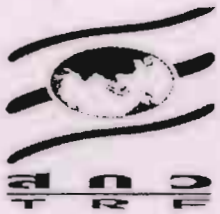

รายงานวิจัยฉบับสมบูรณ์

บทบาทของเซลล์เอ็น เค ในการสร้างสารไซโตไคน์ และคีโมไคน์ ในคนปกติ และผู้ติดเชื้อเอชไอวี

โดย

ดร.สุจินต์ อัศววิทูรทิพย์
ศ.ดร.โกวิท พัฒนาปัญญาสัตย์
ศ.ดร.พิไลพันธ์ พุธวัฒนะ

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สัญญาเลขที่ BGJ/46/2543

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โดย

ดร.สุจินต์ อัศววิทูรทิพย์ [1]
ศ.ดร.โกวิท พัฒนาปัญญาสัตย์ [2]
ศ.ดร.พิไลพันธ์ พุธวัฒนะ [1]

[1] ภาควิชาจุลชีววิทยา
[2] หน่วยเครื่องมือพิเศษเพื่อการวิจัย สถานส่งเสริมการวิจัย
คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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Sujin Assawawitoontip

ABSTRACT

Project Code : BGJ/46/2543

Project Title : Role of the NK cells in cytokine and chemokines production in HIV seronegative and seropositive subjects

Investigators : SUJIN ASSAWAWITOONTIP, Ph.D.[1], KOVIT PATTANAPANYASAT, Ph.D.[2], PILAIPAN PUTHAVATHANA, Ph.D.[1]

[1] Department of Microbiology, [2] the Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University

E-mail Address: mtsaw@mahidol.ac.th, grkpy@mahidol.ac.th, siput@mahidol.ac.th

Project Period : 2 years

This research aimed to investigate the expression of intracellular cytokine and chemokine in natural killer (NK) and $CD8^+$T lymphocytes upon stimulation with PMA and ionomycin. A three color flow cytometric analysis was used to detect intracellular cytokine and chemokine production in 10 HIV seronegative subjects and 28 HIV seropositive subjects (nine had $CD4^+$T cell count < 14%; and 19 had $CD4^+$T cell count $\geq$ 14%). Spontaneous synthesis of IFN-γ, RANTES, MIP-1α and MIP-1β without PMA and ionomycin stimulation was clearly observed in $CD8^+$T cells, but not in NK cells. Upon stimulation, both NK and $CD8^+$T cells were shown to be good sources of IFN-γ and MIP-1β production; whereas RANTES and MIP-1α were mainly produced from $CD8^+$T cells. Furthermore, this study demonstrated that number of IFN γ, RANTES, MIP-Iα and MIP-Iβ expressing $CD8^+$T and NK cells of the HIV infected subjects were not significantly different from those of the HIV seronegative subjects.

KEYWORD: CYTOKINE/ CHEMOKINE/ NK CELLS/ HIV

บทคัดย่อ

รหัสโครงการ : BJG/46/2543

ชื่อโครงการ : บทบาทของเซลล์เอ็น เค ในการสร้างสารไซโตไคน์ และคีโมไคน์ ในคนปกติ และผู้ติดเชื้อเอช ไอ วี

ชื่อนักวิจัย : สุจินต์ อัศววิทูรทิพย์, ปร.ด.[1], โกวิท พัฒนาปัญญาสัตย์, Ph.D.[2], พิไลพันธ์ พุธวัฒนะ, ปร.ด.[1]

[1]ภาควิชาจุลชีววิทยา, [2]หน่วยเครื่องมือพิเศษเพื่อการวิจัย สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

E-mail Address: siput@mahidol.ac.th, mtsaw@mahidol.ac.th, grkpy@mahidol.ac.th

ระยะเวลาโครงการ : 2 ปี

โครงการวิจัยนี้ มีวัตถุประสงค์เพื่อศึกษาความสามารถในการสร้างสารไซโตไคน์และคีโมไคน์ใน NK และ $CD8^+$T lymphocytes ด้วยการกระตุ้นด้วยสาร PMA และ ionomycin โดยวิธี flow cytometry ด้วยการย้อม 3 สี ในคนไทยที่มีสุขภาพดี ไม่มีการติดเชื้อเอชไอวี จำนวน 10 คน และในผู้ป่วยติดเชื้อเอชไอวี จำนวน 28 คน พบว่า การสังเคราะห์สาร IFN-γ, RANTES, MIP-1α, และ MIP-1β โดยปราศจากการกระตุ้นด้วยสาร PMA และ ionomycin เกิดขึ้นได้เฉพาะใน $CD8^+$T cells และจากการใช้สารกระตุ้นพบว่า ทั้ง NK และ $CD8^+$T cells นั้น เป็นแหล่งผลิตสาร IFN-γ และ MIP-1β ในขณะที่เซลล์ซึ่งสร้างสาร RANTES และ MIP-1α ส่วนใหญ่เป็น $CD8^+$T cells ยิ่งไปกว่านั้น การศึกษานี้แสดงให้เห็นว่า $CD8^+$T และ NK cells ในคนปกติ และผู้ติดเชื้อเอชไอวี ไม่มีความแตกต่างจากคนปกติในด้านความสามารถในการผลิตสาร IFN-γ, RANTES, MIP-1α, และ MIP-1β

KEYWORD: CYTOKINE/ CHEMOKINE/ NK CELLS/ HIV

CONTENTS

OBJECTIVE

This study aimed to determine the synthesis of IFN-γ, RANTES, MIP-Iα and MIP-Iβ in different lymphocyte subsets ($CD3^+T$, $CD8^+T$ and NK cells) from HIV seronegative and HIV seropositive subjects with different levels of $CD4^+T$ lymphocyte count

BACKGROUND

NK cells are one component of the innate immune system and have the ability both to lyse target cells and to provide an early source of immunoregulatory cytokines. Human NK cells are defined phenotypically by their expression of CD16, CD56 and lack of expression of CD3; and comprise about 15% of all lymphocytes (1-3). However Thai people have higher number of NK cells as compared to the Caucasians (4). NK cells are central in the innate immune response against tumor, parasites and cells infected with virus and intracellular bacteria (5, 6). Several studies have correlated high NK cell activity with the reduced susceptibility of certain individuals to HIV infection (7); and loss of NK cell activity and frequency had been correlated with HIV disease progression, particularly in individuals with opportunistic infections (8-10). As the result of the rapid mutation in HIV-specific CTL epitopes, HIV escape mutants were discovered. Moreover, Nef protein downregulates MHC class I molecules expression on HIV infected cells and escape from CTL destruction (11-13). Antigenic variation of HIV may render themselves more sensitive to NK cells than CTL mediated cytotoxicity. So NK cell may serve as a second line of defense against HIV-infected cells which evade the T cell mediated immune response as well as a protective mechanism against an array of secondary opportunistic infections and malignant manifestation of HIV infection. The NK cells display at least two effector functions that can contribute to control of infection : first, they can kill infected cells ; second, they are a rich source of inflammatory cytokines, especially IFNγ and chemokines such as RANTES, MIP-1α and MIP-1β with antiviral activity (14).

