CHAPTER V

RESULTS

1. Demographic data

The demographic characteristics were summarised in Table 2. For the demographic of HIV-infected subjects with HLA-A11 patients (Table 3), all 18 patients were asymptomatic and antiretrovirals naïve. These patients included 11 women and 7 men, with a mean age of 33.3 years (ranging from 27 to 45 years). Majority of patients was heterosexuals (15/18 or 83%) and only 17% (2/18) of them were homosexuals. At the time of enrolment, these individuals have been diagnosed HIV-positive for 2 to 11 years, with a mean of 5 years. There were 9 patients whose HIV subtype was identified. Eight patients were subtype A/E (88%), whereas only two patients were subtype B. The CD4+ T lymphocytes were ranged from 325 to 730 cells/ mm^3 with median of 463 cells/ mm^3. Median plasma viral load was 10875 copies/ml (ranging from 3118 to 67,000 copies/ml). For 5 HLA-A11 negative HIV-infected patients, age of the patients ranged from 18 to 47 years with mean of 33.3 years. The time which these individual had been diagnosed HIV-positive ranged from 2 to 10 years with a mean of 4 years. HIV-subtype of these patients was not identified. The CD4+ T lymphocytes ranged from 404 to 711 cells/mm^3 with median of 563 cells/mm^3. Median plasma viral load was 3673 copies/ml (ranging from 155 to 9410 copies/ml). In addition, HIV- seronegative HLA-A11-positive patients, these patients included 3 women and 2 men with a mean age of 39.4 years (ranging from 35 to 51 years). For the HLA typing data of HIV-infected patients, eighteen of twenty three patients were HLA-A11 (82.14%) (Table 4).

Overall donors	HIV^+, $HLA\ A11^+$	HIV^+, $HLA\ A11^-$	HIV^-, $HLA\ A11^+$
Age (Mean ± SD)	33.3 ± 4.72	31.67 ± 14.57	39.4±7.02
Male/female	7/11	2/3	3/2
CD4 [median (range)]	463 (325-730)	563 (404-711)	N/A
Viral load [median (range)]	10875 (3118-67000)	3673 (155-9410)	N/A

Table 2 Demographic data of twenty eight donors. N/A indicates data not available.

Patients No.	Initial name	Gender	Age (year)	Serodiagnosed	Risk factor	HIV-1 Subtype	Visit1 (Date)	CD4+T cells (cells/mm^3)	Viral load (copies/ml)	Visit2 (Date)	CD4+T cells (cells/mm^3)	Viral load (copies/ml)	Visit3 (Date)	CD4+T cells (cells/mm^3)	Viral load (copies/ml)
1	PC	F	37	1997	heterosexual	A/E	22/7/02	518	5460	31/3/03	522	13240	26/5/04	576	113695
2	PN	F	35	1997	heterosexual	A/E	29/7/02	475	9736	11/12/02	374	21631	31/3/03	438	19218
3	CT	M	36	1994	homosexual	A/E	6/8/02	427	67000	26/2/03	471	154947	20/5/03	165	58570
4	RM	F	33	2000	heterosexual	A/E	14/8/02	730	10257	9/4/03	542	6723	26/7/03	440	30535
5	NI	F	29	2001	heterosexual	N/A	19/8/02	632	3118	12/3/03	571	6495	27/1/04	379	12019
6	PS	M	37	1998	heterosexual	A/E	19/8/02	387	8360	20/1/03	398	4987	29/5/04	294	23142
7	KL	M	34	2002	heterosexual	N/A	26/8/02	623	21759	1/4/03	364	101602	3/9/03	375	102101
8	OK	M	28	1996	homosexual	B	4/9/02	403	5911	6/3/03	472	6752	26/1/04	346	1251
9	JL	F	35	1995	heterosexual	A/E	4/9/02	374	16444	31/3/03	354	69361	1/9/03	298	95102
10	SL	F	34	2000	heterosexual	N/A	10/9/02	425	8297	21/1/03	472	6433	29/7/03	506	10861
11	UP	F	43	1999	heterosexual	N/A	10/9/02	539	15585	28/1/03	487	22736	25/5/04	153	>500000
12	KP	F	32	1993	heterosexual	A/E	11/9/02	616	25902	12/3/03	736	7884	20/1/04	707	11387
13	JN	F	28	1996	heterosexual	A/E	17/9/02	343	5231	25/2/03	390	48755	5/8/03	284	58312
14	PY	F	38	2002	heterosexual	N/A	18/11/02	489	35483	10/3/03	538	65235	25/6/03	503	20544
15	WY	M	45	2002	heterosexual	N/A	18/11/02	612	61360	10/3/03	635	380722	25/6/03	286	>500000
16	SR	F	32	1999	heterosexual	N/A	2/12/02	451	11493	26/2/03	471	24966	27/5/03	344	16014
17	MW	M	27	2002	heterosexual	N/A	21/1/02	325	5039	28/5/03	306	81550	18/2/04	263	13324
18	AT	M	28	2002	homosexual	B	31/3/03	413	16639	5/8/03	332	16407	7/4/04	312	68487

