CHAPTER IV

RESULTS

1. Study population

In total, seventeen HIV discordant couples and 2 individual HEPS persons, whose HIV-1 seropositive sex partners died before the time of the first visit, were recruited for this study, and followed up every 3 months for 1 year. The HEPS group (codes H01-H19, n=19) ranged in age from 20-45 years (median=36), and their HIV-seropositive sex partners (codes H01P-H04P, H06P-H16P,H18P-H19P, n=17) from 24-55 years (median=37). Fifteen (80%) HEPS persons were female and 4 (20%) were male, and all of them reported high-risk sexual activities with an HIV-infected sex partner. Two of the HIV-1 seropositive sex partners (H05P and H17P) died before the time of the first visit. Seventeen (90%) of the HIV-infected sexual partners were also enrolled in the longitudinal study. Three HIV-infected sex partners (17.64%) (H04P, H06P and H12P) were lost to follow up as a result of HIV-related complications and insufficient information e.g. intracellular cytokines staining was not performed. Fifteen normal subjects (codes N01-N15, n=15), who were non reactive for anti-HCV, HBsAg, and anti-HIV-1/HIV-2, were studied as a healthy control group. Nine (60%) of the normal control group were male and 5 (40%) were female. The normal control group ranged in age from 19-50 years (median=29). Significantly decreased CD4+ T-lymphocyte counts were observed at the initial visit in HIV-seropositive sex partners in comparison with HEPS and the normal control group (**Table 5**). On the other hand, CD8+ T-lymphocyte counts at the initial visit were higher in HIV-seropositive sex partners than in HEPS persons and the normal control group. The CD4+ cell counts of HEPS have been consistently in the normal range, with no decline over time. The median CD4+ cell count was 222 cells/ml (range, 9–1,354) in HIV-infected sex partners, 747.7 cells/ml (range, 281–1,429) in HEPS persons and 721 cells/ml (range, 633–1,338) in the normal control group. Of the 11 HIV-seropositive sex partners, who had been infected for >1 year, seven had opportunistic infection and four were treated with antiretroviral drugs during this study. Two of 17 HIV-seropositive sex partners received PCP prophylaxis. Two HIV-seropositive sex partners and 3 HEPS persons were infected with hepatitis.

The general clinical characteristics of the cohort and infectious disease in HIV-1–infected sex partners and HEPS groups are shown in **Table 6.**

Table 5. Percentage of CD4+ and CD8+ T cells in HIV-exposed persistently seronegative, HIV-seropositive sex partners and a normal control group.

Group	CD4		CD8	
	%	Absolute cell count	%	Absolute cell count
HEPS persons	37.46±9.28 [b]	747.70±284.23 [b]	28.10±7.59 [b]	566.46±259.27 [b]
HIV-seropositive sex partners	11.27±9.31 [a]	222.59±292.44 [a]	56.83±12.54 [a]	874.05±428.11 [a]
Normal control	38.29±10.17	1208.31±849.94	28.73±13.64	602.18±591.04

[a] $p<0.005$, vs. Normal control subjects.
[b] $p>0.005$, vs. Normal control subjects.

Table 6. Characteristics of clinical disease and infectious disease in HIV-1–infected sex partners and HEPS groups.

Group	Subject	Sex	Age (yr)	Years of infection	Infectious Disease			Antiretroviral therapy	CD4 cell count (cells/mL)*	CD8 cell count (cells/mL)*
					STD	OI	Hepatitis			
HIV+	H 01P	F	24	3	No	No	No	None	1354	1278
sex partners	H 02P	M	41	Unk	No	PCP	No	None	36	475
	H 03P	F	30	2	No	Herpes zoster,TB	No	Yes	66	604
	H 04P	M	47	Unk	No	TB	No	None	13	416
	H 06P	M	40	Unk	Unk	No	**HBV**	None	99	1134
	H 07P	M	32	1	No	No	No	None	13	416
	H 08P	M	27	5	No	Herpes zoster,TB	No	None	130	1042
	H 09P	M	39	Unk	Unk	No	**HCV**	None	25	678
	H 10P	M	42	3	Unk	No	No	Yes	22	814
	H 11P	F	38	2	No	TB	No	Yes	342	827
	H 12P	F	31	Unk	Unk	No	No	PCP prophylaxis	9	248
	H 13P	M	39	4	No	TB	No	None	232	1878
	H 14P	M	36	3	No	TB	No	Yes	148	563
	H 15P	M	55	2	Unk	No	No	None	317	744
	H 16P	M	36	1	Unk	No	No	None	206	747
	H 18P	M	33	1	Unk	No	No	None	49	1065
	H 19P	M	35	Unk	No	No	No	PCP prophylaxis	97	698
HEPS	H 01	M	26	No	No	No	No	None	281	370
	H 02	F	36	No	No	No	No	None	1181	950
	H 03	M	Unk	No	Unk	Unk	**HBV**	None	508	446
	H 04	F	40	No	No	No	No	None	633	837

Table 6. Continued

Group	Subject	Sex	Age (yr)	Years of infection	Infectious Disease			Antiretroviral therapy	CD4 cell count (cells/mL)	CD8 cell count (cells/mL)
					STD	OI	Hepatitis			
	H 05	F	39	No	No	No	**HCV**	None	855	488
	H 06	F	Unk	No	Unk	Unk	No	None	848	527
	H 07	F	Unk	No	Unk	Unk	No	None	604	412
	H 08	F	20	No	No	No	No	None	887	400
	H 09	F	40	No	No	No	No	None	601	1043
	H 10	F	40	No	No	No	No	None	612	442
	H 11	M	Unk	No	Unk	Unk	No	None	1429	690
	H 12	M	42	No	No	No	No	None	644	800
	H 13	F	Unk	No	No	No	No	None	428	428
	H 14	F	38	No	No	No	No	None	524	1646
	H 15	F	Unk	No	Unk	Unk	No	None	803	273
	H 16	F	35	No	No	No	No	None	1019	384
	H 17	F	45	No	No	No	No	None	409	370
	H 18	F	Unk	No	No	No	**HBV**	None	413	346
	H 19	F	Unk	No	No	No	No	None	765	490

NOTE. TB, Tuberculosis; PCP, *Pneumocystis carinii* Pneumonia; HBV, Hepatitis B virus; HCV, hepatitis C virus; Unk, Unkown; M. Male; F, Female.

* CD4+ T cell count at the first visit for cytokine detection (visit3; 6 months follow up) (Dettrairat S.)

2. Detection of HIV antibodies by ELISA (MEIA or EIA) and gelatin particle agglutination (GPA)

All HEPS persons, their sex partners and the normal control group were tested for HIV-1/HIV-2 antibody by IMX HIV-1/HIV-2 third generation Plus (MEIA) or Abbott HIV-1/HIV-2 third generation EIA and gelatin particle agglutination (GPA). HIV antibodies were tested at every visit (5 times) throughout the study period of 12 months (0-12 months), at 0, 3, 6, 9 and 12 months of follow-up. Results of HIV antibody tests by two ELISA assays was in accordance with the gelatin particle agglutination (GPA) test. Nineteen HEPS individuals (100%) remained free of HIV infection by serologic tests. In this study, no HEPS persons were reactive with HIV antibodies, although 5 HEPS persons were lost (H07, H08, H09, H12 and H15) to visit 4 (9 months) and 5 (12 months) of the follow-up. All 17 HIV-infected sex partners (100%) were positive to HIV antibodies, whereas all 15 healthy adults (100%) were negative as shown in **Table 7.**

Table7. Number of reactive subjects to HIV-1/HIV-2 antibody tests in HEPS persons, HIV-infected sex partner and the normal controls (0, 3, 6, 9 and 12 months follow up).

Subject	Assay	Months of testing				
		0	3	6	9	12
HEPS persons (n=19)	MEIA [a]	0/19	0/19	0/19	0/14*	0/15*
	GPA [c]	0/19	0/19	0/19	0/14*	0/15*
HIV-seropositive sex partners (n=17	MEIA [a]	17/17	16/16*	14/14*	10/10*	11/11*
	GPA [c]	17/17	16/16*	14/14*	10/10*	11/11*
Normal control (n=15)	EIA [b]	0/15	**NT**	**NT**	**NT**	**NT**
	GPA [c]	0/15	**NT**	**NT**	**NT**	**NT**

NOTE. NT, No tested

a MEIA, Microparticle Enzyme immunoassay test; *, subjects lost to follow up;

b EIA, Enzyme Immunoassay test;

c GPA, Serodia HIV-1/2 Gelatin Particla Agglutination test.

3. Detection of HIV antibodies (IgG, IgA and IgM) in plasma by Flow Cytometric Immunofluorescence Assay (FIFA)

3.1 Study of HIV-coated latex bead gating for HIV-antibody detected by Flow Cytometric Immunofluorescence Assay (FIFA)

The experiment was performed to study class of HIV antibody (IgG, IgA and IgM) responses in each subject. The HIV-coated latex beads were bound with HIV-antibodies in the serum or plasma of the subjects; FITC-conjugated anti-human IgG, FITC-conjugated anti-human IgA and FITC-conjugated anti-human IgM was subsequently added to bind with each antibody class. The side scatter (SSC) and forward scatter (FSC) of HIV-coated latex beads were collected at ≥10,000 beads events (2,000 beads in a gate) for analyses. **Figure 6** shows the scatter of dot plot of the side scatter (SSC) and forward scatter (FSC) and the area of the gate (No gate, gate R1, R2 and R3). Fluorescence intensity of the beads was shown in histogram patterns after gating on the bead. In comparisons between each gate in the HIV-seropositive group, fluorescence intensity of gate R1 was higher than No gate, R2 and R3. There was a clear difference of fluorescence intensity in comparison between HIV-seropositive and HIV-seronegative samples.

In this study, gate R1 of HIV-coated latex beads was gated to detect HIV-antibodies. The value of fluorescence intensity of ≥50 was positive for the IgG class, while ≥20 was positive for the IgA and IgM.