Cytokines are important mediators generated during immune response and are produced by a variety of activated cells, such as $CD8^+T$ cells, T_H0 and T_H1 $CD4^+T$ cells and NK cells (15,16). They play an important role in regulating the immune response and determining resistance or susceptibility to disease. Chemokines constitute a large family of small cytokines with four conserved cysteins linked by disulfide bonds. When chemokines bind to HIV coreceptors, they are potent and selective inhibitors of HIV infection. Cytokines are a family of proteins that mediated many of responses of innate and adaptive immunity (17). The same cytokines may be produced by many cell types and individual cytokine often acts on diverse cell types. They are synthesized in response to inflammation or antigenic stimuli and usually act locally, in an autocrine or paracrine fashion, by binding to high affinity receptors on target cells (18). Thus, cytokines serve many functions that are critical to host defense against the pathogens and provide link between innate and adaptive immunity (17). Depending on the nature of the infecting virus, the infected cells may be triggered to produce anti-viral cytokines and chemokines that inhibit one or more steps in the viral life cycle (19), thereby limiting the extent of the infection. The complex network of cytokines involved in inflammatory and immunoregulatory responses has an important role in several components of the pathogenesis of HIV infection and AIDS (20).

Recently, several cytokines, e.g., IFN-γ, IFN-α, IFN-β, IL–2, IL–12 and IL-16 have been demonstrated to profoundly affect the state of HIV expression, overall spreading, and suppress HIV infection. In addition, some chemokines e.g., RANTES, MIP-Iα, MIP-Iβ, SDF-1, MCP-1, MCP-2, MCP-3 and IP-10 function as the HIV-suppressive factors by interacting with chemokine receptors. When viruses infect cells, production of IFN is induced, and consequently, viral replication is inhibited. Interferon is believed to be part of the body's first line of defense against viral infection and to be the first cytokine to be recognized. Interferon regulate humoral and cellular immunity and has broad cell growth regulatory activities (15,18,20,21).

IFNs are cytokines that play a complex and central roles in the resistance of mammalian host to pathogens. IFNs, although best known for their anti-viral properties, are potent regulators of cell growth and have immunomodulatory activity (22-24). IFN-γ is the principle macrophage-activating cytokine and serves critical functions in innate immunity and in specific cell-mediated immunity. It has some

anti-viral activity and it functions mainly as an effector cytokine of immune response. IFN-γ is a homodimeric protein produced by NK, $CD4^+T_H0$, and T_H1, and $CD8^+T$ cells. In innate immunity, NK cells secrete IFN-γ in response to recognition of unknown components of microbes or in response to IL-12; in this setting IFN-γ functions as a mediator. In adaptive immunity, T cells produce IFN-γ in response to antigen recognition, and this is enhanced by IL-12. The sequence of reactions involving IL-12 and IFN-γ is central to cell-mediated immunity against intracellular microbes. IFN-γ functions by ; 1) providing the T lymphocytes and NK cells to activate macrophages to kill phagocytosed microbes (23, 24) stimulation of expression of class I and class II MHC molecules (25); 2) acting as costimulator on antigen presenting cells (17); 3) promoting the differentiation of naïve $CD4^+$ T cells to the T_H1 subset and inhibiting the proliferation of T_H2 (25, 26); 4) acting on B cells to promote switching to certain IgG subclasses; 5) activating of neutrophils (23); and 6) stimulating the cytolytic activity of NK cells (27).

Several studies have described an elevation of IFN-γ production in HIV-infected individual; however, there are conflicting reports on the status of IFN–γ production by cells isolated from HIV infected patients. Highly increased constitutive production of IFN-γ has been observed in PBMCs of asymptomatic HIV-infected patients with CDC group II/III as well as patients who have progressed to CDC group IV, as compared to healthy subjects (28, 29). However some studies demonstrated that PBMCs from HIV-infected individuals produce less IFN-γ in response to the stimuli (30, 31). Vitale *et al.* demonstrated that NK cells from both the HIV uninfected and HIV-infected subjects, produced IFN-γ in a higher percentages than T cells (32).

Chemokines are the 8-10 kd peptides that are the product of distinct genes clustered to human chromosomes 4 and 17. Chemokines regulate to selectively promote the rapid adhesion, chemotaxis and activation of leukocyte effector subpopulations (33, 34). In addition, some are able to regulate the proliferative potential of hematopoietic progenitor cells, endothelial cells, and certain types of transformed cells (35, 36). Chemokines act via a family of G-protein-coupled receptors and these types of receptor are usually large ligand (60 amino acids). Chemokine can be divided into two groups, α and β by the arrangement of the first two of four conserved cysteins. Members of the β chemokines posses the first two

conserved cysteins which are adjacent. The gene for the β chemokines is on human chromosome 17, and they generally activate monocytes, lymphocytes, basophils and eosinophils. The major human chemokine β family includes MIP-1α, MIP-1 β and RANTES which have been identified as the major M-tropic HIV suppressive factors (37). The biological properties of MIP-1 α, MIP-1 β and RANTES have been characterized in Table 1 (23, 35, 38).

β chemokines act by competitive binding to a coreceptor, CC chemokine receptors (CCR5), that is also required by macrophage tropic HIV-1 strains as a coreceptor for entry into the host cell. This receptor-ligand binding renders CCR5 unavailable to the virus either by competitively blocking virus-cell interactions or by causing down-regulation from host cell surface (39-43).

The α chemokine, also known as the CXC chemokines, contain a single amino acid between the first and second cystein residues. The gene that instructs synthesis of the α chemokine is on human chromosome 4. The major α chemokine is stroma cell-derived cofactor (SDF-1) which is an extremely efficacious chemoattractant for T lymphocytes and play a prominent role in B cell development. SDF-1 is a 352 amino acid proteins, mostly closely related to the receptor for IL-8 (44, 45). In addition, α chemokine (SDF-1) was shown to specifically inhibit CXCR4 mediated infection by T cell line tropic HIV-1. SDF-1 has been found to be responsible for competitive binding to a coreceptor CXCR4 which was identified as a specific coreceptor essential for entry of T-tropic HIV-1 into target cells (45).

The accumulating data have suggested that HIV infection that augmented production of RANTES, MIP-1 α and MIP-1 β may limit viral replication *in vivo* (46) and provided evidence for a significant inverse correlation between level of these chemokines and a reduction of virus burden *in vivo*. A specific immune response involving a high production of β chemokine by T cells seems to play role of protection in exposed uninfected individuals (47) and may contribute to the control of viral replication in long-term nonprogressors (48). Several studies demonstrated that PBMCs from HIV-1 infected, long term nonprogressors often produce higher levels of β-chemokines upon antigen stimulation *in vitro* than do PBMCs from rapid progressors (49, 50); and higher production of MIP-1β by PBMCs has been associated with an asymptomatic status and a decreased risk of disease progression (51).

Infection without disease progression for longer than 10 years, even though occurs in a minority of HIV-infected people, may be attributable from chemokine production, chemokine receptor polymorphisms including host factors (52). The previous studies have demonstrated that $CD8^+$ T cells, $CD4^+$ T cells and macrophages, as well as several other cell types, secrete cytokines and CC-chemokines productions (53, 54). In addition, a few studies also showed the roles of NK cells for cytokine and chemokine production.