Table 3 Clinical information of HIV-1 infected Thai patients.

M = Male; F = Female; N/A = data not available

	sample ID	Anti-HIV	HLA-A		HLA-B		Bw		HLA-C		DRB1		DRB3/4/5		DQB1	
1	PC	+	24	11	1517	51	BW4		0701/06	1602	4	13	DRB3	DRB4	6	0301/4
2	PN	+	33(03/06)	11	44(032/07)	5201	Bw4		0701	0702	15(02/08)	1404	DRB5	DRB4	5	
3	CT	+	0201	1102	4601		Bw6		1		12	14	DRB3		5	
4	RM	+	2	11	13(01/06))	13	Bw6		03	0406	15		DRB5		5	6
5	NI	+	11		15	35(10/13)	Bw6		04(01/05)	12(03/06)	4	11	DRB4	DRB3(02)		03(02/07)
6	PS	+	1(01/06)	11	57(01/06)	55/5608	Bw4	Bw6	1	06(02/03)	07(01/05)	0901/2	DRB4		03/(03/12)	3
7	KL	+	24	11	07(05/06)	40	Bw6		15		1101	11	DRB3		5	3
8	OK	+	3001	11	4001	13	Bw4	Bw6	6	0702/10	12		DRB3	DRB4	2	0301/4
9	JL	+	24	11	5801	1502	Bw4	Bw6	8	0302	12	15	DR51	DR52	?	?
10	SL	+	2	11	18	46	Bw6		1	07(04/11)	15(02/8)	12	DRB5	DRB3	5	03(01/04)
11	UP	+	11		1502		Bw6		08(01/03)		15(01/06)	12	DRB5	DRB3	0601	03(01/04)
12	KP	+	30	11	13	3915	Bw4	Bw6	6	15	7	15	DR51	DR53	2	5
13	JN	+	1102		58	51	Bw4		3	14	4	8	DRB3	DRB4	2	
14	PY	+	11		27(04/10)	46	Bw4	Bw6	1	12(02/08)	8	11	DRB3(02)		0601	03(01/04)
15	WY	+	11	31	15(20/25)	51	Bw4	Bw6	04(03/07)	14	07(01/05)	11(04/06)	DRB3(02)	DRB4	2	03(01/04)
16	SR	+	2	11	13(01/06)	51(012/06)	Bw4		04(01/05)	03(04/05)	4	11(04/06)	DRB3(02)	DRB4	4	03(01/04)
17	MW	+	24	11	35(05/10)		Bw6		04(01/05)	4	12	14(04/28)	DRB3(01)	DRB3	15	03(01/04)
18	AT	+	2	11	55/5608	46	Bw6		1	04(03/07)	14(01/26)	0901/2	DRB3(02)	DRB4	5	
19	PM	+	24	33(03/06)	44(032/07)	27	Bw4		0701	0702	07	12	DRB3	DRB4	02	03
20	SY	+	33(03/06)		58		Bw4		03(02/14)		13		DRB3		06	
21	VP	+	24		1502	35	Bw6		04(01/05)	08(01/3)	12		DRB3		03(01/04)	
22	NK	+	24		4002	15	Bw6		0401	0702	04	1101	DRB3	DRB4	0301	0302
23	PK	+	24		5801	1502	Bw4	Bw6	8	0302	3	15	DR51		2	0601
24	AB	-	11	26	52	58	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
25	NS	-	11		27		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
26	PP	-	11	24	18	27	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	AP	-	2	11	13	52	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28	KA	-	11	26	8	60	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 4 HLA typing of HLA-A11 positive HIV-infected patients (1-18), HLA-A11 negative HIV-infected patients (19-23), and HIV seronegative individuals (24-28). N/A indicates data not available.