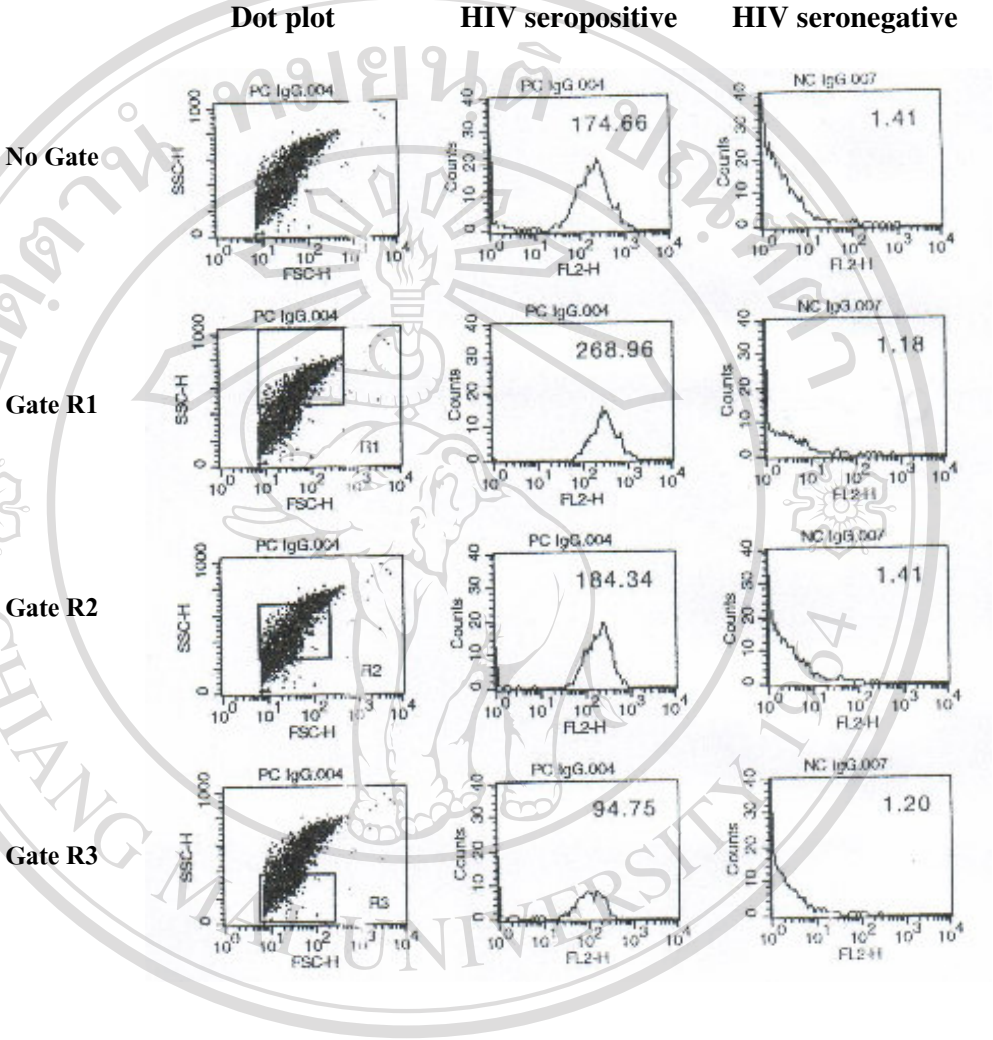

Figure 6. Dot plot of HIV-coated latex beads and histograms of fluorescence intensity of HIV-seropositive and HIV-seronegative samples at No gate and gate R1, R2 and R3.

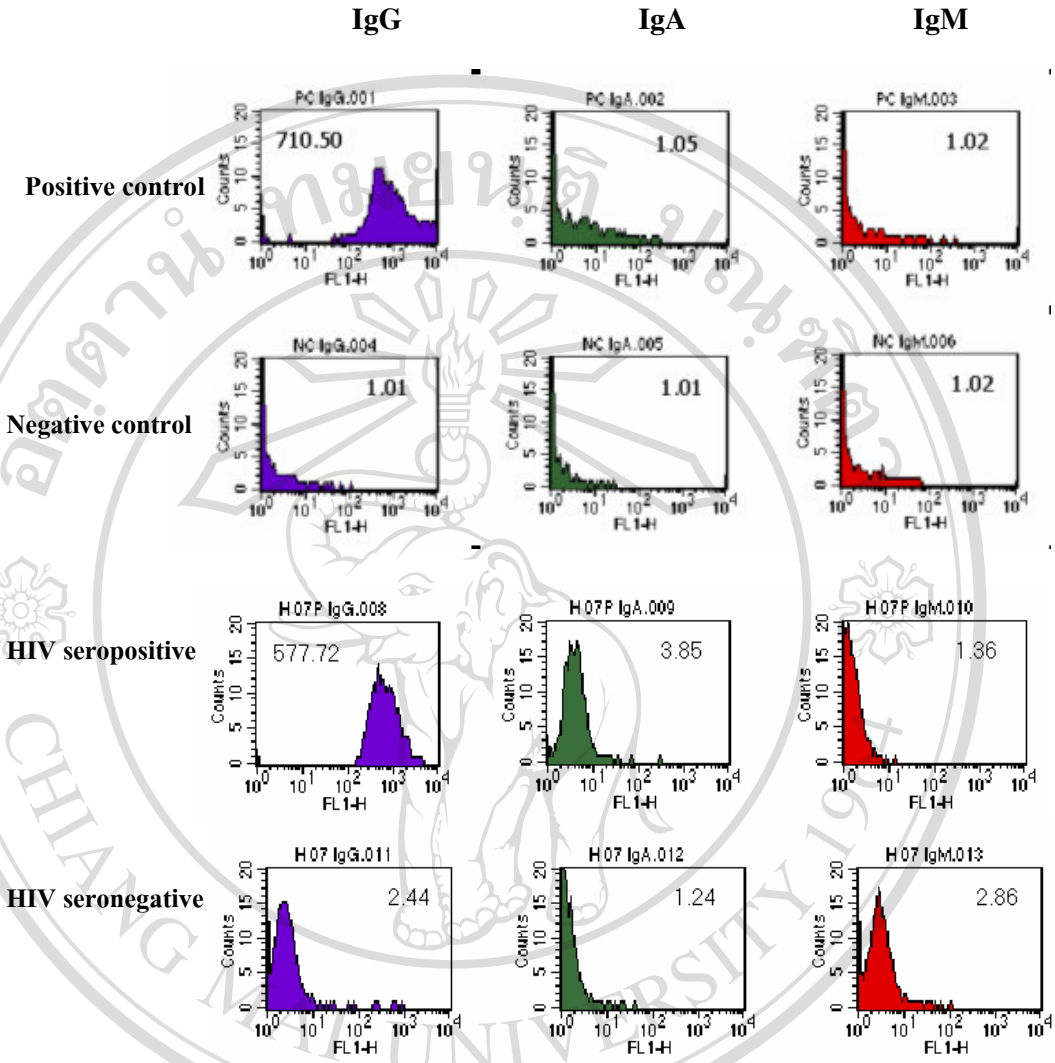

Figure 7. Histogram of HIV antibody class IgG, IgA and IgM by FIFA in positive control, negative control, HIV seropositive (H07P) and HIV seronegative (HEPS, H07).

3.2 Detection of HIV antibody class (IgG, IgA and IgM)

By routine MEIA/EIA or GPA, all HIV-infected subjects were positive with HIV antibody, but no HEPS subjects or healthy controls were positive. Thus, the method used for routine screening was adequate to detect HIV IgG seroconversion. Some previous reports showed that HIV-specific IgA in the absence of IgG was present in the sera of HEPS (Mazzoli S., 1999). In this study, plasma from the HEPS persons and HIV-infected sex partners were analysed for the presence of HIV-antibody class (IgG, IgA and IgM) by FIFA. HIV-antibody responses were classified into classes by each type of conjugated binding. A value of fluorescence intensity ≥50 was positive for the IgG class, and ≥20 was positive for the IgA and IgM class. Negative and positive controls were included in each run.

3.2.1 Detection of HIV antibody class (IgG, IgA and IgM) in HIV-seropositive sex partners

HIV-antibody class IgG was detected in the plasma of all seventeen HIV-infected sex partners (H01P-H04P, H06P-H16P, H18P-H19P). As shown in **Table 10**., the mean levels of serum IgG were significantly greater for HIV-1-seropositive sex partners than those for HEPS (all $p<0.0001$). Using the cut-off criteria described above, fluorescence intensity of serum HIV-1-specific IgA exceeding the cutoff values were present in the serum of 1 out of 19 (5.88%) (H02P) HIV-infected sex partners at visit 2 (3 months) of the follow up. HIV-antibody class IgM was detected in 3 HIV-infected sex partners (17.65%) (H03P, H11P and H18P) at visit 1 (0 month) of the follow up. Only one HIV-infected sex partner (5.88%) (H11P) was positive with HIV-antibody class IgM at every visit, but others were randomized in each subject on each visit (**Table 8, 10**). HIV-antibody class IgM was detected in one HIV-infected sex partner (H15P) at visit 2 (3 months) follow up. None of the negative controls showed any HIV-antibody class (IgG, IgA or IgM) reactivity.

3.2.2 Detection of HIV antibody class (IgG, IgA and IgM) in HEPS

For the 12 months follow up period, FIFA was performed at every visit (3 months) for all HEPS persons (H01-H19). Unfortunately, some HEPS persons were lost during the study, as shown in **Table 9** and **10**. The value of fluorescence intensity in all HEPS individuals was <50 for the IgG class, and <20 for IgA and IgM classes, which corresponded with the mean of negative results. All nineteen samples showed non reactivity for the HIV-1 antibody classes; IgG, IgA and IgM. By definition, all HIV seropositive sex partners, but no HEPS subjects, had an HIV-1-specific IgG response. This data might support non HIV-infection in HEPS individuals. Conversely, humoral immune responses to HIV were not associated with resistance to HIV-infection in this group (**Table 9, 10**).

Table 8. HIV-1/HIV-2 antibody (IgG, IgA and IgM) testing in HIV-infected sex partners by Flow Cytometric Immunofluorescence Assay (FIFA)

Subject	Months of testing														
	0			3			6			9			12		
	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
H 01P	395.96	2.15	6.67	632.09	2.53	5.78	327.81	1.14	5.23	261.80	1.33	3.41	207.21	1.68	6.79
H 02P	982.17	7.77	2.11	1459.02	28.90	3.26	437.14	4.83	2.15	349.12	1.12	1.02	1018.2	3.92	2.69
H 03P	1084.32	8.43	33.4	1213.35	3.19	15.68	557.31	1.25	11.55	365.17	1.78	7.50	406.79	3.28	3.28
H 04P	445.08	6.85	1.73	1700.08	8.43	4.98	NT	NT	NT	NT	NT	NT	NT	NT	NT
H 06P	655.76	2.74	2.74	784.39	2.25	2.29	NT	NT	NT	NT	NT	NT	NT	NT	NT
H 07P	588.21	3.96	1.37	1229.83	5.20	5.67	504.80	5.73	1.95	NT	NT	NT	NT	NT	NT
H 08P	417.92	4.70	8.35	352.27	2.39	1.93	457.25	4.18	3.13	NT	NT	NT	NT	NT	NT
H 09P	756.67	5.14	3.11	2246.79	12.98	3.25	425.51	6.55	2.62	NT	NT	NT	NT	NT	NT
H 10P	408.63	1.63	1.61	951.73	2.27	1.68	358.66	1.12	1.24	259.46	1.39	1.76	421.7	2.39	1.02
H 11P	770.40	3.40	68.54	1064.99	4.18	41.8	368.47	6.76	40.7	365.17	3.16	29.96	1018.2	3.25	41.05
H 12P	349.12	19.4	3.08	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
H 13P	441.09	9.22	1.65	716.92	3.22	1.84	273.84	3.79	2.76	214.80	1.84	1.47	111.40	1.03	1.37
H 14P	964.66	2.97	6.73	1240.94	5.33	6.10	964.66	6.21	8.24	291.64	1.06	1.15	716.92	2.39	1.5

Table 8. Continue

Subject	Months of testing														
	0			3			6			9			12		
	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
H 15P	736.53	2.74	11.24	1094.11	3.28	28.39	582.94	2.55	12.8	NT	NT	NT	716.92	2.39	1.5
H 16P	567.42	1.83	2.09	495.81	4.49	2.48	441.09	1.55	1.18	345.99	1.37	1.06	266.55	1.04	1.73
H 18P	1175.74	14	36.19	449.10	7.99	14.33	491.37	4.57	11.3	421.70	2.39	1.02	435.18	1.19	1.02
H 19P	1046.00	4.14	4.66	763.51	7.07	1.86	403.15	1.20	1.58	327.81	1.35	1.02	305.05	1.04	1.02

NOTE. NT, No tested, Underline value is positive result

Table 9. HIV-1/HIV-2 antibody (IgG, IgA and IgM) testing in HEPS by Flow Cytometric Immunofluorescence Assay (FIFA)