Several novel methods have been developed which facilitate the detection of cytokine and chemokine production by immune cells. Cytokine and chemokines can be assayed by: 1) enzyme-linked immunosorbent assay (ELISA) for quantifying the amount of secreted cytokine and chemokine in serum or cell culture supernatant; 2) determination of specific mRNA in chemokine and cytokine producing cells using polymerase chain reaction (PCR) (55); 3) enumeration of activated cells secreting cytokine by using enzyme-linked immunospot (ELISpot) (56, 57). However, these methods are time consuming by requiring long activation times and they are labour intensive (58). In addition, these methods can not specify cell type which produce cytokine/ chemokine because the same cytokine/ chemokine may be produced from several cell types. For instance, IFNγ may be produced from $CD8^+$, $CD4^+$ and NK cells.

A highly sensitive flow cytometric technique, that is based on the ability to quantitate and phenotypically characterize cytokine expressing cells simultaneously without prior sorting has been developed (59). This technique has the advantage of rapid determining intracellular cytokine production by activating the cells with mitogen in a short incubation time. Subsequently, addition of brefeldin A (BFA) to block the secretary pathway, a process that disrupts intracellular Golgi-mediated transport and allows the cytokines to accumulate and yield an enhanced signal for the detection by flow cytometry. This method has been applied to detect several types of cytokines in every discreted cellular population, and also phenotypically characterize the cytokine producing cells in various diseases such as HIV disease, autoimmunity and allergy. It is also possible to detect intracellular cytokine production for evaluation of human immune response, vaccine efficacy, drug and therapeutic modalities.

Table 1. Biological properties of chemokines

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

MATERIALS AND METHODS

1. Subjects

The subjects enrolled in this study comprised;

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

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

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

1.2.2 19 HIV seropositive subjects with CD≥14% including 11 males and 8 females at age range of 21-53 years (mean±SD=31.9±10.0, median=28.0 years)

2. Intracellular cytokine staining

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

1. IgG_1-PE (Isotype control, Catalog No. 349043) which contains PE labeled anti-IgG_1 mAb

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

3. CD3-PerCP reagent (Leu-4 PerCP, Catalog No. 347344) which contains PerCP labeled anti-CD3 mAb

4. CD8-PerCP reagent (Leu-2a PerCP, Catalog No. 347314) which contains PerCP labeled anti-CD8 mAb

5. CD16-FITC reagent (Leu11a FITC, Catalog No. 347523) which contains FITC labeled anti-CD16 mAb

6. CD56-FITC reagent (NCAM16.2, Catalog No. 340410) which contains FITC labeled anti-CD56 mAb

7. Anti-human IFN-γ mAb-PE (Catalog No. 18905A) which contains PE labeled anti-human IFN γ mAb

8. Anti-human RANTES mAb-PE (Catalog No. 20975A) which contains PE labeled anti-human RANTES mAb

9. Anti-human MIP-1α mAb-PE (Catalog No. 20955A) which contains PE labeled anti-human MIP-1α mAb

10. Anti-human MIP-1β mAb-PE (Catalog No. 23855A) which contains PE labeled anti-human MIP-1β mAb

11. FACS Lysing Solution 10X concentration (Catalog No. 349202)

12. FACS Permeabilizing Solution 10X concentration (Catalog No. 340457) which contains the solution for the permeabilization of lymphocyte membranes prior to intracellular immunofluorescence staining with mAb

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

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

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

2.1 Cell stimulation for cytokine or chemokine production

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

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

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

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

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

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

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

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

9. Centrifuge at 500xg for 5 minutes

10. Discard the supernatant

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

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

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

2.2 Intracellular cytokine and chemokine staining

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

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

Table 2. Monoclonal antibodies to characterize immune cells expressing cytokine and chemokine

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

3. Flow cytometric analysis

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

4. Statistical analysis

Data of each lymphocyte subset and percentage of cytokine and chemokine expressing cells obtained from all subsets were analysed for difference between HIV-1 seronegative and seropositve subjects. Komogorov Smirnov test was used to verified the pattern of distribution of a dataset in order to determine the goodness of fit. When normal distribution of dataset was obtained ($p \geq 0.05$), parametric analyses would be employed by using the independent t-test. On another hand, if distribution of dataset was not normal ($p < 0.05$), non parametric assay would be employed by using Mann whitney-U test.

RESULTS

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

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

The stimulated and unstimulated control blood samples were stained by panel of monoclonal antibodies that determined lymphocyte subset and intracellular cytokines : IFN γ, RANTES, MIP-Iα, MIP-Iβ. The frequencies of cytokine expressing cells were enumerated by 3-color flow cytometric analysis. Gating of lymphocyte subsets and fluorescence intensity of the stained cells are shown in Figures 1-4.