2. HLA-A11-restricted specific CD8+ T cell responses

2.1 HIV-specific CD8+ T cell responses in HIV seronegative HLA-A11-positive individuals and HLA-A11 negative HIV-infected patients

The negative control group was stratified into two groups: five HLA-A11 positive HIV-uninfected donors and five HLA-A11 negative HIV-infected patients.

2.1.1 HIV-specific CD8+ T cell responses in seronegative individuals analysed by ELISpot assay

We enrolled 5 HIV-seronegative HLA-A11 positive donors and confirmed their HIV status by serology. HIV-specific CD8+ T cell of HIV-seronegative HLA-A11 positive donors (AB, NS, PP, AP, and KA) could not be detected by peptide-based ELISpot assays (Table 5).

Peptides		CD8+ T cell responses of HIV-seronegative HLA-A11 positive donors (SFU/10^6 PBMC)				
		AB	NS	PP	AP	KA
1	IATL	-2	-4	8	-6	0
2	GIPH	6	-4	-4	-10	16
3	SVPL	-6	-10	6	-12	2
4	AIFQ	14	-2	44	8	-4
5	QIIE	-6	2	2	0	4
6	QIYA	22	14	8	-6	-8
7	QIYQ	-4	0	12	-6	-2
8	FVNT	0	-8	16	-12	6
9	AVFI	8	6	0	-12	24
10	TQMN	-4	8	-4	10	-12
11	VTVY	0	2	8	-2	2
12	ISLW	-10	-10	-2	-2	-2
13	ITVG	-4	-8	12	2	12
14	RVLK	0	-10	4	-10	2
15	SLCL	-10	0	-8	-16	-4
16	QVPL	6	-2	-8	-12	20
17	GAFD	-2	-6	-2	-10	-10

Table 5 Non responses of HIV-specific CD8+ T cell in HIV-seronegative HLA-A11 positive donors against HLA-A11-restricted peptides. The numbers of spot forming units (SFU) were calculated by subtracting the negative control value from the established SFU count.

2.1.2 HIV-specific CD8+ T cell responses in HLA-A11 negative HIV-infected patients analysed by ELISpot assay

We enrolled 5 HLA-A11 negative HIV-infected patients. HIV-specific CD8+ T cell responses of HLA-A11 negative HIV-infected patients (PM, SY, VP, NK and PK donors) were shown in Table 6. Whilst the patients PM, NK, and PK did not recognise any HLA-A11-restricted peptide, the patients SY and VP, unexpectedly, recognised some peptides. The peptides which the patients SY and VP had HIV-specific T cell responses were ITVGPGQVFY and QVPLRPMTYK peptides, respectively.

	Peptides	CD8+ T cell responses of HIV-seropositive HLA-A11 negative donors (SFU/10^6 PBMC)				
		PM	SY	VP	NK	PK
1	IATL	0	78	6	-16	6
2	GIPH	-18	6	28	4	4
3	SVPL	0	6	26	-2	-6
4	AIFQ	-14	10	36	-2	-6
5	QIIE	-8	6	28	4	2
6	QIYA	-4	10	14	-24	-2
7	QIYQ	-6	10	20	26	0
8	FVNT	0	12	12	-16	-4
9	AVFI	8	12	4	-2	-4
10	TQMN	6	8	8	-12	-4
11	VTVY	-14	20	22	-20	44
12	ISLW	-6	18	22	-10	-2
13	ITVG	-16	**438**	24	-12	-6
14	RVLK	-20	18	20	-8	-2
15	SLCL	-22	28	12	-18	-4
16	QVPL	-6	18	**172**	4	0
17	GAFD	16	9	14	-10	-8

Table 6 HIV-specific CD8+ T cell responses of HLA-A11 negative HIV-infected patients against HLA-A11-restricted peptides. The numbers of SFU were calculated by subtracting the negative control value from the established SFU count.

2.2 HIV-specific CD8+ T cell responses in the HLA-A11 positive HIV-infected patients analysed by ELISpot assay

2.2.1 Frequencies of HLA-A11-restricted HIV-specific CD8+ T cell epitopes recognised by HIV-infected patients

Seventeen HLA-A11 restricted HIV-specific CD8+ T cell epitopes which were reported in the Los Alamos Immunology Database (http://hiv-web.lanl.gov) and other studies were selected to use in this study. In order to determine the frequency of these HLA-A11-restricted CTL epitopes recognised by HIV-infected patients in cross-sectional study, PBMC from eighteen HIV-1-infected patients with HLA-A11 were screened with ELISpot assay upon stimulation with these epitopes.