Subject	Months of testing														
	0			3			6			9			12		
	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
H 01	6.26	1.10	1.88	8.82	1.47	2.57	7.43	1.02	1.57	2.93	1.55	1.80	2.37	1.13	1.98
H 02	3.46	1.25	1.30	3.62	2.31	4.18	12.19	1.05	4.33	10.94	1.01	2.00	5.73	1.22	4.27
H 03	7.37	3.65	7.37	9.14	5.62	5.52	3.02	3.25	6.49	2.50	4.00	6.67	9.14	4.74	5.42
H 04	14.99	2.04	1.70	12.98	2.19	1.93	7.91	1.12	1.50	11.65	1.04	1.31	3.34	1.18	1.46
H 05	16.4	1.04	3.62	1.15	1.32	2.27	2.65	1.15	1.6	1.18	1.14	1.5	1.41	1.19	1.03
H 06	9.91	1.38	4.87	37.52	1.43	2.25	5.99	1.11	2.44	2.5	1.01	1.13	NT	NT	NT
H 07	2.50	1.26	2.92	7.7	2.94	3.28	6.1	1.36	1.72	NT	NT	NT	1.07	1.25	1.13
H 08	1.78	1.00	1.8	1.29	1.41	2.35	1.31	1.5	2.41	NT	NT	NT	NT	NT	NT
H 09	2.29	1.01	6.10	8.20	1.42	6.38	4.24	1.11	10.18	NT	NT	NT	NT	NT	NT
H 10	11.55	1.01	1.78	5.78	1.43	2.15	8.43	1.01	3.59	4.10	1.07	2.29	22.88	1.43	2.17
H 11	1.36	12.3	3.4	7.64	1.36	2.39	6.32	1.06	2.64	3.31	1.03	2.17	7.64	1.36	2.39
H 12	5.62	1.20	10.00	3.82	1.20	3.82	4.22	1.18	2.44	NT	NT	NT	NT	NT	NT
H 13	4.41	1.02	1.49	2.85	1.23	2.01	2.94	1.01	1.38	1.63	1.02	1.29	2.21	1.00	6.04
H 14	2.39	1.02	2.48	3.28	1.63	2.31	5.62	1.29	11.97	1.73	1.01	1.81	2.44	1.24	2.86

Table 9. Continue

Subject	Months of testing														
	0			3			6			9			12		
	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
H 15	2.56	1.06	2.11	9.31	1.65	8.66	3.28	1.18	6.15	NT	NT	NT	2.21	1.01	5.99
H 16	12.30	1.01	2.13	13.46	1.04	3.05	2.44	1.01	1.64	8.74	1.02	1.39	11.14	1.01	1.75
H 17	4.96	1.61	10.75	7.37	1.31	11.55	3.19	1.01	4.47	6.1	1.36	6.26	16.55	1.57	4.07
H 18	7.50	1.26	2.04	9.65	1.05	2.86	5.73	1.03	1.46	8.13	1.7	1.98	11.09	1.6	2.29
H 19	2.69	1.39	1.41	6.15	1.36	1.86	1.49	1.02	1.10	1.29	1.41	1.58	2.74	1.21	1.98

NOTE. NT, No tested

Table 10. HIV-1/HIV-2 antibody (IgG, IgA and IgM) reactivity in HIV-infected sex partners and HEPS by Flow Cytometric Immuno-fluorescence Assay (FIFA)

Visit	HIV-1/HIV-2 antibody Subclass Mean±SD (Range)					
	HIV-infected sex partners (n=17)			HEPS (n=19)		
	IgG	IgA	IgM	IgG	IgA	IgM
visit 1 (0 mo)	693.28±273.43 (349.12-1,175.74)	5.94±4.75 (1.63-19.37)	11.49±18.08 (1.37-68.54)	6.33±4.64 (1.36-16.4)	1.93±2.59 (1.00-12.30)	3.64±2.89 (1.30-10.75)
visit 2 (3 mo)	1024.68±497.49 (352.27-2,246.79)	6.48±6.65 (2.25-28.90)	8.83±11.34 (1.68-41.79)	8.41±7.87 (1.15-37.52)	1.76±1.05 (1.04-5.62)	3.76±2.59 (1.86-11.55)
visit 3 (6 mo)	471.00±165.63 (273.84-964.66)	3.67±2.18 (1.12-6.76)	7.60±10.39 (1.18-40.68)	4.97±2.75 (1.31-12.19)	1.24±0.51 (1.01-3.25)	3.64±3.07 (1.10-11.97)
visit 4 (9 mo)	320.27±62.30 214.8-421.70	1.68±0.65 1.06-3.16	4.94±9.02 1.02-29.96	4.77±3.66 1.18-11.65	1.38±0.79 1.01-4.00	2.37±1.77 1.13-6.67
visit 5 (12 mo)	511.27±312.72 111.40-1,018.15	2.15±1.03 1.03-3.92	5.72±11.84 1.02-41.05	6.80±6.38 1.07-22.88	1.48±0.92 1.00-4.74	2,99±1.73 1.03-6.04

4. Detection of HIV-1 antigen (p24)

The p24 antigen could be detected in serum relatively soon after HIV exposure in many patients, and detection often preceded the process of seroconversion by several weeks. In some cases, unusual patients, who developed AIDS despite testing negative for antibodies to HIV were found. HIV infection was confirmed by the results of p24-antigen assays and polymerase chain reaction amplification of proviral DNA.

The p24 antigen of HIV was not found in any of the HEPS individuals or HIV-infected sex partners. Despite 12 months follow up, no HIV p24 antigen in both HEPS and HIV-infected sex partners was found as shown in **Table 11.**

Table 11. HIV-1 antigen reactivity in HIV-infected sex partners, HEPS and the Normal Control group

Visit	Group		
	HIV-infected sex partners	HEPS	Normal Control
visit 1 (0 mo)	0/17 (0%)	0/19 (0%)	0/15 (0%)
visit 2 (3 mo)	0/16 (0%)*	0/19 (0%)	0/15 (0%)
visit 3 (6 mo)	0/14 (0%)*	0/19 (0%)	0/15 (0%)
visit 4 (9 mo)	0/10 (0%)*	0/14 (0%)*	0/15 (0%)
visit 5 (12 mo)	0/11 (0%)*	0/15 (0%)*	0/15 (0%)

NOTE. *, subjects lost to follow up

5. Detection of proviral DNA from whole blood by Multiplex Nested PCR

Nineteen HEPS and 17 HIV-infected sex partners had DNA extracted from whole blood trimonthly during 12 months follow-up. HIV-1 proviral DNA was amplified from DNA lysate by the nested PCR primers (National HIV Repository and Bioinformatic Center -Thailand), SK 380/390 & SK 38/39 and POL O1/O2 & POL I1/I2, specified for *gag* and *pol* genes, respectively.

Amplification of HIV-1 proviral DNA

Performance of experiments and the evaluation of Multiplex-nested PCR with 19 HEPS persons and 17 HIV-infected sex partners were performed trimonthly for a follow up period of 12 months. In this experiment, primary and secondary PCR products were obtained and measured by gel electrophoresis. No primary PCR products could be detected from this experiment, but only secondary PCR products could be found as shown in **Figure 8.** HIV-1 proviral DNA was detected in all of the HIV-infected sex partners. PCR products of the expected size (260 bp for β-globin, 180 bp for gag and/or 115 bp for pol) were obtained by the Multiplex-nested PCR assay in all 17 (100%) HIV-infected sex partners, but no amplification was observed in the 19 HEPS individuals. The positive PCR results were 100% and 63.23% by *gag* and *pol* genes, respectively. No PCR product corresponding to the band size for 180 bp for gag and/or 115 bp for pol was obtained in any of the 19 HEPS samples tested, despite 12 month's follow up. HIV-infection was not found in any HEPS subjects and there was no false positive PCR from any of these primers. All PCR reactions gave positive results for β-globlin primer, which meant that there was enough DNA to start PCR and no inhibitor in DNA extract. The result is shown in **Table 12, 13 and 14.** This data support that HEPS subjects were uninfected with HIV.

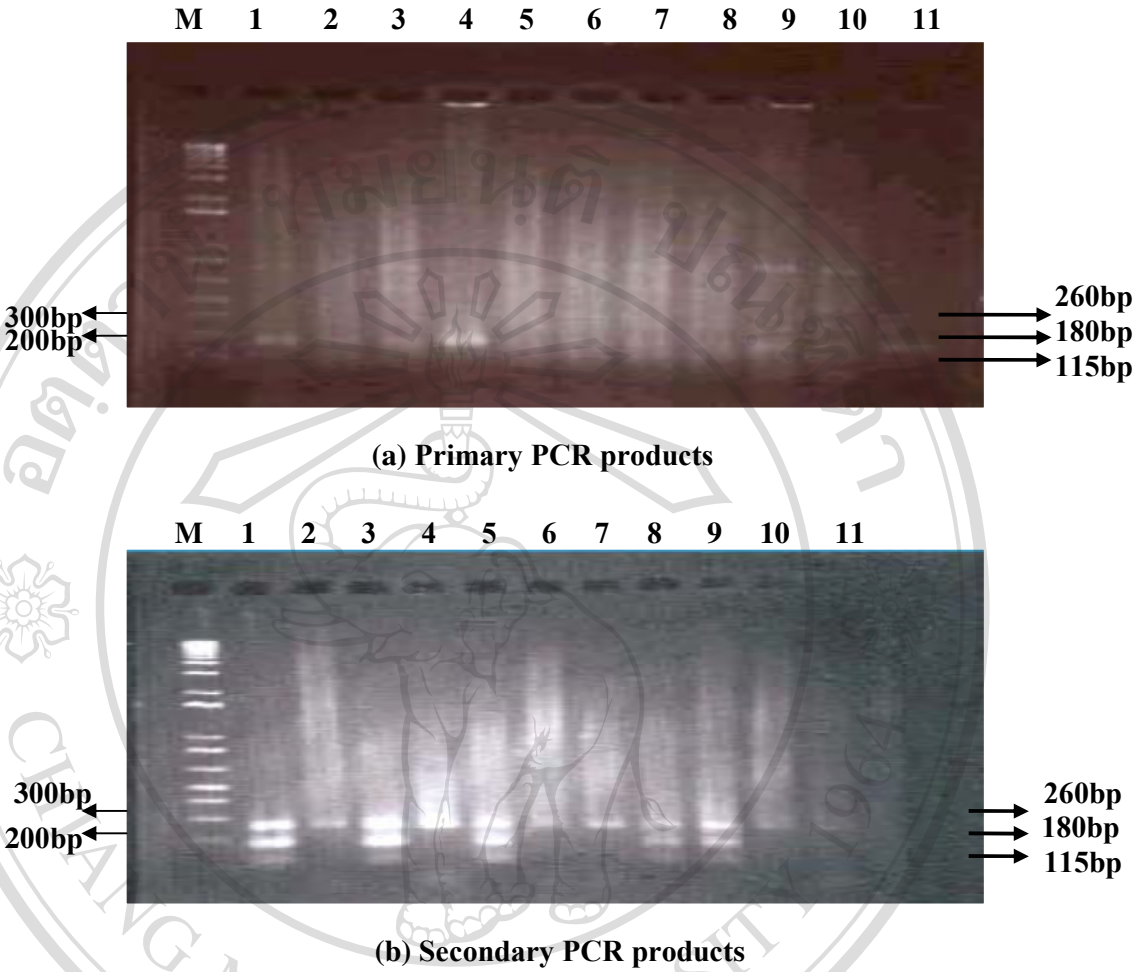

Fig. 8. Agarose gel electrophoretic analysis of primary and secondary PCR products obtained from multiplex-nested PCR amplification of DNA extracts of HIV-infected sex partners and HEPS. No primary PCR products could be detected from this experiment, but only secondary PCR products could be found, (a) primary PCR products, (b) secondary PCR products. A 1 Kb Plus DNA Ladder was used as a marker (lane M). Lane 1 shows the PCR product from the positive control, lane 2 shows the PCR product from the negative control, lane 3, 5, 8 and 9 show the PCR product from HIV-infected sex partners (H11P, H02P, H01P and H 07P, respectively), lane 4, 6, 7 and 10 show the PCR product from HEPS subjects (H11, H02, H03 and H 18, respectively).