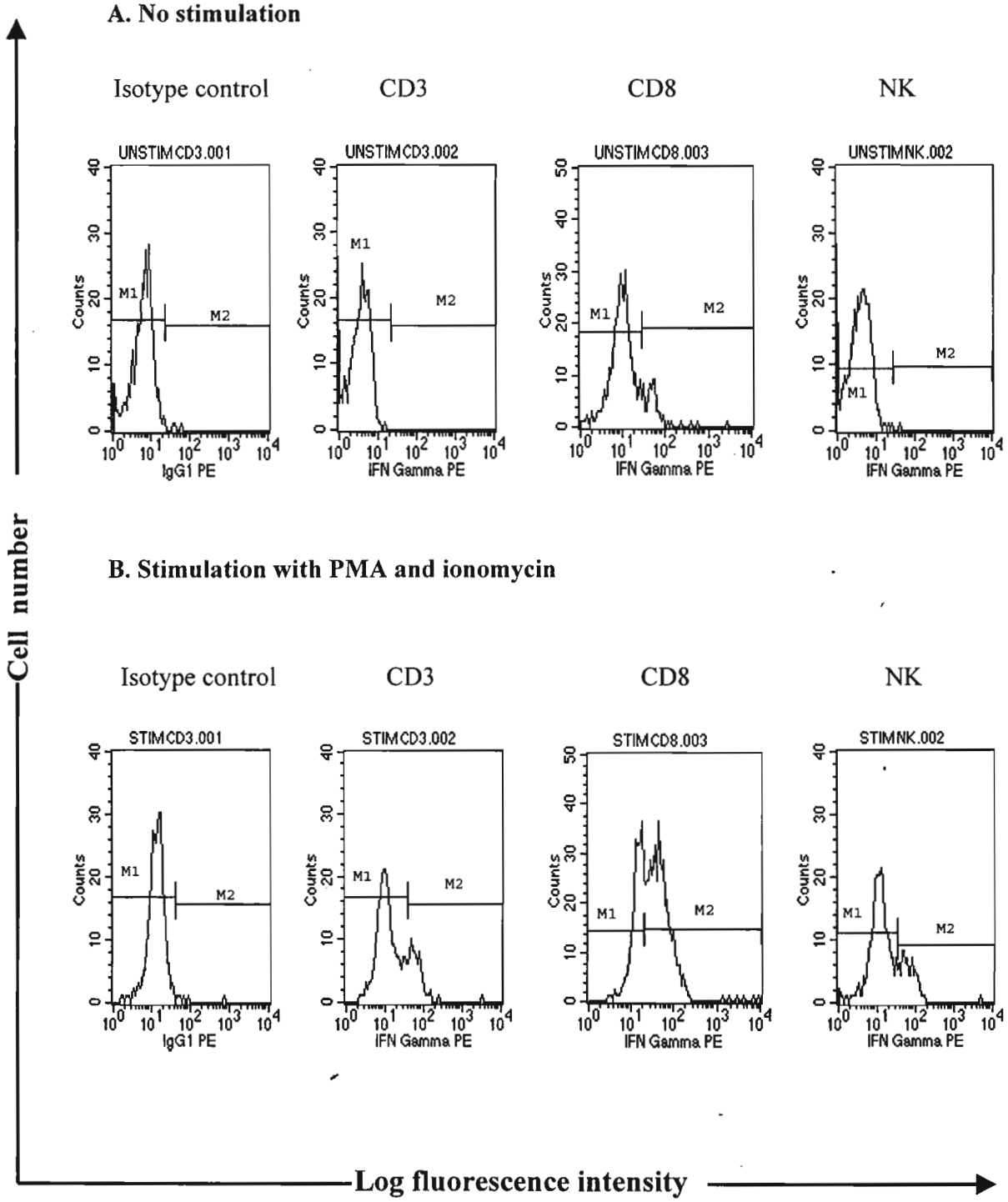

Figure 1. Flow cytometric three-color analysis of IFN γ expressing CD3, CD8 and NK cells from A: unstimulated control; and B: PMA and ionomycin stimulation

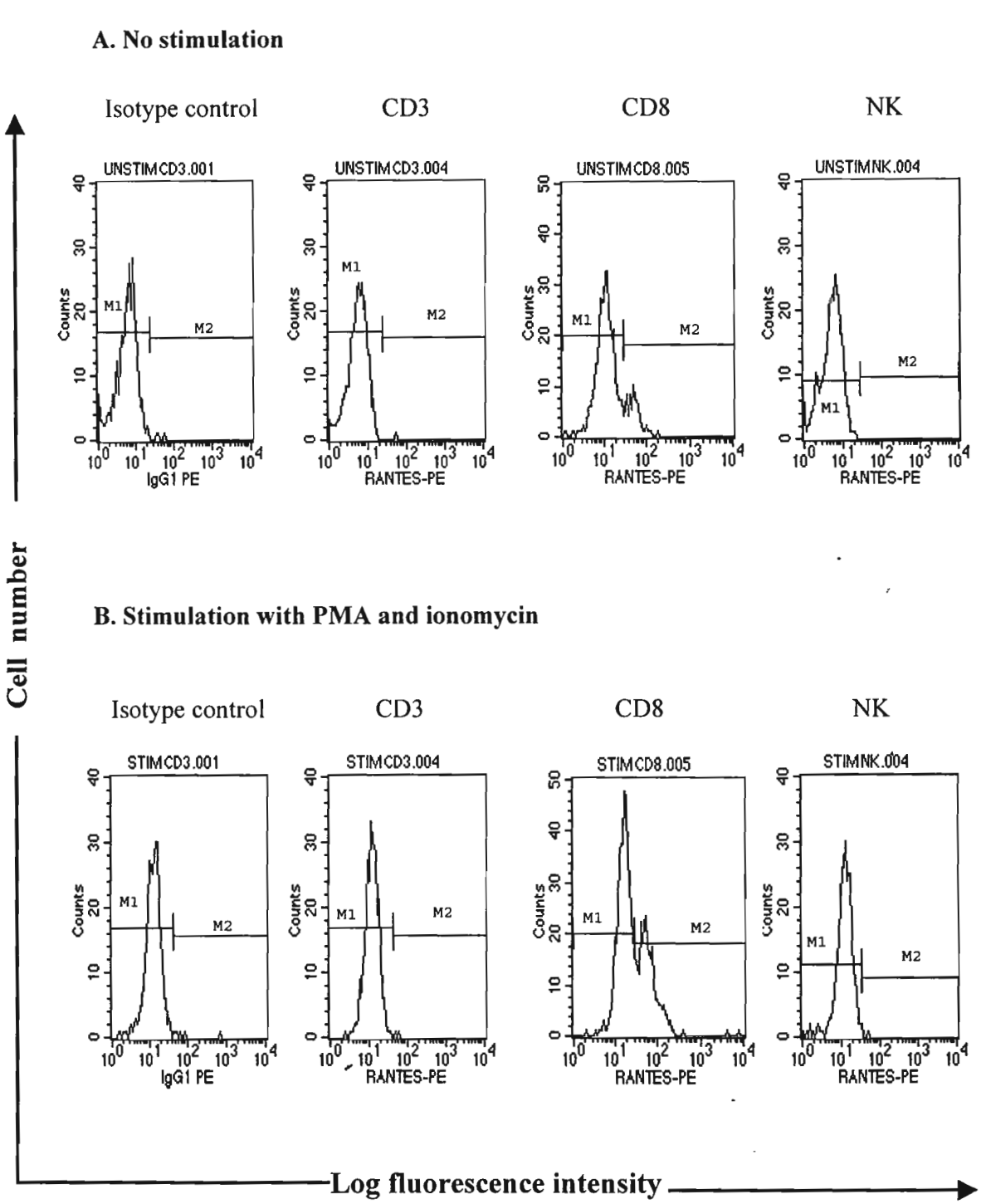

Figure 2. Flow cytometric three-color analysis of RANTES expressing CD3, CD8 and NK cells from A): unstimulated control; and B): PMA and ionomycin stimulation