HLA-A11-positive patients had HIV-specific T cell responses targeting at least two peptides. A total of 12 out of 17 peptides were recognised by HLA-A11-positive patients (Table 7). The number of peptides recognised per subject ranged from two to eight peptides. The broadest T cell responses were demonstrated in patient UP who recognised eight of seventeen peptides comprising Gag protein (IATLWCVHQR), Pol protein (SVPLDESFRK, AIFQSSMTK, QIIEQLIKK, QIYAGIKVK, and AVFIHNFKRK), Env protein (ITVGPGQVFY), and Nef protein (GAFDLSFFLK). In addition, patient UP had the highest magnitude of HIV-specific CD8+ T cell response in this study. The response was directed against QIIEQLIKK peptide (5497 SFU/10^6 PBMC). The results showed five most frequently recognised peptides which also had high level of CTL responses. These peptides were QVPLRPMTYK, GAFDLSFFLK, AIFQSSMTK, QIYAGIKVK, and QIYQEPFKNLK. Indeed, the Nef epitope (QVPLRPMTYK) was the most commonly-recognised epitope in this study whereby 16 of 18 patients had responses to this epitope.

Peptides		1 PC	2 PN	3 CT	4 RM	5 NI	6 PS	7 KL	8 OK	9 JL	10 SL	11 UP	12 KP	13 JN	14 PY	15 WY	16 SR	17 MW	18 AT	Total patients	Median	Min	Max
Gag epitope																					**713**	**266**	**870**
1	IATL					750	676	870				266								4	**713**	266	870
Pol epitope																					**585**	**146**	**5497**
2	GIPH																			0	**0**	0	0
3	SVPL	752										3360								2	**2056**	752	3360
4	AIFQ		438	310		1542	492			252	1184	2744	404	1202						9	**492**	252	2744
5	QIIE								146			5497							332	3	**332**	146	5497
6	QIYA		384	278	606	2134	612	554		2270	2144	210	522			330			564	12	**559**	210	2270
7	QIYQ					642	680				700		446	822	256	468				7	**642**	256	822
8	FVNT																			0	**0**	0	0
9	AVFI											616								1	**616**	616	616
Env epitope																					**307**	**116**	**448**
10	TQMN																			0	**0**	0	0
11	VTVY			448										392		116				3	**392**	116	448
12	ISLW																			0	**0**	0	0
13	ITVG				400							222								2	**311**	222	400
14	RVLK																			0	**0**	0	0
15	SLCL					208														1	**208**	208	208
Nef epitope																					**828**	**152**	**2568**
16	QVPL	752	262	494	152	2006		1020	466	1526	892	944	952	992	218		550	362	764	16	**758**	152	2006
17	GAFD	392		654	1956	220	1164	432		966	1604		2568	1778	1544	2516	518	450		14	**1065**	220	2568
Total peptides		3	3	5	4	7	5	4	2	4	5	8	5	5	3	4	2	2	3		609	116	5497

Table 7 Frequency of HLA-A11-restricted HIV-specific T cell in HIV-infected HLA-A11 positive patients using ELISpot assay.

2.2.2 HIV-specific CD8+ T cell responses against each HIV-1 protein

We analysed the T cell recognition of HLA-A11-positive HIV-infected patients against HLA-A11-restricted HIV-specific CD8+ T cell responses epitopes in this crossectional study (Table 7). HIV-1-specific CD8+ T cell responses for each protein were demonstrated. The result showed that Nef was most frequently targeted protein (100%) followed by Pol (88%), Env (33%), and Gag (22%) (Figure 4).

Only 4 of 18 HIV-infected patients recognised Gag epitope (IATLWCVHQR) with a magnitude of response ranged from 266 to 870 SFU/10^6 PBMC with a median of 713 SFU/10^6 PBMC. There were 16 out of 18 HIV-infected patients recognising Pol epitopes. 6 out of 8 Pol peptides were recognised by HIV-infected patients. The number of Pol peptides recognised per subject ranged from one to five peptides with a median of two peptides. Three Pol peptides (AIFQSSMTK, QIYAGIKVK, and QIYQEPFKNLK) were most frequently targeted by HLA-A11-positive patients. For Env epitopes, there were 6 out of 18 HIV-infected patients recognising Env epitopes. Only 3 out of 6 peptides were recognised by HLA-A11 positive HIV-infected patient. These epitopes were VIVYYGVPVWR, ITVGPGQVFY, and SLCLFSYHR. Nef appeared to be the most immunodominant protein, whereby all patients had responses against this protein. The number of patients recognising QVPLRPMTYK and GAFDLSFFLK were 16 (94%) and 14 (82%), respectively. The number of peptides recognised per subject ranged from one to two peptides with a median of two peptides.

