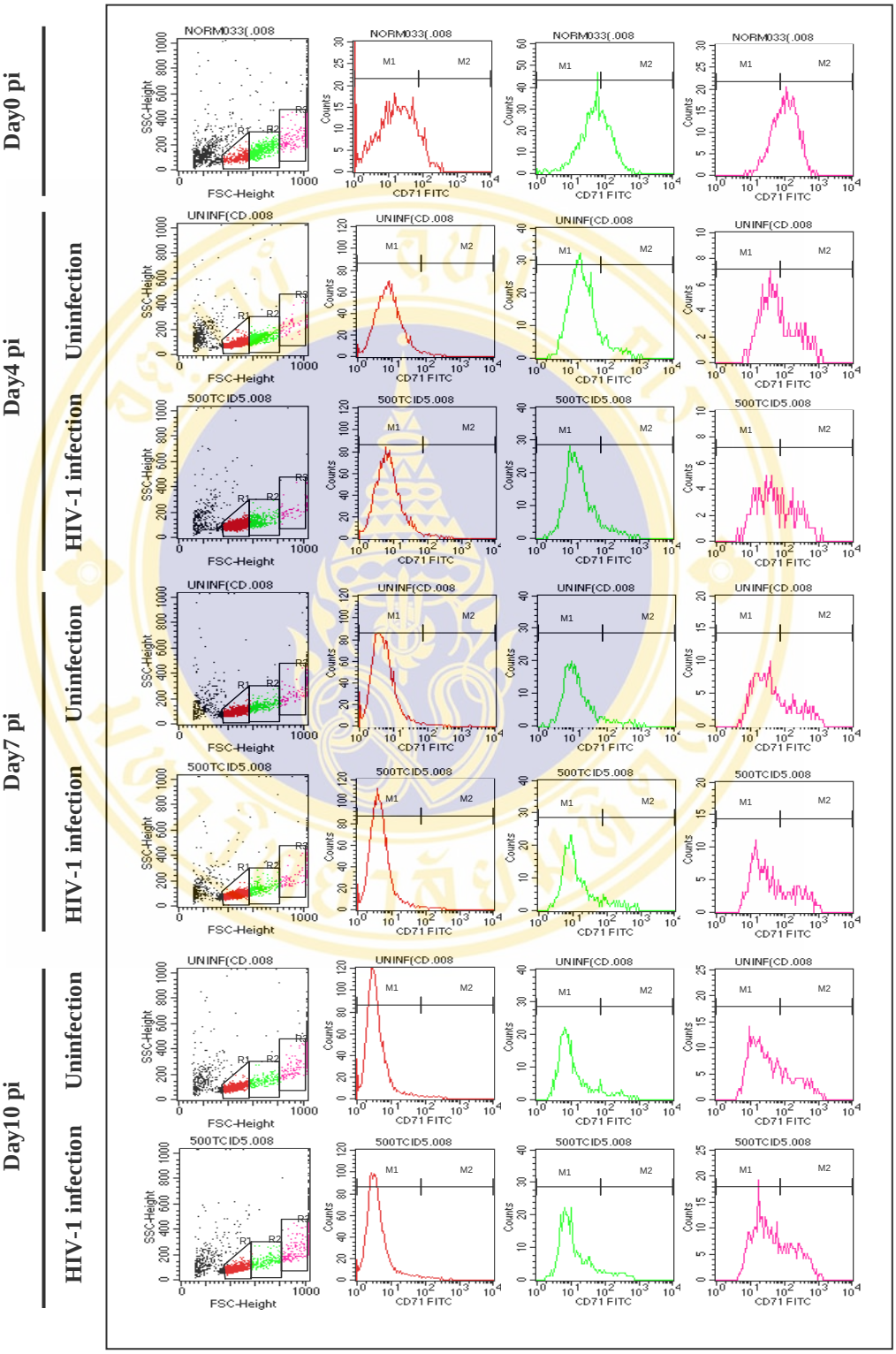
a.



b.



c.



Gate1 **Gate2** **Gate3**

CD71

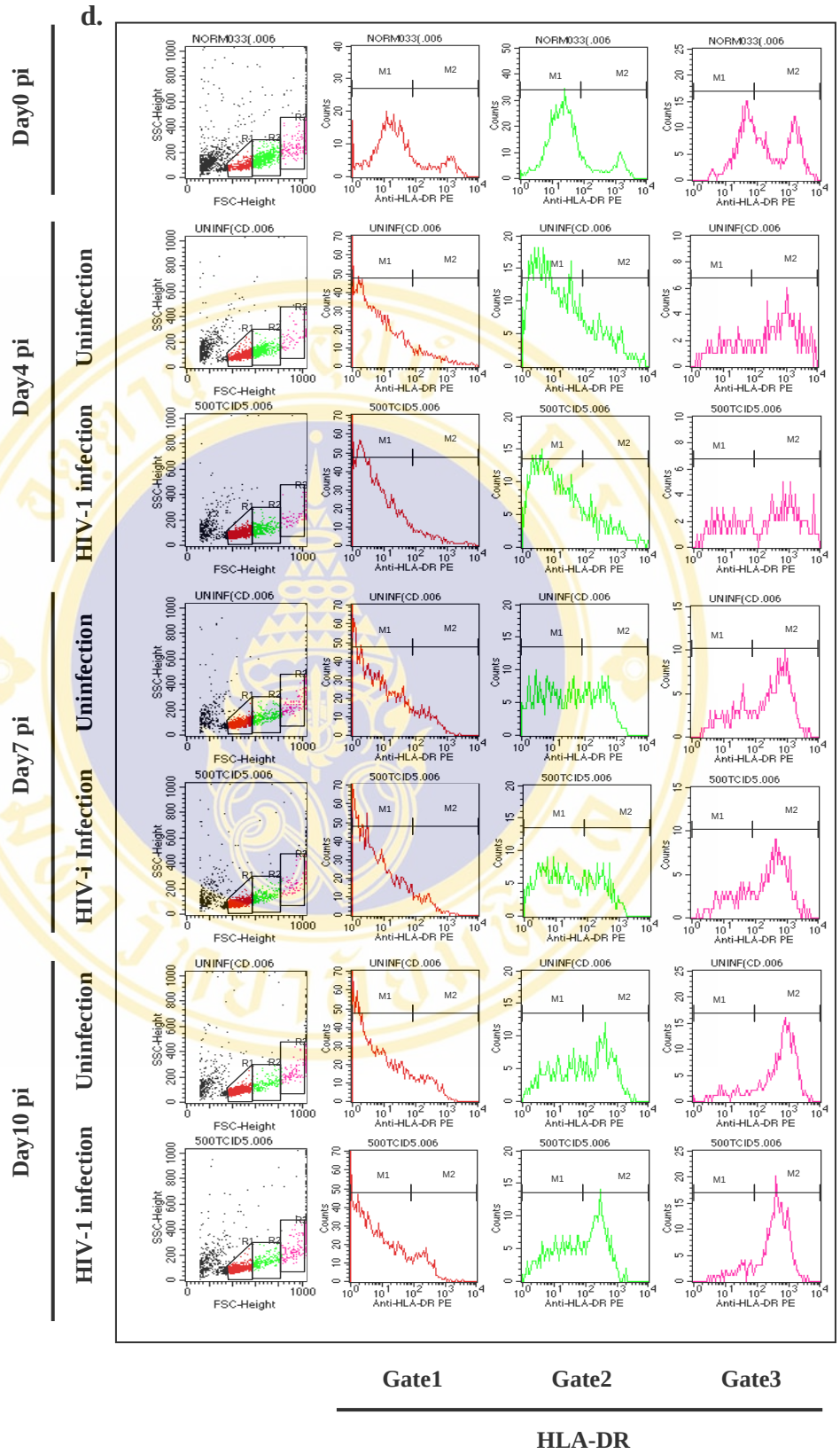


Figure 25. Flow cytometry analysis of a) CD25, b) CD69, c) CD71, and d) HLA-DR expression in lymphocyte gate on both uninfected and HIV-1 infected PBMC during the period of HIV-1 infection.