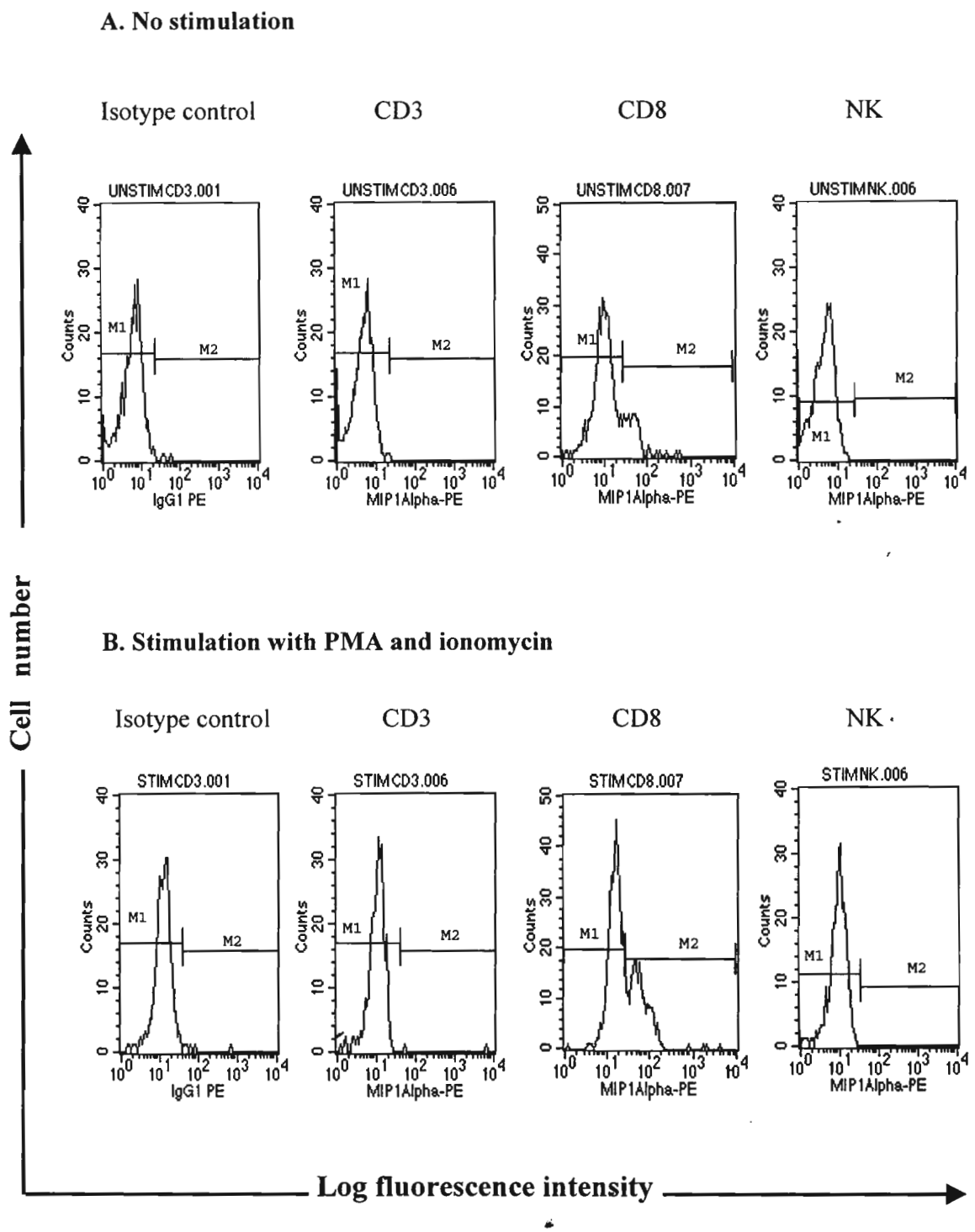

Figure 3. Flow cytometric three-color analysis of MIP-1α expressing CD3, CD8 and NK cells from A): unstimulated control and; B: PMA and ionomycin stimulation

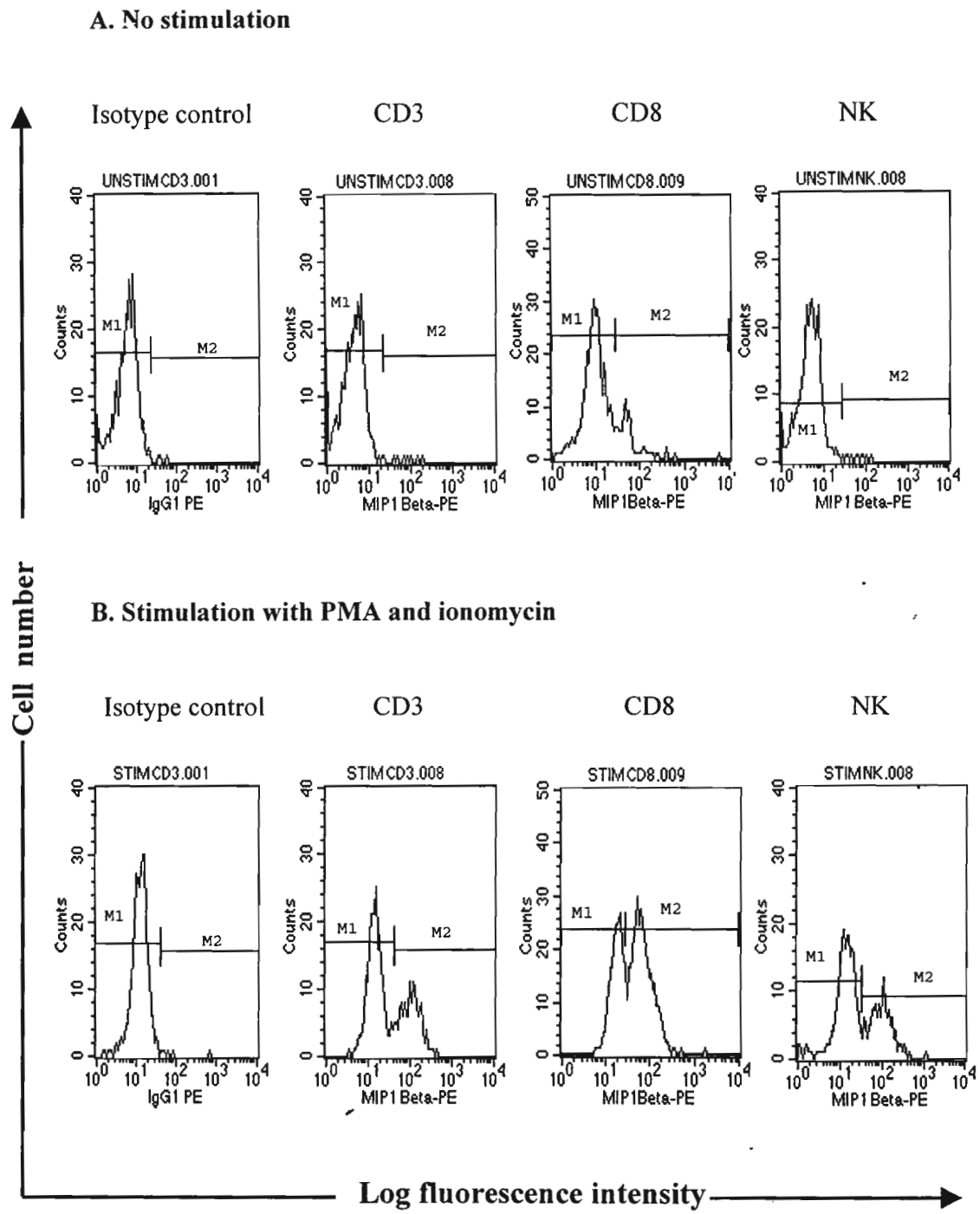

Figure 4. Flow cytometric three-color analysis of MIP-1β expressing in CD3, CD8 and NK cells from A): unstimulated control and; B): PMA and ionomycin stimulation

2. Spontaneous production of cytokine/ chemokine without antigenic stimulation

Staining of the unstimulated cultures showed that spontaneous synthesis of IFN γ, RANTES, MIP-Iα, MIP-Iβ was clearly observed in $CD8^+$T cells, but not so in $CD3^+$T cells and NK cells (Figs. 5-7 and Table 3). Percentages of cells spontaneously synthesized cytokine/ chemokines was the background value which will be substracted from the percentages of stained cells of the stimulated culture to yield the corrected percentages of cells synthesizing cytokine/ chemokines as the result of PMA and ionomycin activation.