When we analysed on the median of magnitudes of CD8+ T cell responses to each protein, we found that the highest magnitudes of HIV-specific CD8+ T cell responses were to Nef, follow by Gag, Pol, and Env. However, there is only significant difference of the median of responses between Env and Nef when they were analysed by Mann-Whitney test. For Nef-specific epitopes, the magnitude of Nef-specific T cell response ranged from 152 to 2568 SFU/10^6 PBMC with a median of 828 SFU/10^6 PBMC. For Gag protein that had only one peptide, the magnitude of response ranged from 266 to 870 SFU/10^6 PBMC with a median of 713 SFU/10^6 PBMC. In addition, a range of magnitude of Pol-specific CD8+ T cell response was 146-5497 SFU/10^6 PBMC with a median of 585 SFU/10^6 PBMC. Moreover, the magnitude of Env-specific CD8+ T cell response ranged 116 to 448 SFU/10^6 PBMC with a median of 307 SFU/10^6PBMC (Figure 5-6).

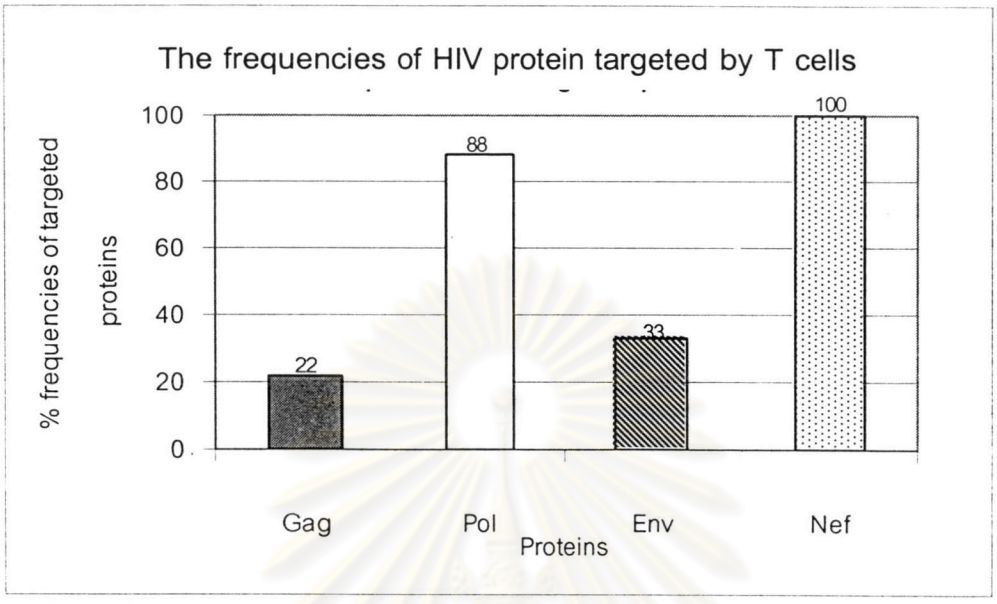

Figure 4 The frequencies of HIV protein targeted by T cells. Results are presented as frequencies of Gag (dark bars), Pol (open bars), Env (hatched bars) and Nef (dot bars) targeted by T cells.