5.1 Determination of CCR5 changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, the percentage of CCR5 (Tables 7, 8A) in gate 1 (small T lymphocytes) significantly increased on day0 versus day4 (p=0.038), day0 versus day7 (p=0.008), day0 versus day10 (p=0.028), and day4 versus day10 (p=0.017). Similarly, significant increase of the CCR5 expression in gate 2 (intermediate T lymphocytes) was observed on day0 versus day4 (p=0.038), day0 versus day7 (p=0.028), day0 versus day10 (p=0.011). However, no significant difference was observed on gate 3 (large T lymphocytes) of uninfected PBMC and all gates of HIV-1 infected PBMC.

5.2 Determination of CXCR4 changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, the percentage of CXCR4 (Tables 7, 8B) in gate 1 significantly decreased on day4 versus day7 (p=0.028) and day4 versus day10 (p=0.008). In gate 2, the percentage of CXCR4 significantly increased on day0 versus day4 (p=0.038), while significant decline was observed on day4 versus day10 (p=0.008) and day7 versus day10 (p=0.011). However, no significant difference was observed on gate 3 of uninfected PBMC. On HIV-1 infected PBMC, HIV-1 infection induced significant decline in the percentage of CXCR4 in all gates. In addition, significant decline in gate 1 was observed on day4 pi versus day10 pi (p=0.008) and day7 pi versus day10 pi (p=0.008). In gate 2, HIV-1 infection induced significant decrease in the percentage of CXCR4 expression on day4 pi versus day10 pi (p=0.008) and day7 pi versus day10 pi (p=0.008). Similarly, significant decline in gate 3 was observed on day4 pi versus day10 pi (p=0.038) and day7 pi versus day10 pi (p=0.021).

Table 7. Expression of chemokine receptors and activation markers divided into three gates during the period of HIV-1 infection.

Chemokine receptors & activation markers	Gate	Uninfection* (%)				HIV-1 infection* (%)		
		Day0	Day 4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
CCR5 (n=10)	Gate1	1.11±1.24	2.39±2.15	4.99±2.70	3.69±2.67	1.68±1.65	3.94±2.60	4.12±2.06
	Gate2	1.49±1.64	3.81±3.39	4.79±4.71	5.96±2.78	3.03±2.76	2.86±2.10	6.11±4.33
	Gate3	2.80±3.95	6.90±7.36	4.93±3.73	3.37±2.61	5.79±5.29	3.21±1.83	5.08±4.02
CXCR4 (n=10)	Gate1	60.28±27.09	75.94±13.08	67.18±17.59	52.08±17.60	76.65±13.59	70.25±18.99	30.91±14.99
	Gate2	63.48±24.58	78.90±15.68	74.23±17.98	59.55±19.13	79.54±14.25	73.92±20.38	56.22±22.56
	Gate3	86.74±7.84	88.44±9.81	83.73±8.86	71.61±26.38	88.56±10.39	84.67±12.80	67.42±34.25
CD25 (n=9)	Gate1	80.57±15.69	61.61±24.03	27.32±11.00	13.73±9.42	60.85±23.65	25.61±8.50	11.41±8.61
	Gate2	90.13±12.10	64.77±20.44	47.23±20.81	25.36±14.60	64.34±18.80	46.75±18.59	23.81±13.06
	Gate3	96.09±5.46	83.22±17.52	70.00±23.86	52.10±20.86	83.97±15.50	69.36±21.56	50.84±18.29
CD69 (n=9)	Gate1	22.96±17.24	9.74±12.70	20.82±17.41	22.89±21.84	10.35±13.39	20.43±18.74	17.31±13.76
	Gate2	16.14±11.60	15.10±16.03	25.79±20.65	20.65±12.21	17.03±21.63	26.24±23.55	20.73±12.15
	Gate3	43.39±37.54	22.59±23.30	31.50±17.58	32.38±25.59	23.38±22.39	35.69±24.33	30.24±21.83
CD71 (n=9)	Gate1	69.18±16.63	3.13±2.29	2.25±1.98	4.79±4.38	3.20±2.38	2.22±1.42	4.92±4.69
	Gate2	89.42±14.09	11.82±3.80	13.36±9.61	12.95±5.38	19.40±24.61	14.19±8.19	13.21±5.20
	Gate3	93.29±12.79	34.51±12.59	39.60±18.93	23.35±12.00	31.01±12.57	42.18±16.64	26.22±12.45
HLA-DR (n=9)	Gate1	17.58±13.11	8.48±5.85	22.05±13.15	28.77±16.54	8.34±5.95	22.91±15.32	31.98±17.79
	Gate2	17.85±11.12	21.09±13.75	46.81±20.68	54.84±21.21	20.54±11.98	48.94±22.14	59.73±20.75
	Gate3	43.76±17.21	58.94±19.93	82.43±14.52	82.45±18.35	58.22±18.85	82.77±13.73	87.14±11.55

* mean ± SD; gate1 = small T lymphocyte; gate2 = intermediate T lymphocyte; gate3 = large T lymphocyte

Table 8. Statistical comparison of chemokine receptors CCR5 (A) and CXCR4 (B) that are divided into three gates during the course of HIV-1 infection.

A. CCR5

			Statistical comparison					
		Day	Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	0.038	0.008	0.028			
		Day4		0.017	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS
Gate2	Uninfection	Day0	0.038	0.028	0.011			
		Day4		NS	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS
Gate3	Uninfection	Day0	NS	NS	NS			
		Day4		NS	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS

B. CXCR4

			Statistical comparison					
		Day	Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	NS	NS	NS			
		Day4		0.028	0.008	NS		
		Day7			NS		NS	
		Day10						0.024
	HIV-1 infection	Day4 pi					NS	0.008
		Day7 pi						0.008
Gate2	Uninfection	Day0	0.038	NS	NS			
		Day4		NS	0.008	NS		
		Day7			0.011		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	0.008
		Day7 pi						0.008
Gate3	Uninfection	Day0	NS	NS	NS			
		Day4		NS	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	0.038
		Day7 pi						0.021

n=10; NS = no significance; gate1 = small T lymphocyte; gate2 = intermediate T lymphocyte; gate3 = large T lymphocyte

5.3 Determination of CD25 changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, the percentage of CD25 (Tables 7, 9A) in gate 1 showed significant decrement on day0 versus day7 (p=0.008) day0 versus day10 (p=0.008), day4 versus day7 (p=0.008), day4 versus day10 (p=0.008), and day7 versus day10 (p=0.008). Significant decline of CD25 expression in gate 2 was observed on day0 versus day4 (p=0.021), day0 versus day7 (p=0.008), day0 versus day10 (p=0.008), day4 versus day7 (p=0.011), day4 versus day10 (p=0.008), and day7 versus day10 (p=0.008). Significant decrease of CD25 in gate 3 was observed on day0 versus day4 (p=0.038), day0 versus day7 (p=0.008), day0 versus day10 (p=0.008), day4 versus day7 (p=0.021), day4 versus day10 (p=0.008), and day7 versus day10 (p=0.011).