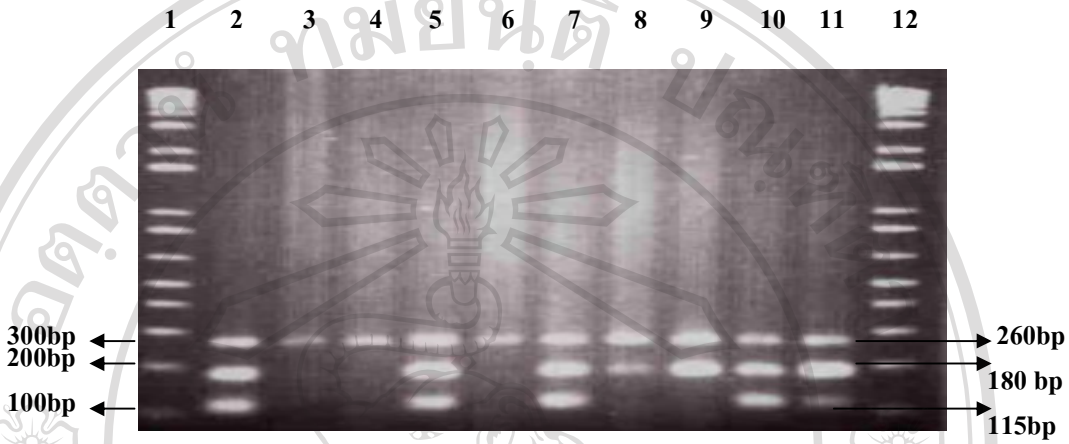

Fig. 9. Agarose gel electrophoretic analysis of secondary PCR products obtained from multiplex-nested PCR. Lanes: 1 and 12, base-pair ladder; 2, positive control; 3, negative control; 4, HEPS (H16); 5, H16P; 6, HEPS (H14); H02P, H01P, H15P, 07P and 08P (lane 7-11, respectively). The positive PCR results were 100% and 62.31% by *gag* and *pol* genes, respectively.

Table 12. Detection of proviral DNA in HIV-infected sex partners by Multiplex Nested PCR

Couple	visit 1 (0 mo)				visit 2 (3 mo)				visit 3 (06mo)				visit 4 (09mo)				visit 5 (12mo)			
	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result
no.	260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp	
H 01P	+	+	-	Pos.	+	+	-	Pos.	+	+	+	Pos.	+	+	-	Pos.	+	+	-	Pos.
H 02P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.
H 03P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.
H 04P	+	+	-	Pos.	+	+	+	Pos.	died				died				died			
H 06P	+	+	-	Pos.	+	+	+	Pos.	died				died				died			
H 07P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	ND	ND	ND	Pos.	ND	ND	ND	Pos.
H 08P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	ND	ND	ND	Pos.	ND	ND	ND	Pos.
H 09P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	ND	ND	ND	Pos.	ND	ND	ND	Pos.
H 10P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.
H 11P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	-	Pos.	+	+	-	Pos.
H 12P	+	+	-	Pos.	died				died				died				died			
H 13P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.
H 14P	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	-	Pos.	+	+	-	Pos.
H 15P	+	+	+	Pos.	+	+	-	Pos.	+	+	-	Pos.	ND	ND	ND	Pos.	+	+	+	Pos.
H 16P	+	+	-	Pos.	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.
H 18P	+	+	+	Pos.	+	+	+	Pos.	+	+	-	Pos.	+	+	+	Pos.	+	+	+	Pos.

Table 12. Detection of proviral DNA in HIV-infected sex partners by Multiplex Nested PCR (continued.)

(continued.)

Couple	visit 1 (0 mo)				visit 2 (3 mo)				visit 3 (06mo)				visit 4 (09mo)				visit 5 (12mo)			
	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result
no.	260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp	
H 19P	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.	+	+	+	Pos.

NOTE. +,Positive; Pos., Positive; -, Negative; Neg., Negative; mo., month; ND, Not Done

Table 13. Detection of proviral DNA in HIV highly- exposed persistently seronegative (HEPS) persons by Multiplex Nested PCR

Couple	visit 1 (0 mo)				visit 2 (3 mo)				visit 3 (06mo)				visit 4 (09mo)				visit 5 (12mo)			
	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result
no.	260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp	
H 01	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 02	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 03	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 04	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 05	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 06	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 07	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	ND	ND	ND		+	-	-	Neg.
H 08	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	ND	ND	ND		ND	ND	ND	
H 09	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	ND	ND	ND		ND	ND	ND	
H 10	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 11	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 12	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	ND	ND	ND		ND	ND	ND	
H 13	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 14	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 15	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	ND	ND	ND		+	-	-	Neg.
H 16	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.

Table 13. Detection of proviral DNA in HIV highly- exposed persistently seronegative (HEPS) persons by Multiplex Nested PCR (continued.)

Couple no.	visit 1 (0 mo)				visit 2 (3 mo)				visit 3 (06mo)				visit 4 (09mo)				visit 5 (12mo)			
	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result	reactivity band			Result
	260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp		260bp	180bp	115bp	
H 17	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 18	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.
H 19	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.	+	-	-	Neg.

NOTE. +,Positive; Pos., Positive; -, Negative; Neg., Negative; mo., month; ND, Not Done

Table 14. Detection of proviral DNA in HIV-infected sex partners and HIV highly- exposed persistently seronegative (HEPS) persons by Multiplex Nested PCR

Samples	months follow	*n*	nested PCR primers		
			gag (SK380/390 & 38/39)	pol (POL O1/O2 & I1/I2)	*β*-globin gene
HIV-infected sex partners	0	17	17	3	17
	3	16	16	13	16
	6	14	14	12	15
	9	10	10	7	10
	12	11	11	8	11
HEPS	0	19	0/19	0/19	19/19
	3	19	0/19	0/19	19/19
	6	19	0/19	0/19	19/19
	9	14	0/14	0/14	14/14
	12	15	0/15	0/15	15/15

6. Intracellular Cytokine Staining; ICCS

6.1 Flow cytometric dot-plots of cell surface staining

The frequencies of cytokine producing cells were performed with PMA and ionomycin stimulated whole blood in the presence of brefeldin-A. In these experiments, the negative control cells were unstimulated whole blood from the same subjects in the presence of brefeldin-A. Cells were stained for cytokine, CD69, CD4 and CD3 expression. The size and granularity of the cells were dramatically changed in the PMA-ionomycin stimulated cells compared to the unstimulated cells (**Figure 10A and B**).

In HIV infection, CD4 cells held the majority for the prognosis and progression of the disease, since they were stained directly in this study. Although the use of PMA and ionomycin resulted in a much larger down-regulation of CD4 than CD8, CD4 was chosen as a marker in these experiments, and CD4+ cells were main subjects to analyse cytokine response. CD4+ lymphocytes remained easy to distinguish, with a minimal decrease in the percentage of positive cells (Pala P., 2000). The problem of autofluorescence was detected by isotype control (**Figure 11**). Thus, the separation of positive and negative populations was enhanced.

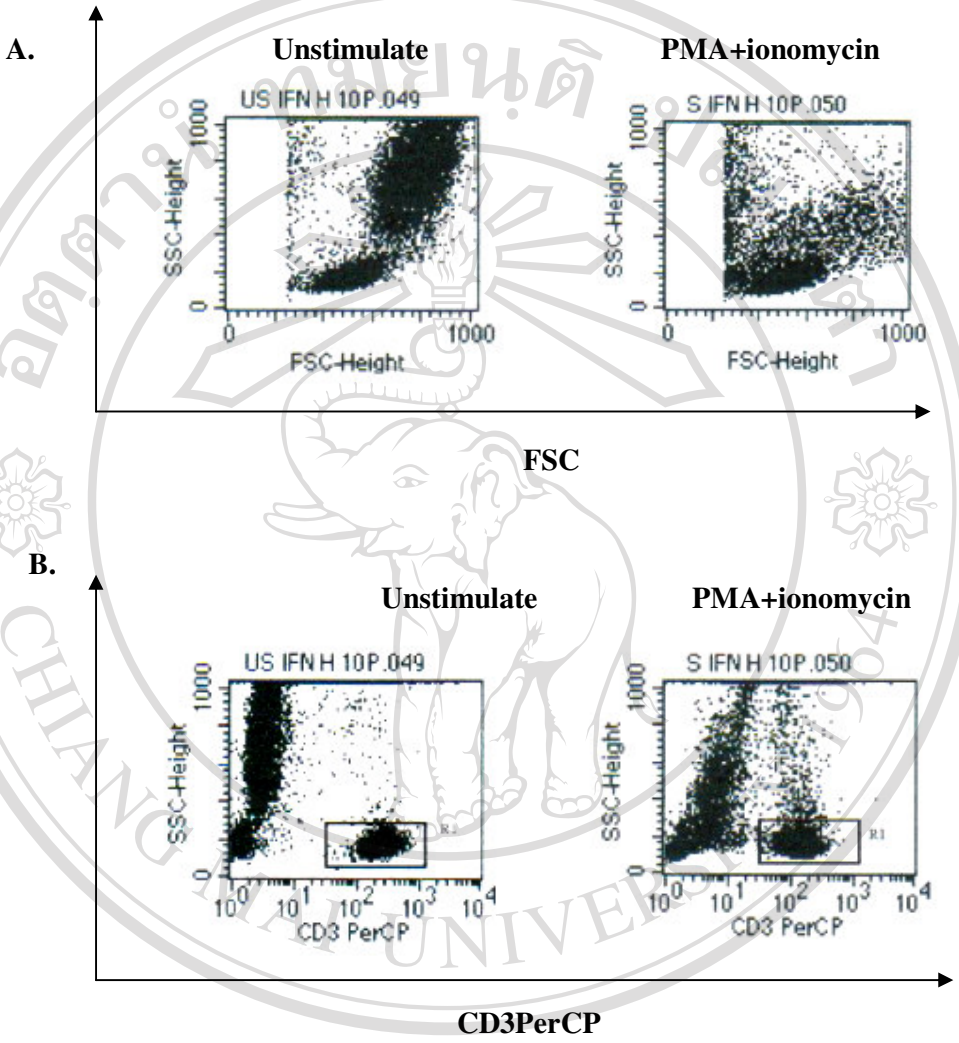

Figure 10. Experiments were performed to study cytokines expression in fresh whole blood (within 6 hours) after PMA and ionomycin stimulation for 6 hours. The cells were stained for CD3, CD4, CD69/cytokines, simultaneously. The scatter properties of cells of side scatter (SSC) and forward scatter (FSC) were changed after stimulation (**A**). The CD3 T cells were gated (**B**).