Table 3. The background value of cytokine expressing cell without antigenic stimulation

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

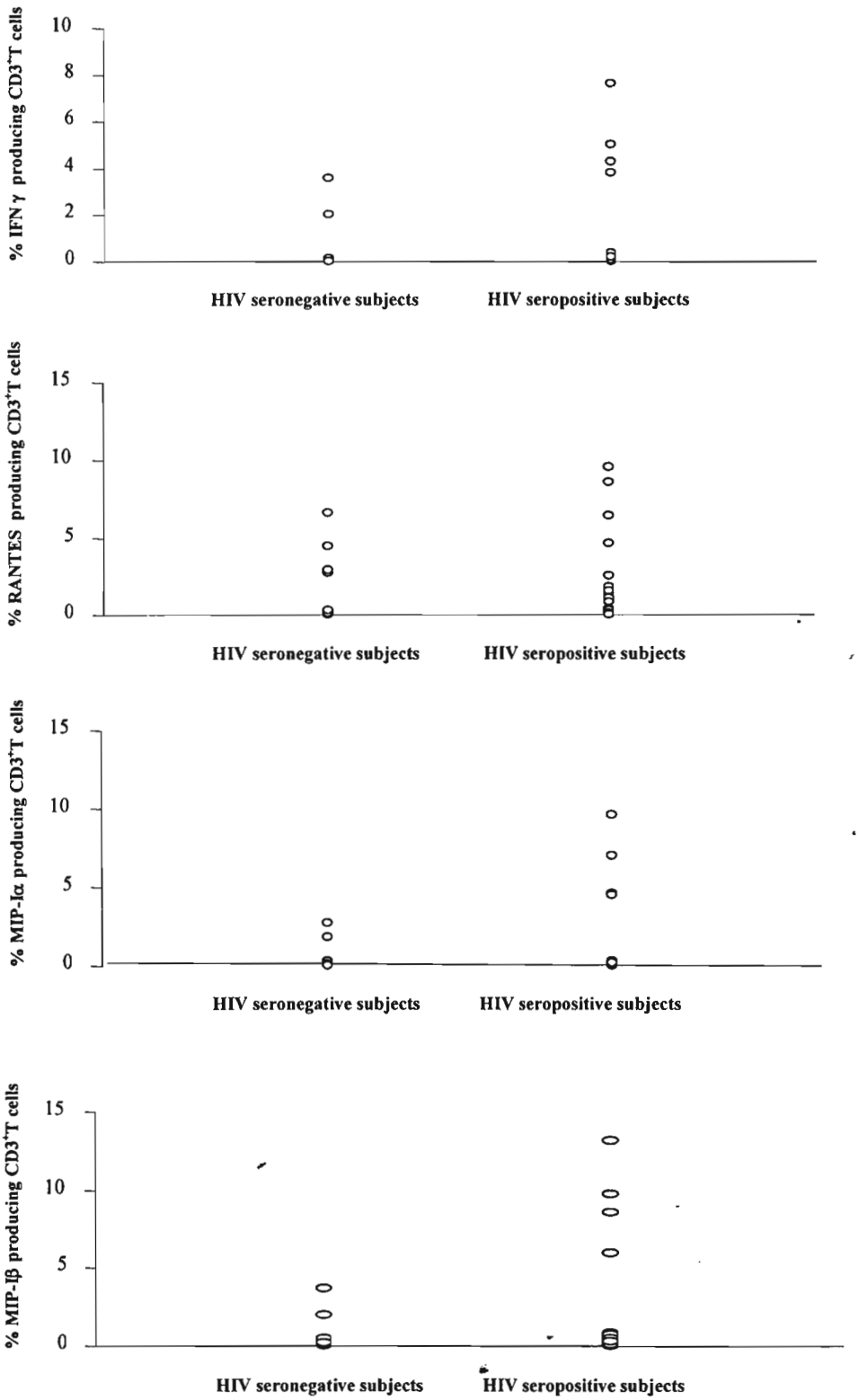

Figure 5. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated $CD3^+$T cells from HIV seronegative (n=10) and seropositive subjects (n=28)

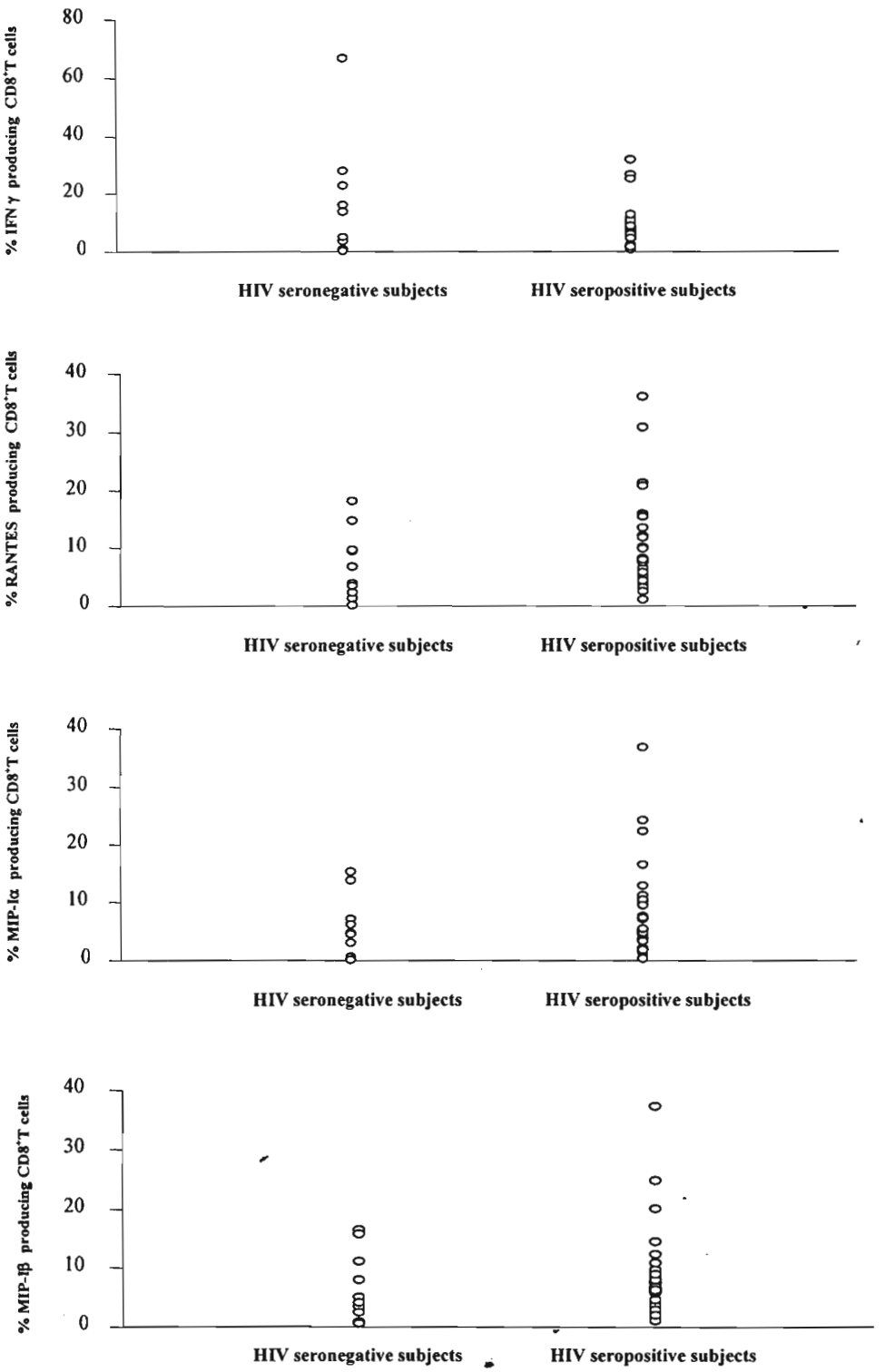

Figure 6. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated $CD8^+$ T cells from HIV seronegative (n=10) and seropositive subjects (n=28)