In HIV-1 infected proliferative lymphocytes, the percentage of CD25 in gate 1 significantly decreased on day4 pi versus day7 pi (p=0.008), day4 pi versus day10 pi (p=0.008), and day7 pi versus day10 pi (p=0.008). Significant decline was found in gate 2 of day4 pi versus day7 pi (p<0.011), day4 pi versus day10 pi (p=0.008), and day7 pi versus day10 pi (p=0.008). In addition, the largest size of CD25 proliferative lymphocytes in gate 3 also significantly decreased on day4 pi versus day7 pi (p=0.028), day4 pi versus day10 pi (p=0.008), and day7 pi versus day10 pi (p=0.011).

5.4 Determination of CD69 changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, the percentage of CD69 (Tables 7, 9B) in gate 1 showed significant decrement on day0 versus day4 (p=0.021), while significant increment was observed on day4 versus day10 (p=0.008), and day7 versus day10 (p=0.008). Significant increment of CD69 expression in gate 2 was observed on day4 versus day7 (p=0.015). In HIV-1 infected PBMC, the percentage of CD69 in gate 1 significantly increased on day4 pi versus day7 pi (p=0.008). Significant increment was found in gate 2 of day4 pi versus day7 pi (p=0.008). However, no significant difference was observed in the percentage of CD69 on both uninfected and HIV-1 infected PBMC in gate 3.

Table 9. Statistical comparison of activation markers CD25 (A), CD69 (B), CD71 (C), and HLA-DR (D), that are divided into three gates during the course of HIV-1 infection.

A. CD25

			Statistical comparison					
		Day	Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	NS	0.008	0.008			
		Day4		0.008	0.008	NS		
		Day7			0.008		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	0.008
		Day7 pi						0.008
Gate2	Uninfection	Day0	0.021	0.008	0.008			
		Day4		0.011	0.008	NS		
		Day7			0.008		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.011	0.008
		Day7 pi						0.008
Gate3	Uninfection	Day0	0.038	0.008	0.008			
		Day4		0.021	0.008	NS		
		Day7			0.011		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.028	0.008
		Day7 pi						0.011

B. CD69

			Statistical comparison					
		Day	Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	0.021	NS	NS			
		Day4		0.008	0.008	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	NS
		Day7 pi						NS
Gate2	Uninfection	Day0	NS	NS	NS			
		Day4		0.015	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	NS
		Day7 pi						NS
Gate3	Uninfection	Day0	NS	NS	NS			
		Day4		NS	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS

C. CD71

		Day	Statistical comparison					
			Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	0.008	0.008	0.008			
		Day4		NS	NS	NS		
		Day7			0.021		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS
Gate2	Uninfection	Day0	0.008	0.008	0.008			
		Day4		NS	NS	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	NS
		Day7 pi						NS
Gate3	Uninfection	Day0	0.008	0.008	0.008			
		Day4		NS	0.008	NS		
		Day7			0.011		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					NS	0.028
		Day7 pi						0.011

D. HLA-DR

		Day	Statistical comparison					
			Uninfected PBMC			HIV-1 infected PBMC		
			Day4	Day7	Day10	Day4 pi	Day7 pi	Day10 pi
T lymphocytes								
Gate1	Uninfection	Day0	NS	NS	NS			
		Day4		0.008	0.008	NS		
		Day7			0.038		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	0.008
		Day7 pi						0.011
Gate2	Uninfection	Day0	NS	0.011	0.011			
		Day4		0.008	0.008	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	0.008
		Day7 pi						0.028
Gate3	Uninfection	Day0	NS	0.008	0.008			
		Day4		0.008	0.008	NS		
		Day7			NS		NS	
		Day10						NS
	HIV-1 infection	Day4 pi					0.008	0.008
		Day7 pi						NS

n=9; NS = no significance; gate1 = small T lymphocyte; gate2 = intermediate T lymphocyte; gate3 = large T lymphocyte

5.5 Determination of CD71 changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, significant decline of CD71 expression (Tables 7, 9C) in gate 1 was observed on the day comparison between day0 versus day4 (p=0.008), day0 versus day7 (p=0.008), and day0 versus day10 (p=0.008). While the percentage of CD71 significantly increased on day7 versus day10 (p=0.021). The level of CD71 in gate 2 decreased on day0 versus day4 (p=0.008), day0 versus day7 (p=0.008), and day0 versus day10 (p=0.008). Similarly, CD71 expression in gate 3 significantly decreased on day0 versus day4 (p=0.008), day0 versus day7 (p=0.008), day0 versus day10 (p=0.008), day4 versus day10 (p=0.008), and day7 versus day10 (p=0.011).

In contrast, HIV-1 infection did not induce any significant differences in the percentage of CD71 proliferative lymphocytes in gate 1 and gate 2. Significant decline in gate 3 was observed on both day4 pi versus day10 pi (p=0.028) and day7 pi versus day10 pi (p=0.011).

5.6 Determination of HLA-DR changes on PBMC in each gate during the period of HIV-1 infection

In uninfected PBMC, the significant increment of HLA-DR expression (Tables 7, 9D) in gate 1 was found on day4 versus day7 (p=0.008), day4 versus day10 (p=0.008), and day7 versus day10 (p=0.038). The percentage of HLA-DR in gate 2 significantly increased on day0 versus day7 (p=0.011), day0 versus day10 (p=0.011), day4 versus day7 (p=0.008), and day4 versus day10 (p=0.008). Significant increase of HLA-DR in gate 3 was observed on day0 pi versus day7 pi (p=0.008), day0 versus day10 (p=0.008), day4 versus day7 (p=0.008), and day4 versus day10 (p=0.008).

In HIV-1 infected PBMC, the result of HLA-DR in gate 1 significantly increased on day4 pi versus day7 pi (p=0.008), day4 pi versus day10 pi (p=0.008), and day7 pi versus day10 pi (p=0.011). Similarly, HLA-DR expression in gate 2 was induced significant increment by the period of HIV-1 culture on day4 pi versus day7 pi (p=0.008), day4 pi versus day10 pi (p=0.008), and day7pi versus day10 pi (p=0.028). The percentage of HLA-DR in gate 3 also significantly increased between the day comparison of day4 pi versus day7 pi (p=0.008) and day4 pi versus day10 pi (p=0.008).

5.7 Determination of chemokine receptor and activation marker changes in each gate comparison between uninfected and HIV-1 infected PBMC on days 4 pi, 7 pi, and 10 pi

The data from flow cytometry was divided into three gates by the size of proliferative lymphocytes. The percentage of chemokine receptors (Figure 24) and activation markers (Figure 25) of each gate was analyzed and compared between uninfected and HIV-1 infected PBMC that was observed on days 4 pi, 7 pi, and 10 pi.

Significant decline of CXCR4 (Tables 7, 8B) expression was observed in gate 1 of day10 pi. However, HIV-1 infection did not induce any significant differences in the percentage of CCR5 (Tables 7, 8A), CD25 (Tables 7, 9A), CD69 (Tables 7, 9B), CD71 (Tables 7, 9C), and HLA-DR (Tables 7, 9D) on days 4, 7, and 10 post HIV-1 infection.

6. Expression of chemokine receptors and activation markers and p24 antigen on T lymphocyte gate after infection with CRF01_AE in Thai donor cells

6.1 Correlation of flow cytometric percent intracellular p24 determination and chemokine receptors on both day7 pi and day10 pi

No significant differences were observed for the correlation of flow cytometric percent intracellular p24 (Figure 26) determination and chemokine receptors on both day7 pi and day10 pi (Tables 10-11, Figures 24,25).