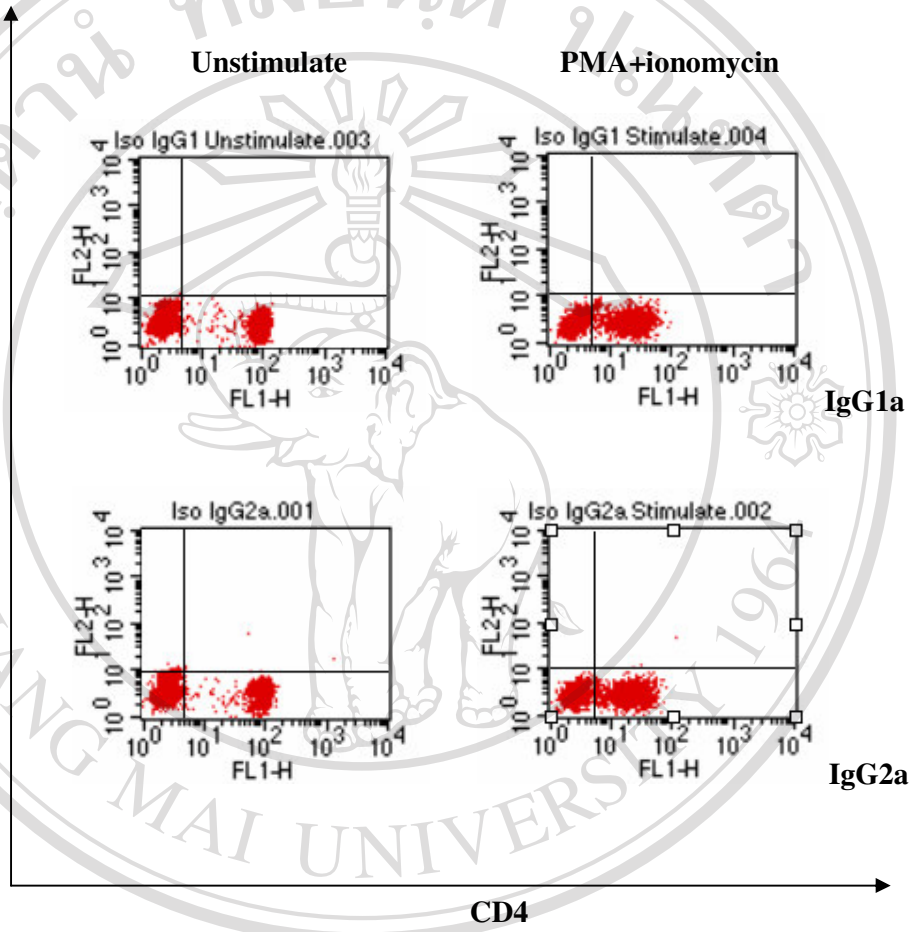

Figure 11. The problem of autofluorescence was detected by isotype control; IgG1a and IgG2a clones were used. Different patterns of CD4+ cells between those unstimulated and stimulated with PMA and ionomycin were shown are shown.

6.2 Detection of CD69 on T Cells Stimulated with PMA and ionomycin

CD69 expression and intracellular cytokine production were measured after 6 hours incubation. Unstimulated CD4+/CD4- cells did not show any expression of cytokines or the early activation marker, CD69. Furthermore, stimulation by PMA and ionomycin led to a high upregulation of CD69 and a proportion of cytokine expressing cells (**Figure 12A.**). CD69 was analysed and shown to be expressed in >95% of the T cells in all the experiments. Brefeldin-A amplified the positive signal, and the incidence of cytokine expressing cells was readily detected (**Figure 12B.**). Thus, the percentage of cytokine-producing cells after 6 hours in these experiments represented the true overall potential for cytokine production in cell populations.

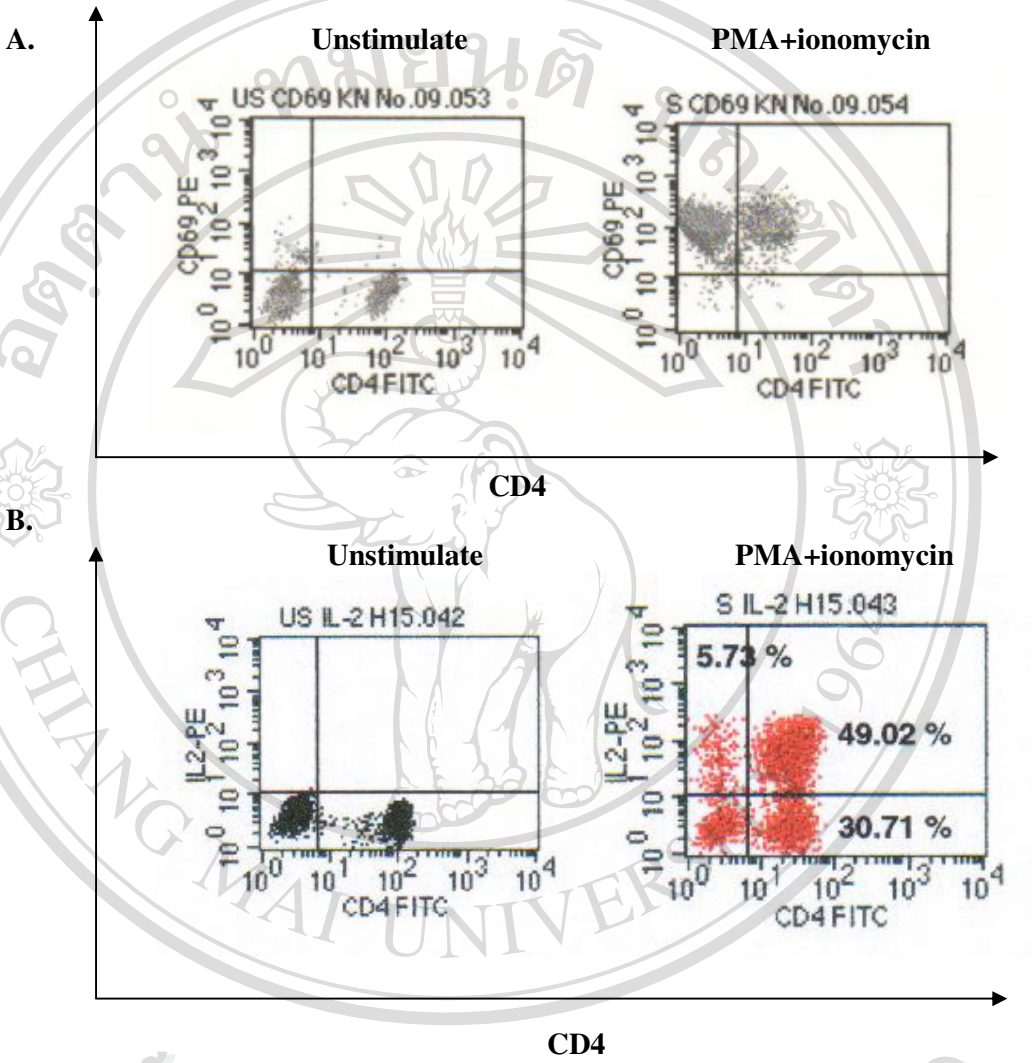

Figure 12. Flow cytometric dot plots of CD69 expression after 6 hours on unstimulated and PMA/ionomycin stimulated CD4 T lymphocytes in the presence of brefeldin-A from a representative healthy individual. Unstimulated cells served as controls (**A**). Unstimulated T cells did not show any CD69 or cytokine expression. PMA–ionomycin stimulation generated a vast activation of T cells as indicated by the high expression of CD69. The cytokine expression seen in the Golgi-compartment of the cells resulted in a number of positive cells detected by flow cytometry. The addition of brefeldin-A significantly enhanced the staining signals for cytokine expressing cells and a high number of positive cells were detected (**B**).

6.3 Flow cytometric dot-plots of intracellular cytokine staining

Patterns of cytokine production in whole blood after 6-hours stimulation with PMA and ionomycin were detected at the single cell level using intracellular staining and flow cytometry. Typical dot-plots of intracellular IL-2, IL-4, and IFN-γ production by cells from a HEPS code H03 are shown in **Figure 13**. Co-staining with CD4+ enabled CD3+ T cells to be subdivided into CD4+ and CD4- cells (**Figure 13**). Those cells that did not stain for CD4 (left-hand quadrants) were assumed to represent the CD4- (CD8) T helper T cell population. Blood samples from all HEPS subjects, HIV-infected sex partners and the normal control group were similarly analyzed, and the percentage of each cytokine-producing cell population in a total population of CD4+ cells and CD4-(CD8) cells was calculated. The number shown in each quadrant plot represents the percentage of cytokine producing cells. HEPS persons and HIV-infected sex partners were studied at 6, 9 and 12 months follow up for intracellular cytokine production within the CD4 T cell subset and compared with the normal control group.

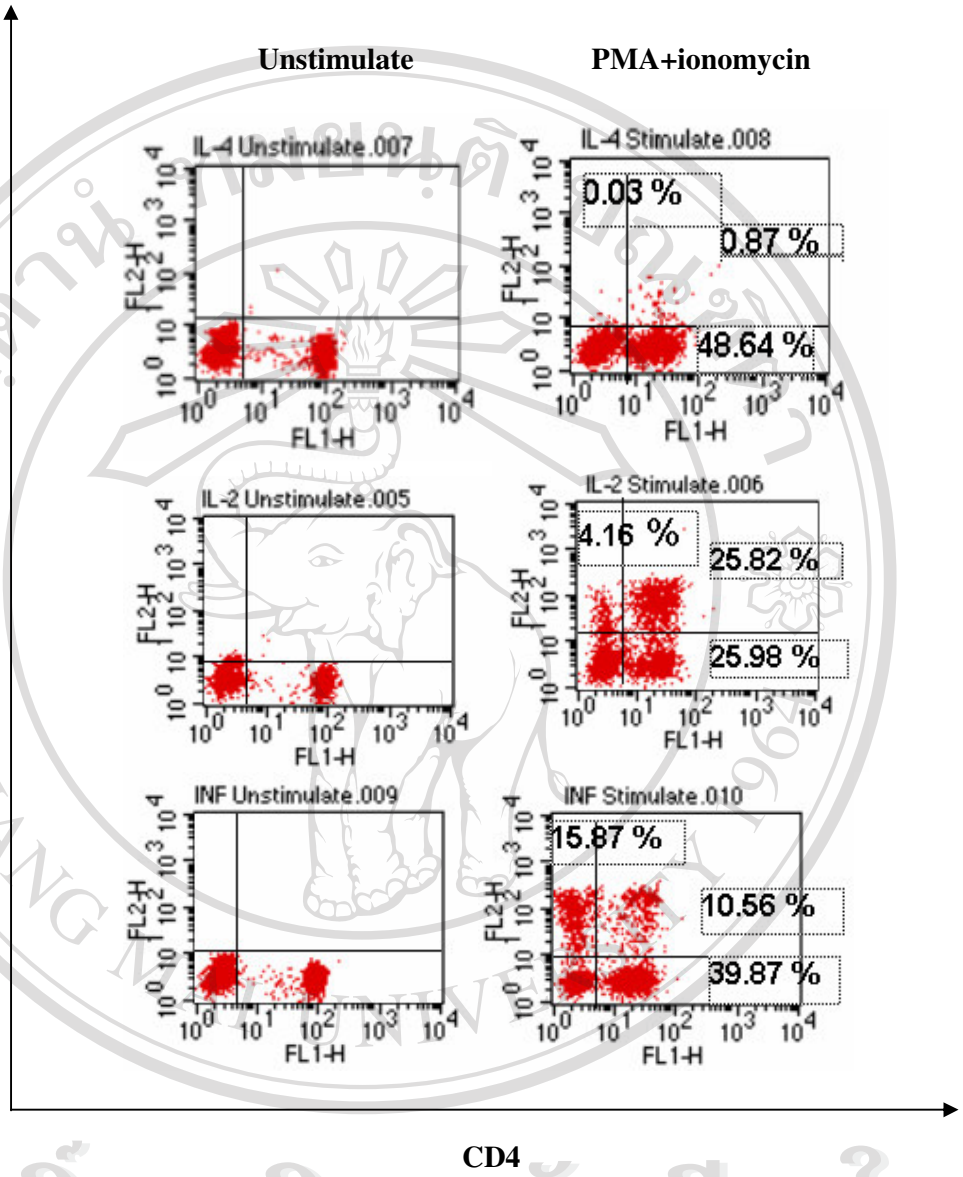

Figure13. Typical flow cytometric analysis of IL-2, IL-4 and IFN-γ production. Whole blood was stimulated with PMA and ionomycin for 6 hours, and their production of IL-2, IL-4 and IFN-γ was determined at the single cell level by intracellular cytokine staining and flow cytometry. The percentages in the upper and lower right quadrants represent the percentage of CD4+ cells with or without expression of IL-2, IL-4 and IFN-γ in the total lymphocyte population gated, whereas, the percentages in the upper left quadrants represent the percentage of CD4- (CD8+) with expression of IL-2, IL-4 and IFN-γ in the total lymphocyte population gated.