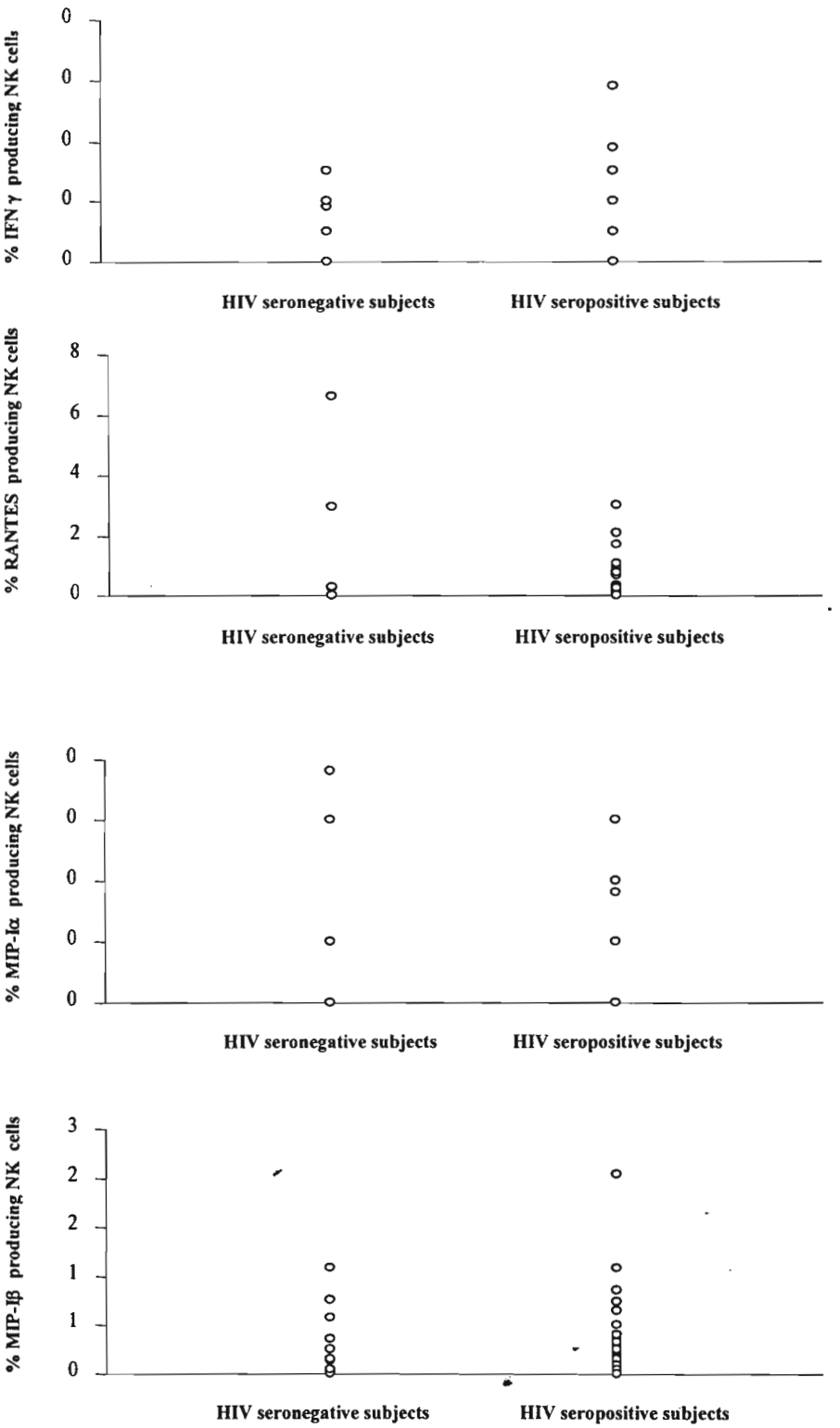

Figure 7. Production of intracellular cytokines, e.g., IFN γ, RANTES, MIP-I α and MIP-I β in non-activated NK cells from HIV seronegative (n=10) and seropositive subjects (n=28)

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

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

The results of cytokine/ chemokine production in $CD3^+T$ cells, $CD8^+T$ cells and NK cells of HIV seronegative and HIV seropositive subjects are shown in Figure 8. together with the summarization in Table 4.