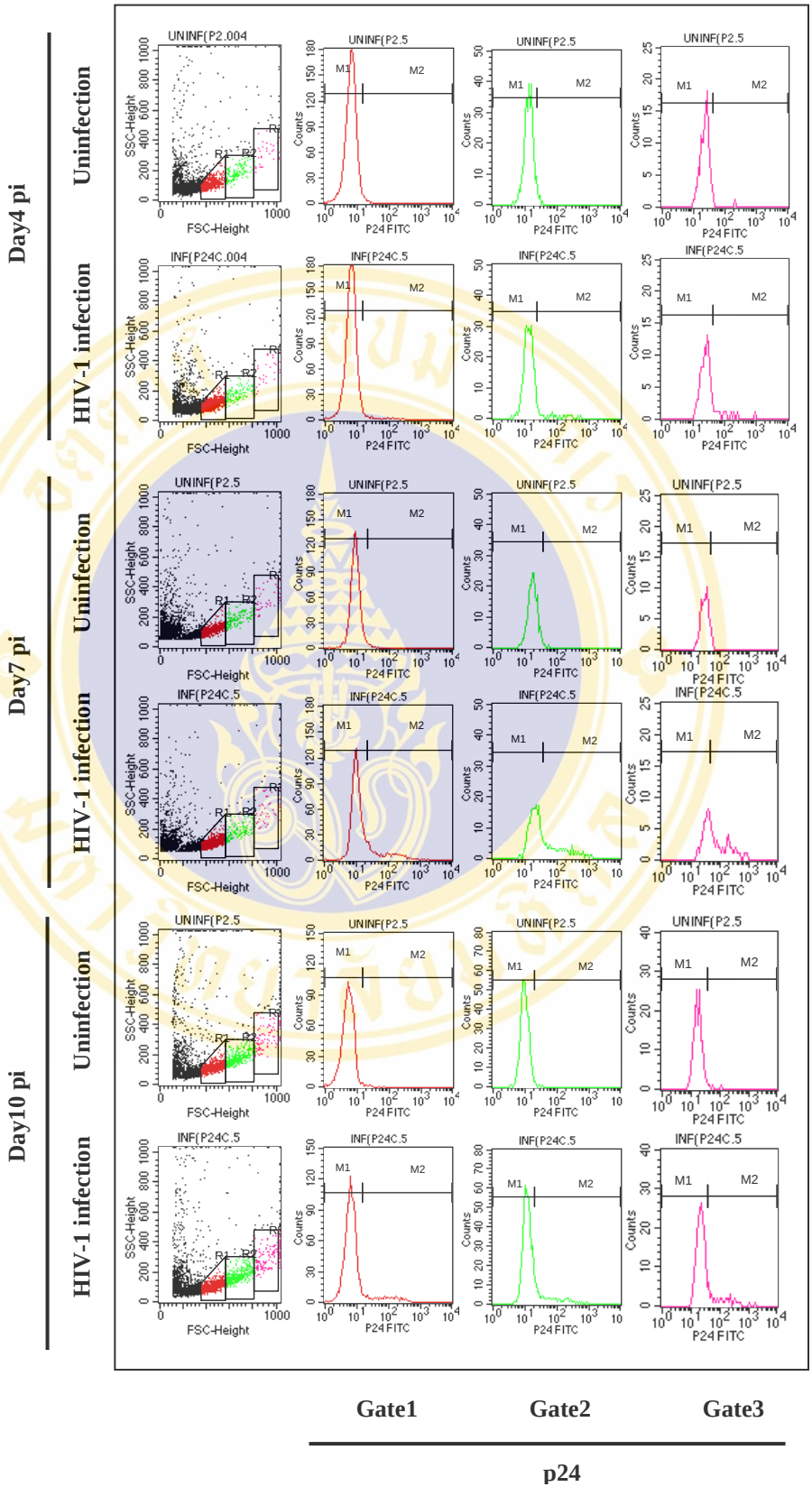


Figure 26. Flow cytometry analysis of percent intracellular p24+ cells in lymphocyte gate during the period of HIV-1 infection.

Table 10. Expression of chemokine receptors, activation markers and percent intracellular p24 antigen divided into three gates during the course of HIV-1 infection.

Chemokine receptors, activation markers & p24	Gate	HIV-1 infection (Mean ± SD)	
		Day7 pi	Day10 pi
CCR5	Gate1	4.33 ± 2.87	3.52 ± 1.93
	Gate2	2.87 ± 2.43	5.32 ± 4.64
	Gate3	2.71 ± 1.75	4.25 ± 4.04
CXCR4	Gate1	64.60 ± 17.65	36.34 ± 12.04
	Gate2	71.22 ± 22.64	53.54 ± 25.31
	Gate3	81.63 ± 13.03	62.29 ± 37.68
CD25	Gate1	27.81 ± 8.29	12.34 ± 9.71
	Gate2	52.85 ± 16.24	24.54 ± 14.94
	Gate3	75.34 ± 20.30	55.18 ± 18.60
CD69	Gate1	18.88 ± 20.91	12.04 ± 9.77
	Gate2	25.81 ± 27.14	19.55 ± 13.63
	Gate3	33.88 ± 27.69	25.51 ± 22.71
CD71	Gate1	2.53 ± 1.47	5.88 ± 4.94
	Gate2	16.34 ± 8.07	14.76 ± 4.71
	Gate3	47.39 ± 15.01	27.42 ± 13.97
HLA-DR	Gate1	27.63 ± 13.99	37.40 ± 16.35
	Gate2	55.44 ± 20.78	62.57 ± 23.05
	Gate3	85.10 ± 14.87	86.24 ± 13.14
p24	Gate1	3.43 ± 2.94	4.14 ± 2.79
	Gate2	6.29 ± 4.64	5.86 ± 3.39
	Gate3	10.00 ± 9.06	7.00 ± 3.83

n=7

Table 11. Correlation among percent intracellular p24, chemokine receptors and activation markers in lymphocyte gate.

Chemokine receptors & activation markers	p24 (%)			
	day7 pi		day10 pi	
	R	*p* value	R	*p* value
Chemokine receptors				
CCR5	-0.182	0.425	0.184	0.425
CXCR4	0.023	0.921	0.251	0.272
Activation markers				
CD25	0.467	0.033	0.158	0.494
CD69	-0.153	0.508	0.357	0.112
CD71	0.369	0.099	0.309	0.173
HLA-DR	0.454	0.039	0.313	0.167

6.2 Correlation of flow cytometric percent intracellular p24 determination and activation markers on day7 and day10 pi

On day7, although not significant, a slight but consistent correlation was observed between %p24 and CD71 (R=0.369, *p*=0.099) (Table 11 and Figure 27), while direct correlation between %p24 and CD25 (R=0.467, *p*=0.033) was significantly found (Figure 28). Moreover, significant direct correlation between %p24 and HLA-DR (R=0.454, *p*=0.039) was also observed (Figure 29). However, no statistical significance was found for the direct correlation between the percentage of p24 and all four activation markers on day10 pi.