6.4 Detection of cytokine production at the single-cell level within the T cells in normal controls

In this experiment, the 6 hours mitogen stimulation of fresh whole blood with the combination of PMA (25 ng/mL) and an ionomycin (1 μg/mL) in the presence of Brefeldin A (10 μl/mL) resulted in the following percentages of CD4+ cells stained intracellularly: for IL-2-24.00-49.06% (mean=35.64±7.03%), IFN-γ-5.05-14.21% (mean=9.95±3.02%) and IL-4-0.54-2.57% (mean=1.38±0.49%) (**Table 15.**) These results were similar to values previously reported (Picker LJ., 1995, Prussin C., 1995, Jung T., 1993).

6.5 Detection of cytokine production at the single-cell level within the T cells in each group at visit 3, 4 and 5 (6, 9 and 12 months) of the follow up.

In this study, all HEPS individuals and HIV-infected sex partners were found to have IL-2-producing T cells, IFN-γ-producing T cells and IL-4-producing T cells in third, fourth and fifth visit (6, 9 and 12 months) of the follow up, respectively.

Intracellular staining and flow cytometry identified a heterologous pattern of cytokine-producing CD4+ T cells in PMA/I-stimulated whole blood from both the HIV-infected sex partners and the normal controls, but a homologous pattern was identified in both HEPS subjects and normal controls. When comparing each individual in the HEPS and normal control group by the Wilcoxon matched pairs test, there were no significant differences in cytokine production of T cells in each group as shown in **Figure 14, Table 15, 16, 17 and 18.**

Cytokine production patterns at the single-cell level within the CD4 T cell subset.

Interleukin-2 (IL-2)-producing CD4+ T cells at 6, 9 and 12 months follow-up

IL-2-producing CD4 T cells after stimulation with PMA/ionomycin was studied in 19 HEPS individuals, 14 HIV-infected sex partners and 15 healthy controls at 6 months follow up. Unfortunately, 3 HIV-infected sex partners, 7 HIV-infected sex partners and 5 HEPS persons, and 6 HIV-infected sex partners and 4 HEPS persons were lost to follow up at 6, 9 and 12 months, respectively. A highly significant difference in IL-2-producing CD4 T cells was observed between HEPS groups and HIV-infected sex partners ($p<0.001$), and HIV-infected sex partners and healthy groups ($p<0.001$) at 6, 9 and 12 months follow up. The proportion of IL-2-producing CD4 T cells from HEPS individuals was higher than the samples from HIV-infected sex partners (35.15±7.94% vs. 7.35±8.72%; $p<0.001$, 30.62±7.74 % vs. 8.64±6.65; $p<0.001$, 30.30±7.41% vs. 11.25±8.77%; $p<0.001$) at 6, 9 and 12 months follow up, as shown in **Table 15** and **Figure 15.** On the other hand, there was no significant difference in the proportion of IL-2-producing CD4 T cells between HEPS individuals and healthy control groups (35.15±7.94% vs. 35.64±7.03%; $p>0.05$, 30.62±7.74 % vs. 35.64±7.03; $p>0.05$, 30.30±7.41% vs. 35.64±7.03; $p>0.05$) at 6, 9 and 12 months follow up, as shown in **Table 15** and **Figure 15.**

In order to disregard the possibility that the differences in cytokine producing cells between HIV-infected sex partners and the healthy control samples described above were simply due to decreased CD4, the data to show the percentage of CD4+ T cells synthesizing IL-2 as a proportion of the total CD4+ population were normalized (**Table 16**). It is clear that in HIV-infected sex partners, CD4+ T cells had to impaired ability synthesize IL-2 compared with normal controls in response to stimulation with PMA/Ionomycin (0.38±0.19 % vs. 0.62±0.07%; $p<0.001$, 0.42±0.16% vs. 0.62±0.07%; $p<0.001$, 0.42±0.11 % vs. 0.62±0.07%; $p<0.001$; **Table 16**) at 6, 9 and 12 months follow up.

Interferon-gamma (IFN-γ) producing CD4+ T cells at 6, 9 and 12 months follow-up

A similar observation was found in HEPS individuals, HIV-infected sex partners and healthy controls studied here. A highly significant difference in IFN-γ-producing CD4 T cells was observed between HEPS groups, HIV-infected sex partners and healthy groups at 6, 9 and 12 months follow up. The proportion of IFN-γ-producing CD4 T cells from HEPS individuals was higher than those from HIV-infected sex partners (12.08±4.89% vs. 4.39±3.08%; $p<0.001$, 12.68±4.75% vs. 3.81±1.86%; $p<0.001$, 11.20±4.62% vs. 5.43±2.95%; $p<0.05$) at 6, 9 and 12 months follow up, as shown in **Table 15** and **Figure 16.** There was no significant difference in the proportion of IFN-γ-producing CD4 T cells between HEPS persons and healthy controls (12.08±4.89% vs. 9.95±3.02%; $p>0.05$, 12.68±4.75% vs. 9.95±3.02%; $p>0.05$, 11.20±4.62% vs. 9.95±3.02%, $p>0.05$) at 6, 9 and 12 months follow up. There was a significant difference in the proportion of IFN-γ-producing CD4 T cells between HIV sex partners and healthy controls (4.39±3.08% vs. 9.95±3.02%; $p<0.001$, 3.81±1.86% vs. 9.95±3.02%; $p<0.001$, 5.43±2.95% vs. 9.95±3.02%; $p<0.05$) at 6, 9 and 12 months follow up, as shown in **Table 15** and **Figure 16.**

For normalization of the data, the percentage of CD4+ T cells synthesizing IFN-γ was a proportion of the total CD4+ population**.** There was an increased ability of CD4+ T cells to synthesize IFN-γ in HIV positive individuals in comparison with healthy controls in response to stimulation (0.39±0.26 % vs. 0.18±0.04%; $p<0.05$, 0.27±0.11% vs. 0.18±0.04%; $p<0.05$, 0.25±0.08% vs. 0.18±0.04%; $p<0.05$; **Table 16**) at 6, 9 and 12 months follow up.

Interleukin-4 (IL-4) producing CD4+ T cells at 6, 9 and 12 months follow-up

The percentage of IL-4-producing cells in T cells from HEPS individuals did not differ significantly from that in healthy controls (1.13±0.65% vs. 1.38±0.49% ; $p>0.05$, 1.19±0.65% vs. 1.38±0.49%; $p>0.05$, 1.38±1.19 % vs. 1.38±0.49%; $p>0.05$; **Table 15** and **Figure 17**) at 6, 9 and 12 months follow up. However, there was a significantly different proportion of T cells that stained positive for IL-4-producing CD4 T cells between HIV-infected sex partners and healthy controls at 6, 9 and 12 months follow-up (0.45±0.26% vs. 1.38±0.49%; $p<0.05$, 0.74±0.41% vs. 1.38±0.49%; $p<0.001$, 0.72±0.43 % vs. 1.38±0.49%; $p<0.001$; **Table 15** and **Figure 17**).

For normalization of the data, the percentage of CD4+ T cells synthesized IL-4 as a proportion of the total CD4+ population**.** CD4+ T cells had no significant ability to synthesize IL-4 in response to stimulation in HEPS individuals compared with healthy controls (0.02±0.01% vs. 0.02±0.01%; $p>0.05$, 0.02±0.01% vs. 0.02±0.01%; $p>0.05$) at 6 and 9 months follow up, but they did have significant ability to synthesize IL-4 in response to stimulation in HEPS individuals compared with healthy controls (0.02±0.02% vs. 0.02±0.01%; $p<0.05$) at 12 months follow up (**Table 16)**. For HIV-infected sex partners, CD4+ T cells had no significant ability to synthesize IL-4 in response to stimulation in HIV sex partners compared with healthy controls (0.05±0.03% vs. 0.02±0.01%; $p>0.05$, 0.04±0.04 % vs. 0.02±0.01%; $p>0.05$) at 6 and 12 months follow up, but they did have significant ability to synthesize IL-4 in response to stimulation in HEPS individuals compared with healthy controls (0.05±0.04 % vs. 0.02±0.01%; $p<0.05$) at 9 months of follow up.

Intracellular cytokine analysis of CD8 cells in HIV-infected sex partners and healthy controls.

Interleukin-2 (IL-2)-producing CD8+ T cells at 6, 9 and 12 months follow-up

IL-2-producing CD4- T cells (CD8+ T cells) after stimulation with PMA/ionomycin were evaluated in all subjects at 6 months follow up. No significant difference in IL-2-producing CD8 T cells was found between HIV-infected sex partners and normal controls ($p>0.05$), while a lower significant difference in IL-2-producing CD8 T cells was found in HEPS persons when compared with healthy groups ($p<0.001$) at 6, 9 and 12 months follow up. The percentages of IL-2 cells in CD8+ cells of HIV-infected sex partners ranged from 3.72 to 24.95%, 0.34 to 29.38% and 3.73 to 28.35% (mean±SEM were 9.60±6.39%, 9.59±8.01%, and 9.65±6.80%) at 6, 9 and 12 months follow up, respectively as shown in **Table 17**. The proportion of IL-2-producing CD8 T cells from HEPS individuals was lower than that in the samples from healthy controls (0.17±0.07 vs. 0.24±0.05; $p<0.05$, 0.17±0.06% vs. 0.24±0.05%; $p<0.05$, 0.13±0.07% vs. 0.24±0.05% ; $p<0.001$) at 6, 9 and 12 months follow up as shown in **Table 18.** On the other hand, HIV seropositive subjects had reduced IL-2 production, which was lower than that in the samples from HEPS individuals.

Interferon-gamma (IFN-γ) producing CD8+ T cells at 6, 9 and 12 months follow-up

A highly significant difference in IFN-γ-producing CD8 T cells was observed in HIV-infected sex partners when compared with HEPS and healthy controls. The proportion of IFN-γ-producing CD8 T cells from HIV-infected sex partners was higher than that in the samples from healthy controls (54.85±18.52% vs. 25.73±7.38%; $p<0.001$, 52.47±15.23% vs. 25.73±7.38%; $p<0.001$, 41.98±11.92% vs. 25.73±7.38%; $p<0.001$) at 6, 9 and 12 months follow up as shown in **Table 17.** Likewise, no significant difference in the proportion of IFN-γ-producing CD8 T cells was found between HIV-infected sex partners, HEPS groups and healthy controls as shown in **Table 18**.