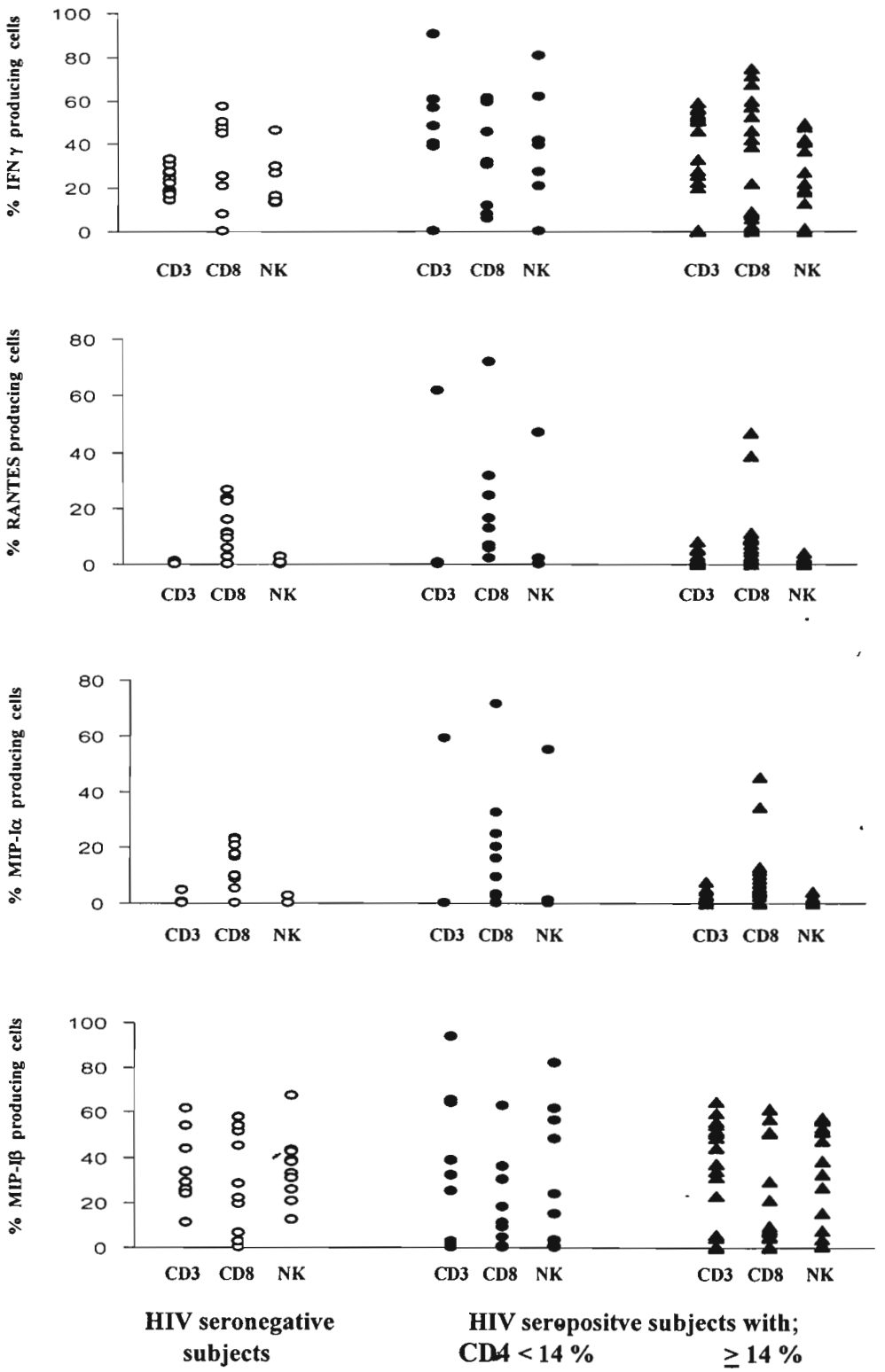

Figure 8. Corrected percentages of IFN γ, RANTES, MIP-Iα and MIP-Iβ expressing $CD3^+T$, $CD8^+T$ and NK lymphocytes in HIV seronegative (n=10) and seropositive subjects (n=28)

Table 4. Flow cytometric analyses on cytokine and chemokine producing cells upon stimulation by PMA and ionomycin

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

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

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

DISCUSSION

NK cell is a critical component of innate immune response and thought to be important during the early stage of HIV infection (6, 60, 61). Although cytokine expressing $CD8^+$T cells have been extensively documented, there have been few studies on cytokine production from NK cells.

Previous group of investigators had found that NK cells are as potent as $CD8^+$T cells in the mediation of suppression of HIV replication, predominantly by secretion of CC-chemokines (62). In addition, Fehniger *et al.* (61) demonstrated that the supernatant from NK cell culture could inhibit the replication not only of M-tropic HIV-1, but also of T-tropic HIV-1 strain. Scharton *et al.* (63) also demonstrated that NK cells could produce IFN-γ during the first week of infection, and the protection occured more rapidly than might be expected for conventional primary T cell response. Oliva *et al.* (64) found that the level of RANTES, MIP-1α and MIP-1β synthesized by NK cells was comparable to those produced by the stimulated $CD8^+$T cells and the study done by Vitale *et al.* (65) reported a higher percentages of IFN-γ producing NK cells as compared to number of IFN-γ producing T cells from both the HIV seronegative and HIV seropositive subjects.

Concerning the methods used for determining cytokine and chemokine production such as ELISA, RT-PCR, T cell cloning and ELISpot, all of these methods are time consuming and labor intensive. Moreover, these techniques require long activation time and cannot determine the frequencies and the phenotypes of the cytokine-producing cells at the single cell level (28, 66, 67-70). The flow cytometric method has been designed to simply and rapidly assess intracellular cytokine production in different leukocyte subsets resided in PBMCs or whole blood samples. These procedures determine the percentages of activated cells and the identification of leukocyte subsets capable of expressing various cytokines and cell surface antigens simultaneously. The ability to assess the key intracellular functional markers by multiparametric flow cytometry offer some unique advantages in a number of clinical applications.

The present study was a further effort to reveal the production of various types of intracellular cytokines in NK and $CD8^+$T cells. We demonstrated that NK and $CD8^+$T cells were the expandable sources of cytokine and chemokine production in healthy subjects and HIV-1 infected ones. Few percentages of cytokine or chemokine producing $CD8^+$T, and NK cells were observed in unstimulated PBMC samples (Figures 5-7 and Table 3). NK cells and $CD8^+$T cells from HIV infected cases were functionally active with regards to the production of cytokine and chemokine in response to PMA and ionomycin as compared to HIV seronegative subjects. Both NK and $CD8^+$T cells from HIV seronegative and HIV seropositive subjects were capable of producing high level of IFN-γ and MIP-1 β; whereas, the production of RANTES and MIP-1α was mainly produced from $CD8^+$T cells (Table 4). Thus, HIV infection of any disease stage may not affect the cell ability to produce cytokines and chemokines. In contrast, Azzoni *et al.* (30) found that NK cells from HIV-infected cases were impaired in their ability to produce IFN-γ as compared to the HIV seronegative donors. Moreover, Peruzzi *et al.* (71) suggested that the gp120 mediated a decrease in IFN-γ production by NK cells may contribute to the cytokine imbalance observed in HIV infection. The different finding may be attributable to the the influence of race on their ability of cytokine expression.

Our study aimed to investigate the role of NK cells in the protection against HIV disease progression based on the advantages that: a) Thai people had higher frequency of NK cells as compared to the Caucasians: b) NK cells could retain their activity on chemokine/cytokine production: c) NK cell function is not antigen specific; therefore, they can overcome the problem of HIV escape from humoral and cell-mediated immune response. Therefore, if NK cell activity can be sustained by any immunostimulator, it should provide an alternate approach for HIV treatment.

CONCLUSION

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

NK cell possess several advantages in the control of HIV disease progression such as: 1) Thai people had high NK cell number; 2) NK cell recognition is antigen non specific so that it can overcome the problem of as escape of HIV mutant from cytotoxic T lymphocytes and neutralizing antibodies which both of them are antigen specific in function. Maintaining cytokine production in NK cells may provide an alternative approach to cope with HIV infection.

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APPENDIX

Reagents for flow cytometry

1. 1% Paraformaldehyde

Paraformaldehyde (sigma)	1	g
1X PBS	100	ml

Filter through 0.1 μm filtered paper, store in the dark bottle at 4 °C.

2. FACS lysing solution

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

Working 1X FACS lysing solution

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

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

3. FACS permeabilizing solution

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

Working FACS permeabilizing solution (1X)

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

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

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

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

PMA stock solution (0.1 mg/ml)

PMA powder (Sigma)	1	mg
DMSO	10	ml

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

PMA working solution (1 ng/ml)

Stock PMA solution	2	µl
Steriled PBS	198	µl

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

5. Ionomycin

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

Ionomycin stock solution (0.5 mg/ml)

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

Store the stock solution in small aliquots (e.g.,20µl) at –20 °C.

Ionomycin working solution (0.05 mg/ml)

Stock ionomycin solution	10	µl
Steriled PBS	90	µl

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

6. Brefeldin A (Sigma B-7651)

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

Brefeldin A stock solution (5 mg/ml)

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

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

Brefeldin A working solution (0.5 mg/ml)

Stock Brefeldin A solution	20	µl
Sterile PBS	180	µl

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