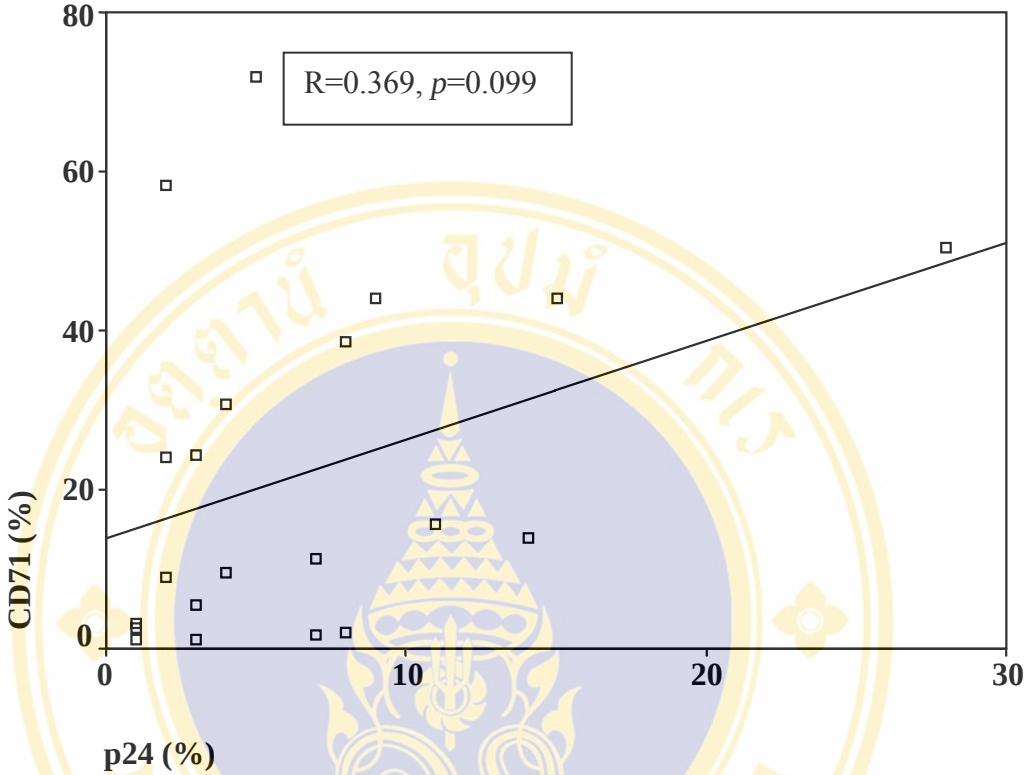


Figure 27. Correlation between intracellular p24 antigen and CD71 in lymphocyte gate on day7 post infection. The percentage of intracellular p24 and surface marker CD71 of infected PBMC were quantitated using flow cytometry. (The data obtained were analyzed using Pearson correlation method, n=7).

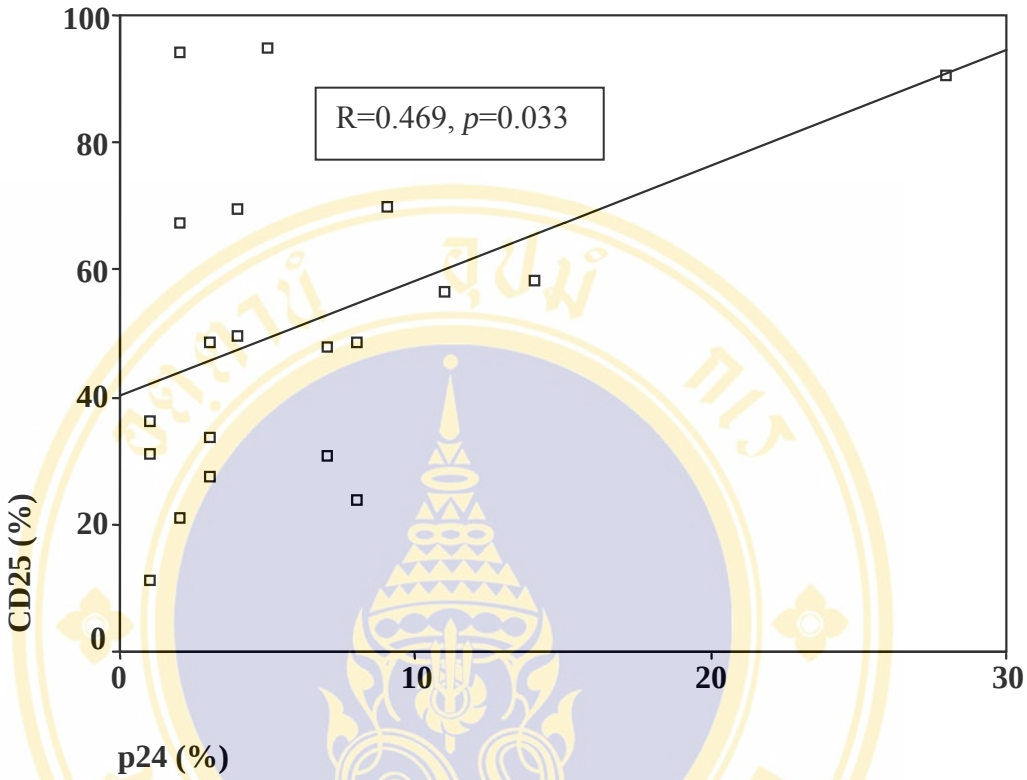


Figure 28. Correlation between intracellular p24 antigen and CD25 in lymphocyte gate on day7 post infection. The percentage of intracellular p24 and surface marker CD25 of infected PBMC were quantitated using flow cytometry. (The data obtained were analyzed using Pearson correlation method, n=7).

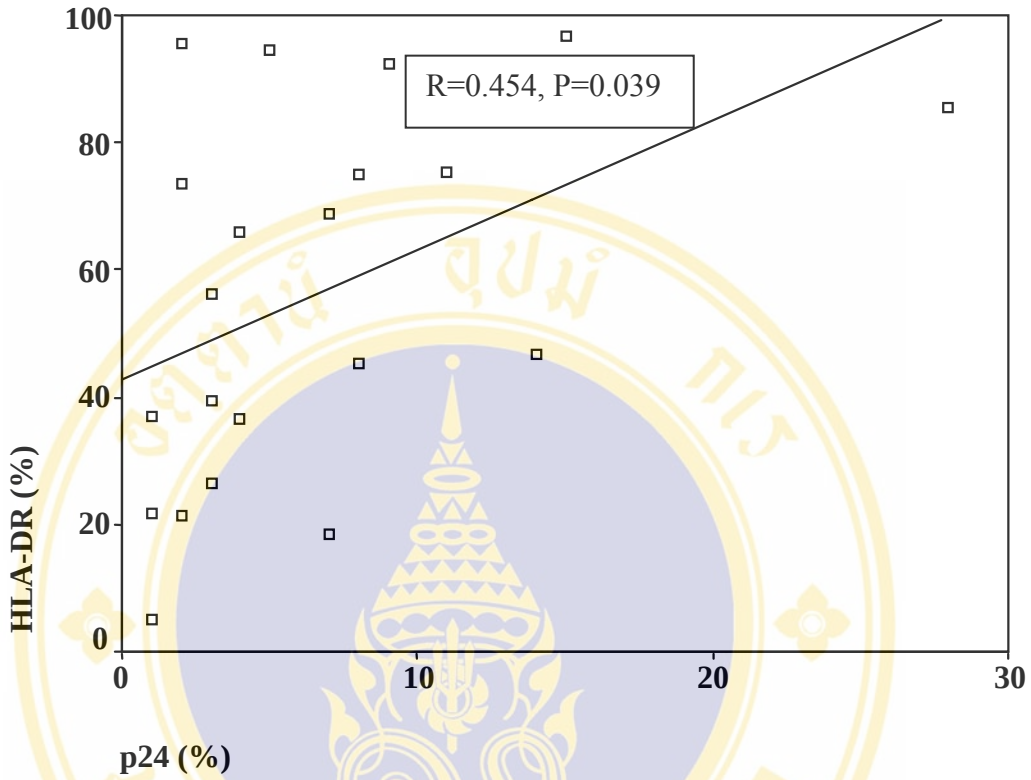


Figure 29. Correlation between intracellular p24 antigen and HLA-DR in lymphocyte gate on day7 post infection. The percentage of intracellular p24 and surface marker HLA-DR of infected PBMC were quantitated using flow cytometry. (The data obtained were analyzed using Pearson correlation method, n=7).