For normalization of the data, the percentage of CD8+ T cells synthesizing IFN-γ was a proportion of the total CD8+ population**.** There was not increased ability of CD8+ T cells to synthesize IFN-γ in HIV positive individuals in comparison with healthy controls in response to stimulation (0.66±0.15 % vs. 0.57±0.09%; $p>0.05$, 0.65±0.13% vs. 0.57±0.09%; $p>0.05$, 0.57±0.16% vs. 0.57±0.09%; $p>0.05$; **Table 18**) at 6, 9 and 12 months follow up.

Interleukin-4 (IL-4) producing CD8+ T cells at 6, 9 and 12 months follow-up

The percentage of IL-4-producing cells in CD8 T cells from HEPS individuals was significantly lower than that in the healthy controls (0.10±0.09% vs. 0.36±0.40%; $p<0.05$, 0.27±0.41% vs. 0.36±0.40%; $p<0.05$; **Table 17**.) at 6 and 12 months follow up, but a significant difference was not found at 9 months follow up (0.14±0.16% vs. 0.36±0.40%; $p>0.05$, **Table 17**). On the other hand, the proportion of IL-4-producing CD8 T cells from HIV-infected sex partners showed no significant difference between HEPS persons, HIV-infected sex partners and healthy controls as shown in **Table 18**.

Table 15. Percentage of interleukin (IL)-2-producing CD4 T cells, interferon gamma (IFN-γ)-producing CD4 T cells and interleukin (IL)-4-producing CD4 T cells in healthy control subjects, HEPS individuals and HIV-seropositive sex partners.

Subject, follow up	*n*	Percentage of cells within the CD4+ subset		
		IL2+	IFN-γ+	IL-4+
Control	15	35.64±7.03	9.95±3.02	1.38±0.49
HEPS				
Visit 3 (6 mo.)	19	35.15±7.94 [c, e]	12.08±4.89 [c, e]	1.13±0.65 [c, d]
Visit 4 (9 mo.)	14	30.62±7.74 [c, e]	12.68±4.75 [c, e]	1.19±0.65 [c, f]
Visit 5 (12 mo.)	15	30.30±7.41 [c, e]	11.20±4.62 [c, d]	1.38±1.19 [c, f]
HIV-seropositive				
Visit 3 (6 mo.)	14	7.35±8.72 [b]	4.39±3.08 [b]	0.45±0.26 [b]
Visit 4 (9 mo.)	10	8.64±6.65 [b]	3.81±1.86 [b]	0.74±0.41 [a]
Visit 5 (12 mo.)	11	11.25±8.77 [b]	5.43±2.95 [a]	0.72±0.43 [a]

NOTE. Data are mean ± SEM.

[a] $p<0.05$, vs. healthy control subjects.

[b] $p<0.001$, vs. healthy control subjects.

[c] $p>0.05$, vs. healthy control subjects.

[d] $p<0.05$, vs. HIV-seropositive.

[e] $p<0.001$, vs. HIV-seropositive.

[f] $p>0.05$ vs. HIV-seropositive.

Table 16. Percentage proportion of interleukin (IL)-2-producing CD4 T cells with the total CD4+ population, interferon gamma (IFN-γ)-producing CD4 T cells with the total CD4+ population and interleukin (IL)-4-producing CD4 T cells with the total CD4+ population in healthy control subjects, HEPS individuals and HIV-seropositive sex partners.

Subject, follow up	***n***	**Percentage of cells within the CD4+ subset**		
		IL2+	**IFN-γ+**	**IL-4+**
Control	15	0.62±0.07	0.18±0.04	0.02±0.01
HEPS				
Visit 3 (6 mo.)	19	0.59±0.07 [c, d]	0.21±0.09 [c, d]	0.02±0.01 [c, d]
Visit 4 (9 mo.)	14	0.54±0.10 [a, d]	0.24±0.09 [c, f]	0.02±0.01 [c, d]
Visit 5 (12 mo.)	15	0.49±0.09 [b, f]	0.19±0.08 [c, d]	0.02±0.02 [a, f]
HIV-seropositive				
Visit 3 (6 mo.)	14	0.38±0.19 [b]	0.39±0.26 [a]	0.05±0.03 [c]
Visit 4 (9 mo.)	10	0.42±0.16 [b]	0.27±0.11 [a]	0.05±0.04 [a]
Visit 5 (12 mo.)	11	0.42±0.11 [b]	0.25±0.08 [a]	0.04±0.04 [c]

NOTE. Data are mean ± SEM.

[a] p<0.05, vs. healthy control subjects.

[b] p<0.001, vs. healthy control subjects.

[c] p>0.05, vs. healthy control subjects.

[d] p<0.05, vs. HIV-seropositive.

[e] p<0.001, vs. HIV-seropositive.

[f] p>0.05, vs. HIV-seropositive.

Table 17. Percentage of interleukin (IL)-2-producing CD8 T cells, interferon gamma (IFN-γ)-producing CD8 T cells and interleukin (IL)-4-producing CD8 T cells in healthy control subjects, HEPS individuals and HIV-seropositive sex partners.

Subject, follow up	*n*	Percentage of cells within the CD8+ subset		
		IL2+	IFN-γ+	IL-4+
Control	15	10.15±3.31	25.73±7.38	0.36±0.40
HEPS				
Visit 3 (6 mo.)	19	6.68±3.07 [a, f]	20.14±9.13 [a, e]	0.10±0.09 [a, e]
Visit 4 (9 mo.)	14	7.26±3.54 [a, f]	24.83±12.99 [c, e]	0.14±0.16 [c, d]
Visit 5 (12 mo.)	15	5.66±3.49 [a, d]	20.88±10.66 [c, e]	0.27±0.41 [c, d]
HIV-seropositive				
Visit 3 (6 mo.)	14	9.60±6.39 [c]	54.85±18.52 [b]	1.24±1.73 [c]
Visit 4 (9 mo.)	10	9.59±8.01 [c]	52.47±15.23 [b]	1.47±2.57 [a]
Visit 5 (12 mo.)	11	9.65±6.80 [c]	41.98±11.92 [b]	1.36±2.16 [c]

NOTE. Data are mean ± SEM.

[a] $p<0.05$, vs. healthy control subjects.

[b] $p<0.001$, vs. healthy control subjects.

[c] $p>0.05$, vs. healthy control subjects.

[d] $p<0.05$, vs. HIV-seropositive.

[e] $p<0.001$, vs. HIV-seropositive.

[f] $p>0.05$ vs. HIV-seropositive.

Table 18. Percentage proportion of interleukin (IL)-2-producing CD8 T cells with the total CD8+ population, interferon gamma (IFN-γ)-producing CD8 T cells with the total CD8+ population and interleukin (IL)-4-producing CD8 T cells with the total CD8+ population in healthy control subjects, HEPS individuals and HIV-seropositive sex partners.

Subject, follow up	*n*	Percentage of cells within the CD8+ subset		
		IL2+	IFN-γ+	IL-4+
Control	15	0.24±0.05	0.57±0.09	0.01±0.01
HEPS				
Visit 3 (6 mo.)	19	0.17±0.07 [a, d]	0.50±0.16 [c, d]	0.002±0.002 [c, d]
Visit 4 (9 mo.)	14	0.17±0.06 [a, f]	0.54±0.18 [c, f]	0.003±0.003 [c, d]
Visit 5 (12 mo.)	15	0.13±0.07 [a, f]	0.48±0.17 [c, f]	0.01±0.01 [c, d]
HIV-seropositive				
Visit 3 (6 mo.)	14	0.12±0.07 [a]	0.66±0.15 [c]	0.013±0.018 [c]
Visit 4 (9 mo.)	10	0.12±0.09 [a]	0.65±0.13 [c]	0.017±0.029 [c]
Visit 5 (12 mo.)	11	0.13±0.08 [a]	0.57±0.16 [c]	0.02±0.02 [c]

NOTE. Data are mean ± SEM.

[a] p<0.05, vs. healthy control subjects.

[b] p<0.001, vs. healthy control subjects.

[c] p>0.05, vs. healthy control subjects.

[d] p<0.05, vs. HIV-seropositive.

[e] p<0.001, vs. HIV-seropositive.

[f] p>0.05 vs. HIV- seropositive.

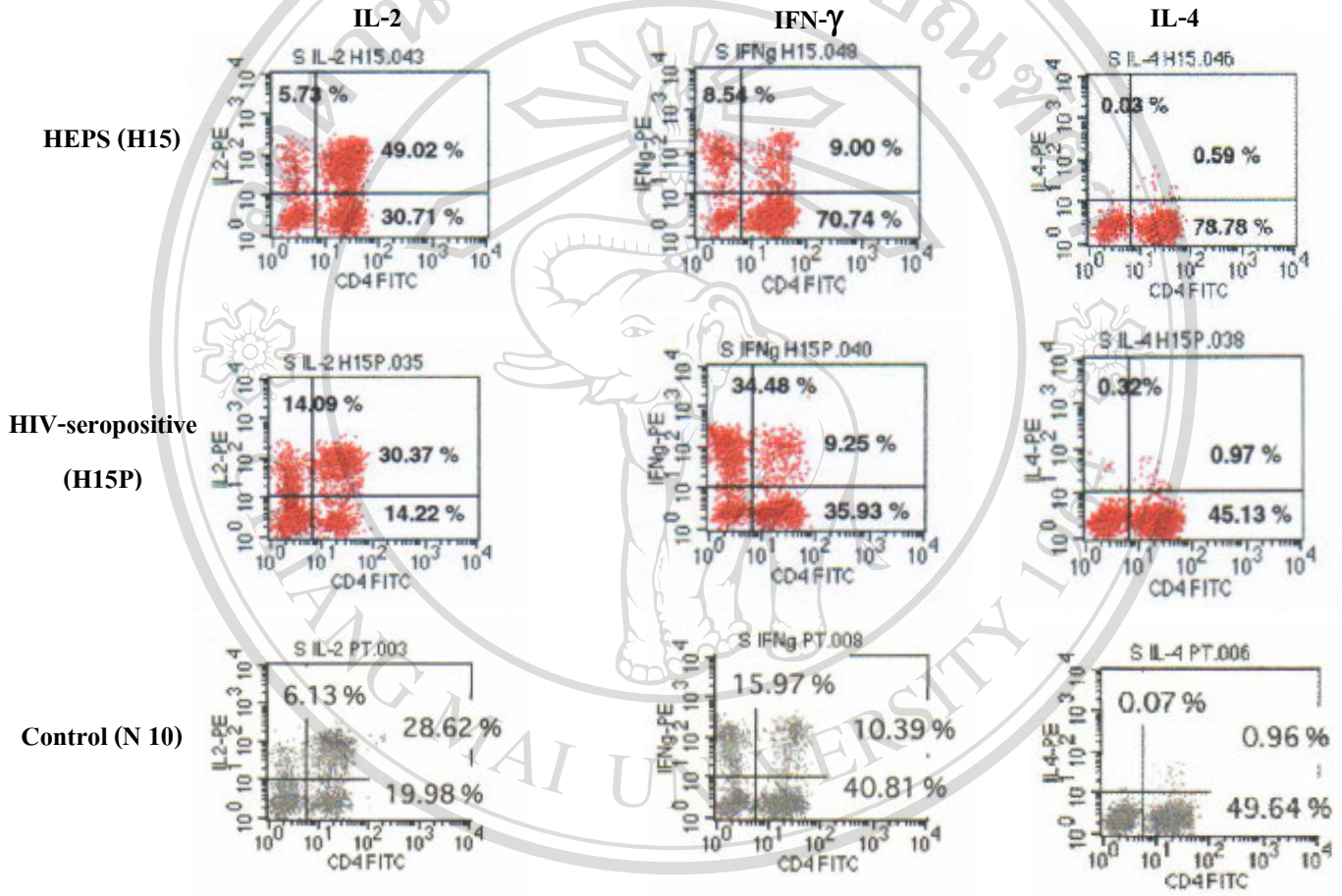

Figure 14. Analysis of interferon-gamma (IFN-γ), interleukin-2 (IL-2) and interleukin-4 (IL-4) production at the single cell level by flow cytometry within the CD4 T cell subset. Representative example of the flow cytometric analysis of CD-gated lymphocytes intracellularly stained for IFN-γ, IL-2 and IL-4 from HEPS individuals, normal controls and HIV-infected sex partners.

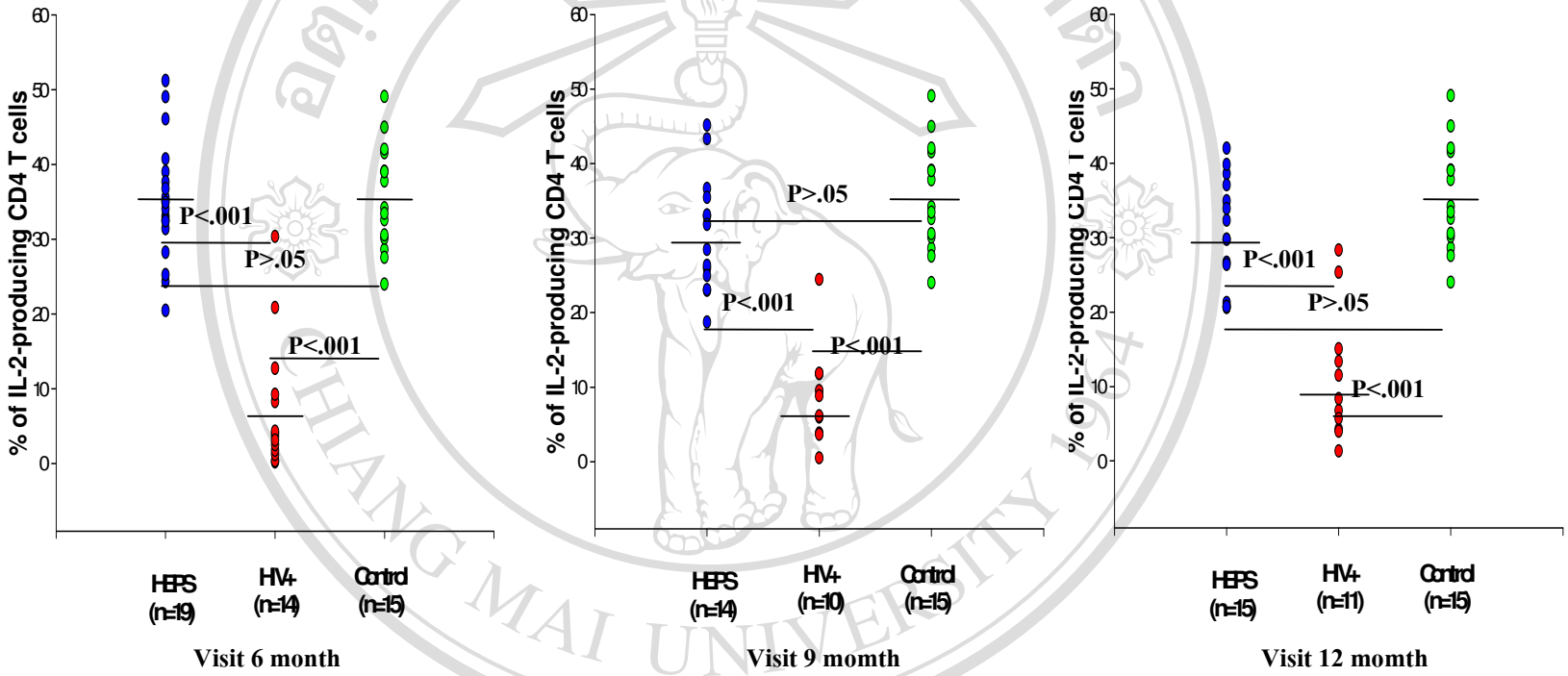

Figure 15. Analysis of interleukin (IL-2) production at the single cell level by flow cytometry within the CD4 T cell subset. Percentage of CD4 T cells able to produce IL-2 in HEPS individuals, normal controls and HIV-infected sex partners. Each symbol represents 1 individual. Bars represent mean. +, Positive; -, Negative; ● , HEPS; ● , HIV seropositive; ● , Normal control.

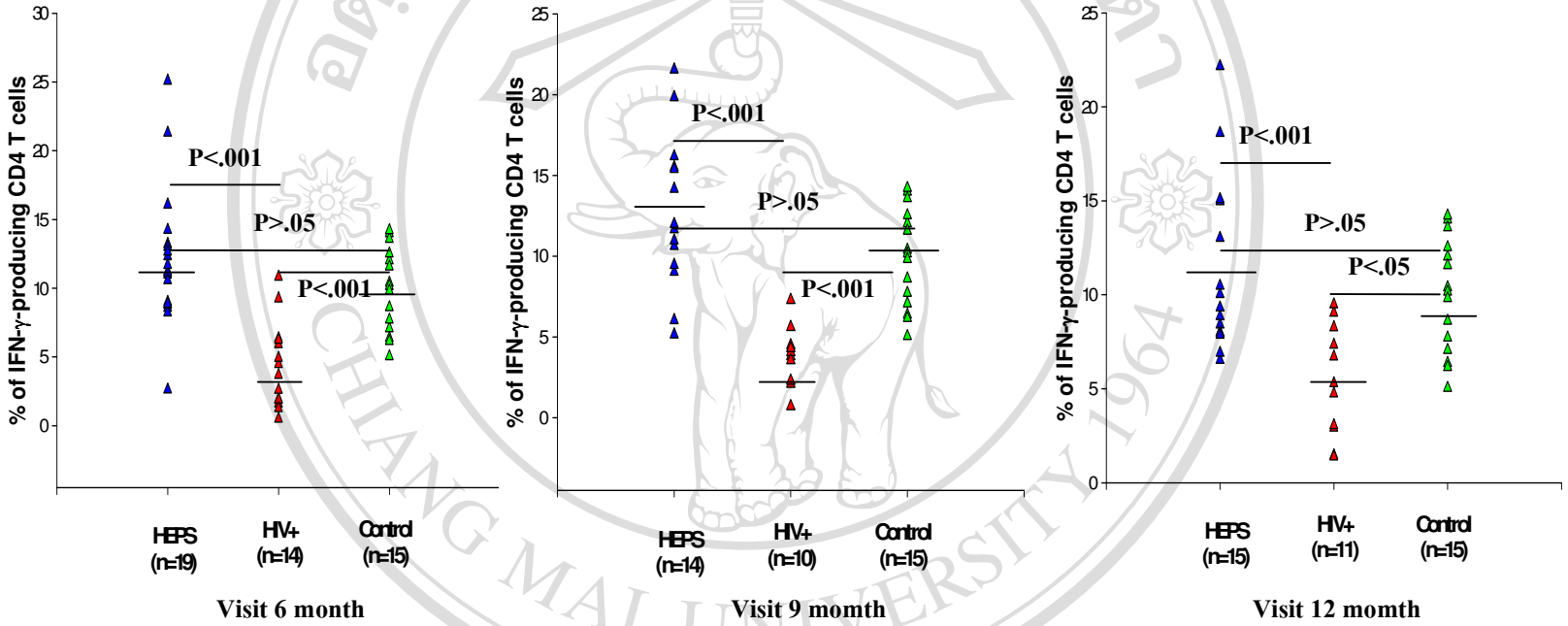

Figure 16. Analysis of interferon-gamma (IFN-γ) production at the single cell level by flow cytometry within the CD4 T cell subset. Percentage of CD4 T cells able to produce IFN-γ in HEPS individuals, normal controls and HIV-infected sex partners. Each symbol represents 1 individual. Bars represent mean. +, Positive; -, Negative; ▲, HEPS; ▲, HIV seropositive; ▲, Normal control.

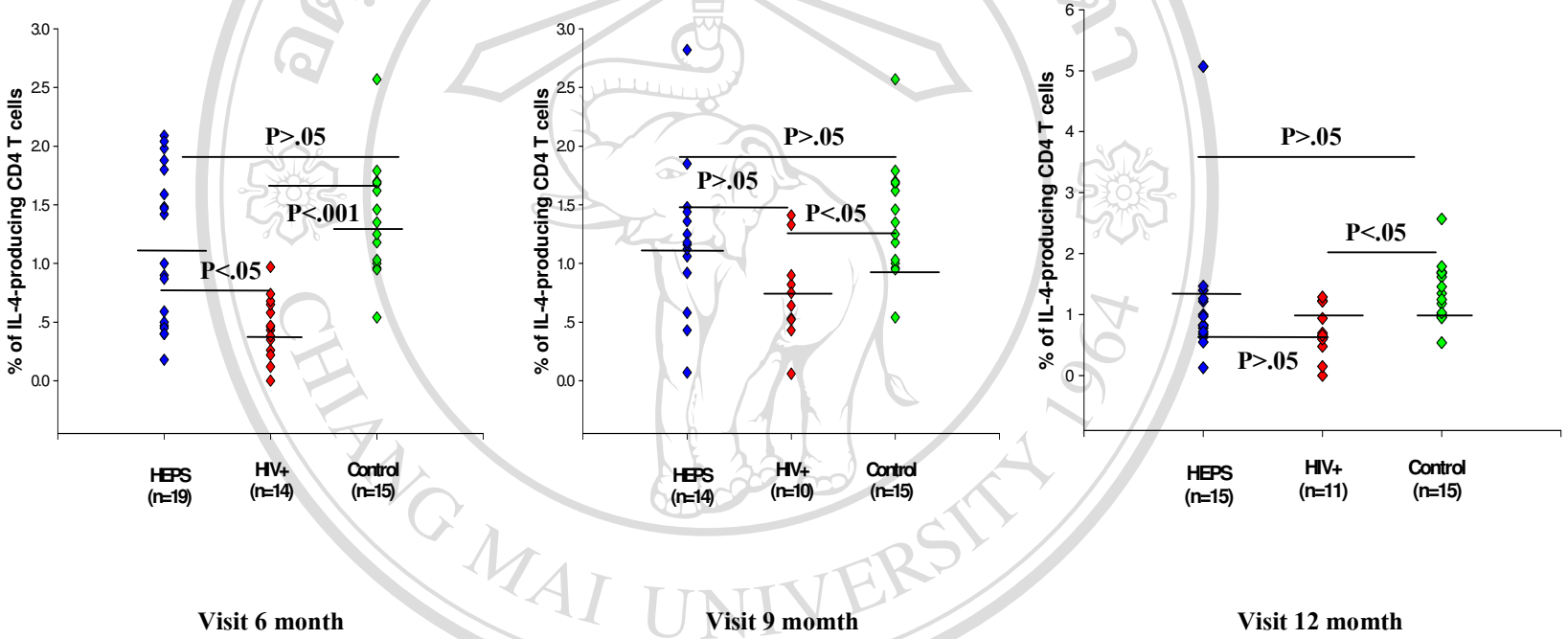

Figure 17. Analysis of interferon-gamma (IL-4) production at the single cell level by flow cytometry within the CD4 T cell subset. Percentage of CD4 T cells able to produce IL-4 in HEPS individuals, normal controls and HIV-infected sex partners. Each symbol represents 1 individual. Bars represent means. +, Positive; -, Negative; , HEPS; , HIV seropositive; , Normal control.