7. Correlation of flow cytometric percent intracellular p24 determination and ELISA extracellular measurement

Data and graphs between p24 antigen (ng/ml) versus percent intracellular p24+ cells of CRF01_AE infection in seven Thai donors at day7 and day10 pi were shown in Table 12 and Figures 29-30. For HIV-1 infection at day 7 pi, direct correlation between p24 antigen (ng/ml) and %p24+ cells (R=0.929, *p*=0.002) was significantly found (Figure 30). Similarly, significant direct correlation was observed between p24 antigen (ng/ml) and %p24+ cells at day10 pi (R=0.871, *p*=0.011) (Figure 31).

Table 12. Results of flow cytometric percent intracellular p24 determination and ELISA extracellular measurement.

DONOR No.	Day7 pi		Day10 pi	
	ELISA (ng/ml)	Intracellular p24 (%)	ELISA (ng/ml)	Intracellular p24 (%)
026	12000	6	29600	5
027	2750	1	13600	2
028	32400	8	44500	7
029	6600	2	26000	4
031	4800	2	3750	1
032	36500	9	50750	5
034	16000	3	37000	6

n=7

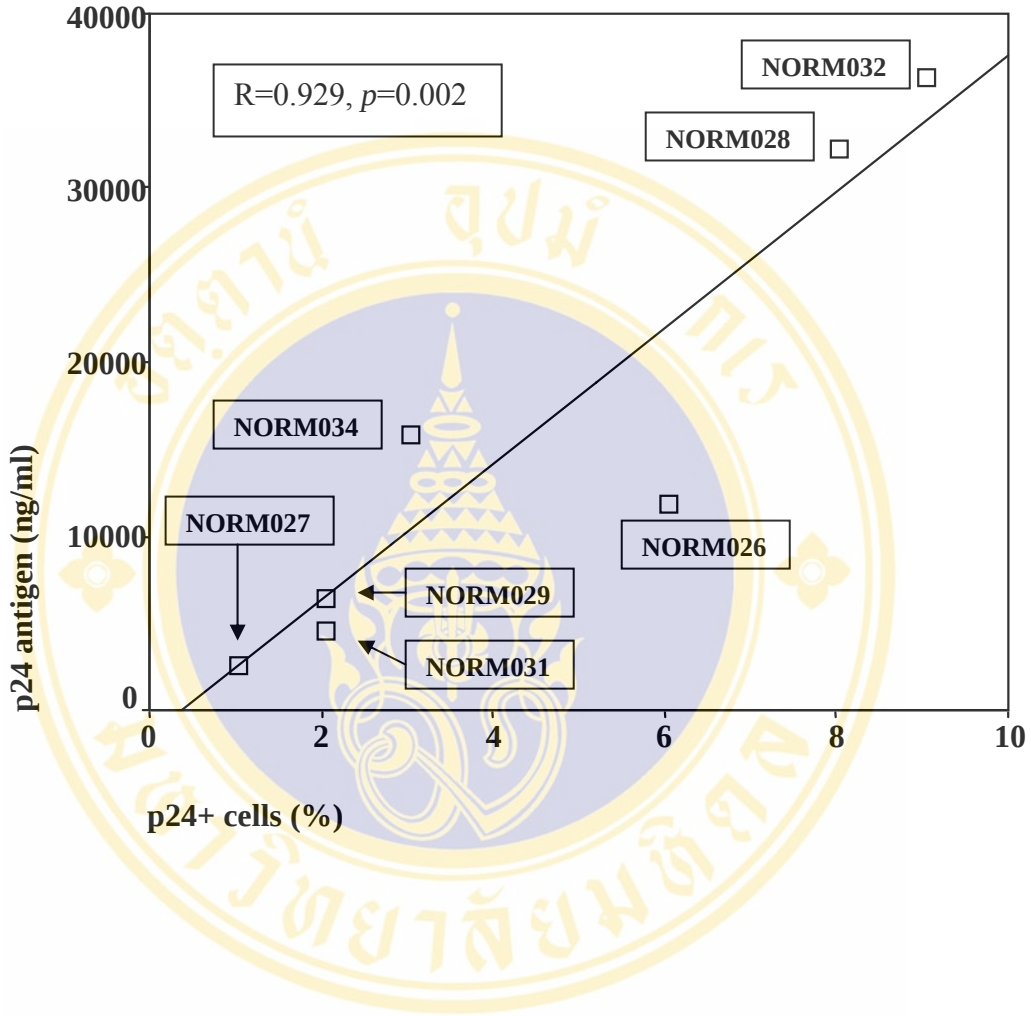


Figure 30. Correlation between intracellular p24 cells and p24 antigen (ng/ml) on day7 post infection. Intracellular and extracellular p24 of infected PBMC were measured using flow cytometry and ELISA, respectively. (The data obtained were analyzed using Pearson correlation method, n=7).

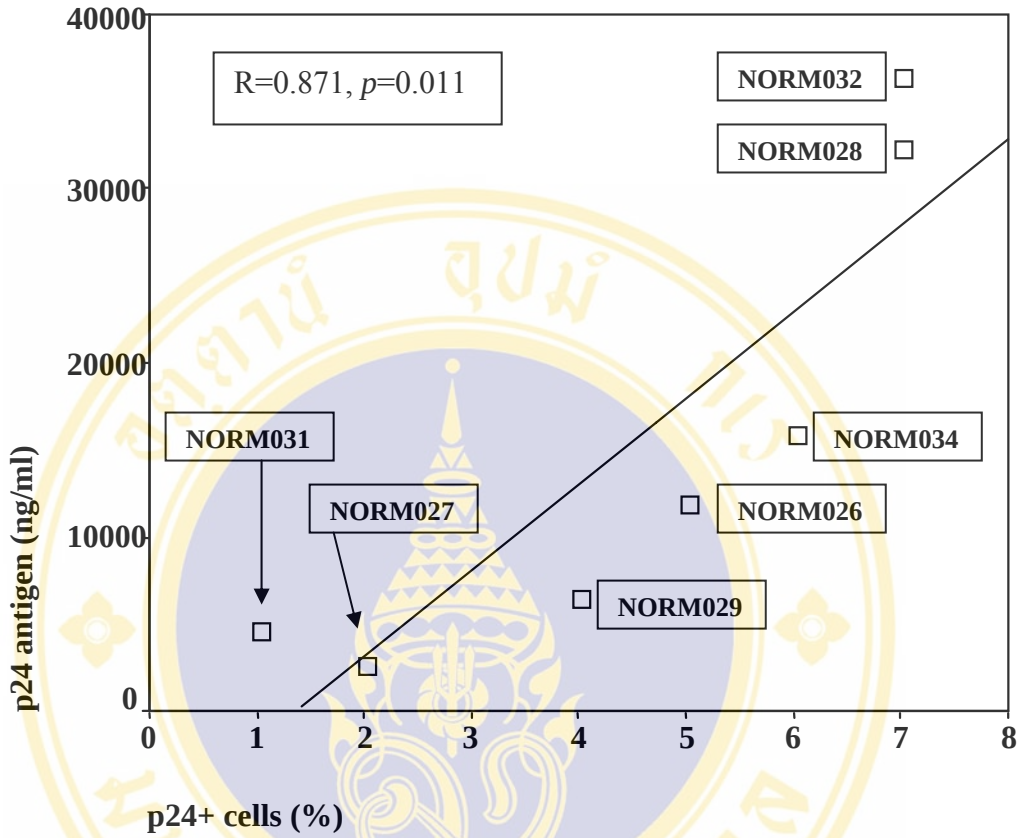


Figure 31. Correlation between intracellular p24 cells and p24 antigen (ng/ml) on day10 post infection. Intracellular and extracellular p24 of infected PBMC were measured using flow cytometry and ELISA, respectively. (The data obtained were analyzed using Pearson correlation method, n=7).

CHAPTER VI
DISCUSSION

In addition to CD4 molecule as primary binding of HIV-1 gp120, productive HIV-1 infection requires a chemokine receptor as coreceptor for entering its target cell. The selective use of the CCR5 or CXCR4 coreceptors is the molecular explanation of the previous phenotypic categorization of HIV-1 isolates (88, 93, 116). CCR5 is the principal co-receptor for HIV-1 variants that are sexually transmitted, usually found since the early infection to symptomatic phase and persist within the majority of infected individuals (R5 isolates). The appearance of variants that use CXCR4 or both coreceptors (X4 and R5X4 isolates) signals accelerated CD4+ T-cell loss and disease progression (25, 117-119). The phenotypic switch from R5 to X4 viruses *in vivo* typically occurs only after several years of infection. Surprisingly, this is slow given that changing only a few residues in gp120 can be sufficient to convert an R5 virus into an R5X4 virus *in vitro* and that such changes have to occur continuously giving the error rate of reverse transcription (120-122). The reason for the selective growth of the R5 strain is not known, but could reflect a replication advantage of R5 viruses over X4 viruses in CD4+ cells. It should be noted that the use of chemokine and its derivatives have to keep in mind that it may accelerate selective pressure to use the more virulent coreceptor (CXCR4). In this study we used R5 virus (NSI/E) to evaluate the levels of CCR5 and CXCR4 expression on uninfected and infected CD4+ and CD8+ T cells by using cryopreserved PBMC. Our results showed that response of cryopreserved PBMC samples from healthy individuals to PHA stimulation can be monitored by flow cytometric assay that is detecting the *in vitro* expression of chemokine receptors (CCR5 and CXCR4) and activation markers (CD25, CD69, CD71, and HLA-DR) on the cell surface. After PHA stimulation, all activation markers and chemokine receptors were significant increase except the percentage of CCR5 was downregulated, which similar to Bleul's group (123). This

may be explained by interleukin 2, that was normally used to stimulate the cells, can downregulate CCR5 expression (124), and the amount of CCR5 on PBMC from each donor and freeze-thaw effect (125). Moreover, it has been known that CD8+ T lymphocytes are involved in the control of HIV infection *in vivo*, either by cytolytic mechanisms or by release of HIV-suppressive factors. The chemokines (RANTES, MIP-1 lα, and MIP-1β) (80, 97, 126) and CD8 antiviral factor (CAF) (127-129) were identified as the major HIV-suppressive factors secreted by CD8+ T cells. Similarly, these chemokines can be observed in HIV culture and may bind to CCR5 molecules. Therefore, these may effect to the expression of CCR5 as well as blocking cellular entry of HIV. In addition, a large range of standard deviation (SD) of both unstimulated and PHA-stimulated PBMC may effect from the different efficacy of individual to express the receptor. From this study, *p* value of CCR5 in CD4+ T cells is 0.074, which is not significant difference. The high variation of SD after PHA stimulation may affect the experiment. The other chemokine receptor, CXCR4, and activation markers (CD25, CD69, CD71, and HLA-DR) were significantly increased. These findings support previous studies that showed significant increment in CXCR4 (123), and activation markers (100).

To compare the expression level of chemokine receptors and activation markers between uninfected and infected PBMC, HIV-1 was added to each PHA-stimulated PBMC. A slight but consistent decrease in the expression of chemokine receptors (CCR5 and CXCR4) and so with the activation markers (CD25, CD69, CD71, and HLA-DR) were observed in days 4 pi, 7 pi, and 10 pi. This lower amount of chemokine receptors and activation markers seen in infected PMBC than uninfected PBMC might be due to HIV infection because CD4+ cells are the targets of HIV infection. However, these were not observed on CD8+ cells. These results show that HIV can affect only CD4+ T cells and this organism causes downregulation in the expression level of CCR5, CXCR4, CD25, CD69, CD71, and HLA-DR on CD4+ T cells.

To compare the expression level of chemokine receptors during the period of HIV-1 culture, the results were compared within uninfected and infected PBMC between each day. The rate of CCR5+ T cells increased on both uninfected and

infected PBMC. More specifically, significant increase was observed on both CD4+ T cells and CD8+ T cells. The rate of CXCR4 decreased on infected CD4+ T cells but it increased on CD8+ T cells along with CD69 and HLA-DR. The increase in expression level of CXCR4, CD69, and HLA-DR on CD8+ T cells may be the result of the mechanism to get rid of HIV infected CD4+ T cell as target cell of HIV infection. After HIV infection, some chemokine receptors are used as its coreceptor. In addition, CD4+ T cells as HIV-target cells may be destroyed by HIV infection. While CD8+ T cells have hardly disturbed by this. The level of CD25 expression decreased from day0 pi to day10 pi. Locher and coworkers found that R5 and X4 viruses were found to reduce the level of proliferation and so with the majority of the activation markers in CD4+ T cells that were stimulated with interleukin 2 (IL-2) (130). Similar to transferrin receptor, CD71, expression decreased during the period of HIV-1 infection on both uninfected and HIV-1 infected of CD4+ and CD8+ T cells.

According to activation level, T cell population was divided into three gates using cell size (small, intermediate, and large). The level of activation markers and chemokine receptors in small T cells were lower than in intermediate size T cells and the highest in large size population. A slight but consistent increase was observed in the percentage of CD71, CD69, and CXCR4. Moreover, significant increase was found in the percentage of CD25 and HLA-DR. The results suggested that the expression of chemokine receptors and activation markers could be increased by maturation of these cells. Direct correlation was found between cell size and percentage of intracellular p24 antigen. The results indicated that HIV has more efficient to infect the high level of T cell activation than the low level of T cell activation.

HIV-1, which causes a progressive breakdown of immunity can be quantified by several different assays. In addition, CD4+ T cell counts, which usually determine the stage of disease, and plasma viral load (VL), which reflects active ongoing viral replication, are used to predict disease progression (131, 132). The impairment of T cell function provides a separate measurement. Impairment occurs early in HIV-1 infection, when CD4+ T cell counts are still within normal ranges, and decreases further as disease progresses (21, 133). The proliferative responses to mitogens, such

as pokeweed (PWM) or phytohemagglutinin (PHA), and to anti-CD3 plus anti-CD28 antibodies are sensitive prognostic markers for AIDS (21, 134). Moreover, PHA-stimulated PBMC was used for this thesis. The altered expression of lymphocyte surface antigens also reflects the dynamic interaction between the immune system and HIV-1. The surface expression of HLA-DR is significantly increased and predicts the progression to AIDS in adults (21, 110). Resino and coworkers found positive correlation between viral load and HLA-DR expression on CD4+ T cells (111). Similarly, we found the positive correlation between the percentage of HLA-DR and the percentage of intracellular p24 antigen on the intermediate and large CD4+ T cells. These may link to the progress of the disease; therefore, HLA-DR may be a sensitive prognostic marker for HIV-1 infected individuals.

All living organisms, from the smallest to the largest ones (microorganisms, plants, and animals) need iron as essential growth mineral and have elaborate mechanisms for its acquisition, transport and storage. Iron exists as two active forms, ferric (Fe3+) and ferrous (Fe2+), which contribute to its flexibility. Indeed, it has the ability to accept and donate electrons readily. Iron is a useful catalytic center for many cellular reactions such as energy metabolism, respiration (105) and DNA synthesis (106). For HIV-1 replication, the virus requires the presence of an iron to the up-regulation of proviral transcription (107). Furthermore, HIV -infected cells use the metal to produce viral particles (108). A direct link can then be established between iron overload and the stimulation of HIV replication (109). If HIV-infected cells required more iron to produce the new viral particles; therefore, the transferrin receptor (CD71), which is used to bind to transferrin, expression on CD4+ T cells should be increased. Similar to this recent study, we found that increase in the percentage of intracellular p24 antigen on CD4+ T cells was positively correlated with the expression level of CD71 in intermediate and large cell size.

In normal T cells, the early activation antigen, CD69, is expressed within hours after activation of T, B, NK cells, and some other cell types but is not expressed on resting lymphocytes (135-137). Krowka and coworkers found that HIV-1 infected donors had significantly lower frequencies of CD4+ and CD8+ lymphocytes expressing CD69 after stimulation with PHA in comparison to uninfected donors (23).

Similar to our studies, we found decrease in the percentage of CD69 on CD4+ T cells was observed. It is possible that HIV-1 infection can impair the function of CD4+ T cells to express CD69 antigen. Taken together with CD71, the percentage of CD69 on day10 pi was positively correlated with the percentage of intracellular p24 antigen in intermediate and large cell size.

CD25 is the part of the IL-2 receptor and it is expressed by lymphocytes within 24-48 hrs after mitogenic stimulation (100, 101, 103). The rapid expression of this activation antigen and its easy measurement by flow cytometry suggest that expression of CD25 molecule may be useful to evaluate the function of lymphocytes. Moreover, immune response in HIV-1 infected individuals was gradually impaired. Imunomodulation with IL-2 is one of the complementary treatments used in combined anti HIV-1 therapy. Administration of IL-2 is expected to improve immune responses and to activate latently infected cells; however, IL-2 may increase the pool of susceptible cells, by triggering, not only proliferation, but also expression of chemokine receptors in CD4 cells. Modern medicine used IL-2 with HAART in HIV-1 infected individuals. So the level expression of CD25 may be the prognostic marker for HIV-1 infected individuals too.

The level of HIV-1 infection was measured by two principles to detect p24 antigen. Furthermore, the two techniques of p24 measurement, flow cytometric percent intracellular p24 detection (%p24) and p24 antigen capture (ELISA) (ng/ml), were compared the level of intracellular and extracellular p24 antigen, respectively. These techniques show the same result. Therefore, flow cytometric determination of percent intracellular p24 can be used for p24 antigen detection the same as p24 antigen capture (ELISA).

CHAPTER VII
CONCLUSION

The present study comprised three major parts: 1) flow cytometric analyses to determine the expression of both chemokine receptors (CCR5 and CXCR4) and activation markers (CD25, CD69, CD71, and HLA-DR) on CD4+ T cells and CD8+ T cells from Thai normal PBMC infected with CRF01_AE on day4, day7, and day10 post infection; 2) to study the relationship between intracellular p24 antigen and level of individual activation markers and chemokine receptors on proliferative lymphocytes; 3) to compare the result of p24 antigen from intracellular p24 antigen flow cytometry and p24 antigen capture ELISA.

After PHA stimulation, all activation markers and chemokine receptors were significant increase except the percentage of CCR5 was downregulated. To compare the expression level of chemokine receptors and activation markers between uninfected and infected PBMC, HIV-1 was added to each PHA-stimulated PBMC. After HIV-1 infection, a slight but consistent decrease in the expression of chemokine receptors (CCR5 and CXCR4) and so with the activation markers (CD25, CD69, CD71, and HLA-DR) were observed in days 4 pi, 7 pi, and 10 pi. These results show that HIV can affect only CD4+ T cells and this organism causes downregulation in the expression level of CCR5, CXCR4, CD25, CD69, CD71, and HLA-DR on CD4+ T cells. To compare the expression level of chemokine receptors during the period of HIV-1 culture, the results were compared within uninfected and infected PBMC between each day. The rate of CCR5+ T cells increased on both uninfected and infected PBMC. More specifically, significant increase was observed on both CD4+ T cells and CD8+ T cells. The rate of CXCR4 decreased on infected CD4+ T cells but it increased on CD8+ T cells along with CD69 and HLA-DR. In contrast, the decreased level of CD25 expression on CD4+ T cells was observed from day0 pi to day10 pi. Similar to CD71, expression was decreased during the period of HIV-1 infection on both uninfected and HIV-1 infected of CD4+ and CD8+ T cells.

Three-color flow cytometric analysis was conducted to study the relationship between intracellular p24 antigen and level of individual activation markers and chemokine receptors on proliferative lymphocytes. According to activation level, T cell population was divided into three gates using cell size (small, intermediate, and large). The level of activation markers and chemokine receptors in small T cells were lower than in intermediate size T cells and the largest size population. A slight but consistent increase was observed in the percentage of CD71, CD69, and CXCR4. Moreover, significant increase was found in the percentage of CD25 and HLA-DR. The results suggested that the expression of chemokine receptors and activation markers could be increased by activation of these cells. Direct correlation was found between cell size and percentage of intracellular p24 antigen. The results indicated that HIV has more efficient to infect the high level of T cell activation than the low level of T cell activation.

The level of HIV-1 infection was measured by two principles to detect p24 antigen. Furthermore, the two techniques of p24 measurement, flow cytometric intracellular p24 detection and p24 antigen capture (ELISA) (%p24), were compared for the level of intracellular and extracellular p24 antigen (ng/ml), respectively. These techniques show the same result on both day7 pi ($R=0.929$, $p=0.002$) and day10 pi ($R=0.871$, $p=0.011$). Therefore, flow cytometric percent intracellular p24 detection can be used to replace for p24 antigen detection using p24 antigen capture (ELISA).

Flow cytometric analyses on CD4+ and CD8+ T cells demonstrated that there was no statistical significant difference in activation markers (CD25, CD69, CD71, and HLA-DR) and chemokine receptors (CCR5 and CXCR4) between uninfection and HIV-1 infection. In exception, a decrease in the percentage of CXCR4 on the infected CD4+ T cells was observed at day10 pi.

This research suggested that the expression of activation markers and chemokine receptors might be used as surrogate markers to use as prognostic factors for HIV infected individuals and to monitor the disease progression. Extensive study is needed in order to ascertain this point of view.

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APPENDIX

APPENDIX

A. Reagents for cell cultivation

A.1 Growth media for peripheral blood mononuclear cells (PBMC) cell line

A.1.1 5X RPMI 1640

Dissolve RPMI 1640 powder 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°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)	1.25	ml
Streptomycin (40,000 units/ml)	1.25	ml

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

A.1.3 Interleukin-2 media (IL-2 media) (100 ml)

1X RPMI 1640	78	ml
IL-2	10	ml
Fetal calf serum (FCS)	10	ml
Pen-Strep	1	ml
L-Glutamine	1	ml
Fungizone	100	μl

A.1.4 Tissue culture media (TC media) (100 ml)

1X RPMI 1640	88	ml
FCS	10	ml
Pen-Strep	1	ml
L-Glutamine	1	ml
Fungizone	100	µl

A.1.5 Wash media (100 ml)

1X RPMI 1640	96	ml
FCS	2	ml
Pen-Strep	1	ml
L-Glutamine	1	ml
Fungizone	100	µl

A.2 Cell freezing media {10% dimethylsulfoxide (DMSO) in 20% fetal bovine serum (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	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°C under pressure of 15 lbs/square inch for 15 minutes, then, store at room temperature.

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	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	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 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	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 .

C. Reagents for PBMC preparation

C.1 Standard Ficoll-hypaque

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

BIOGRAPHY

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