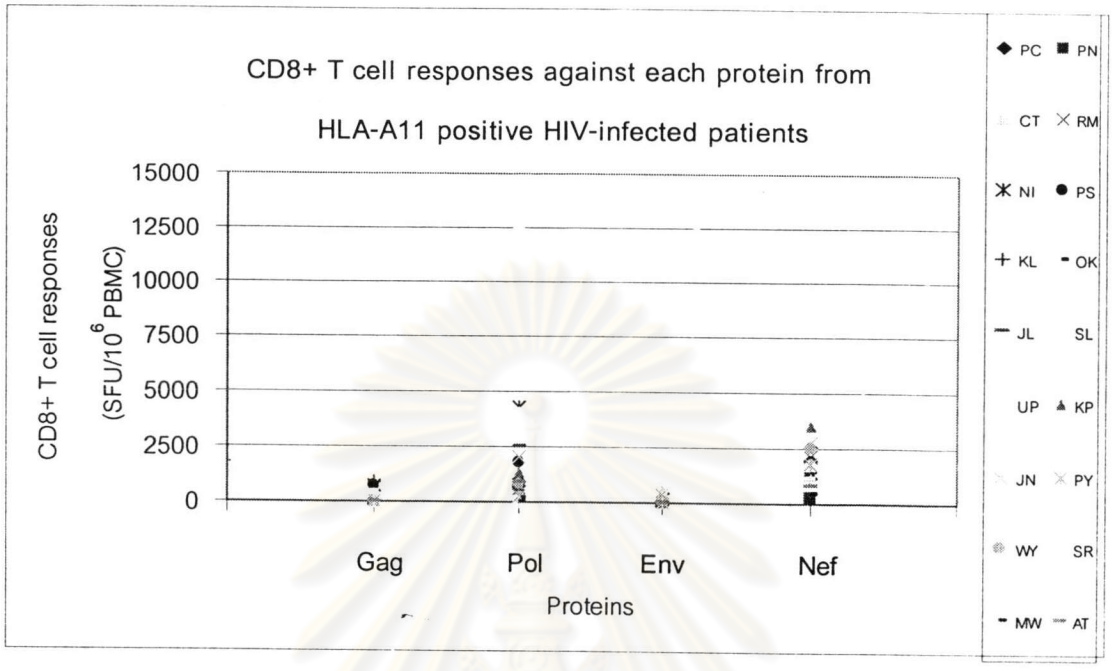

Figure 5 HIV-specific CD8+ T cell responses of HLA-A11 positive HIV-infected patients against each protein.

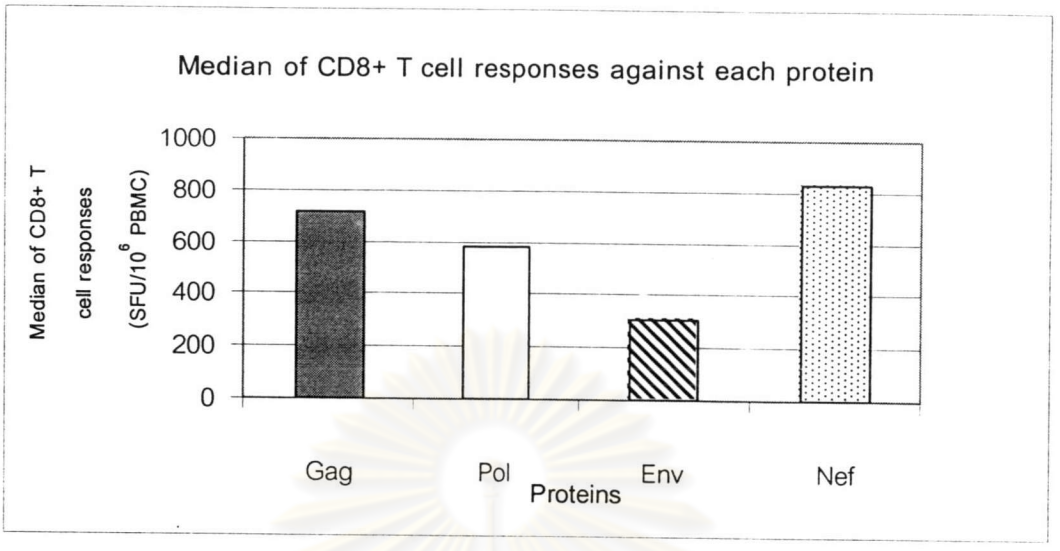

Figure 6 The median of HIV-specific CD8+ T cell responses of HLA-A11 positive HIV-infected patients against each protein. Results are presented as median of CD8+ T cell responses against Gag (dark bars), Pol (open bars), Env (hatched bars) and Nef (dot bars).

2.2.3 Longitudinal analysis of HIV-1-specific CD8+ T cell responses against HLA-A11-restricted HIV-specific CD8+ T cell epitopes

The HIV-specific T cell responses in these patients were longitudinally analysed by ELISpot assays to establish dynamic relationship between HIV viral loads and the T cell responses. The CD8+ T cell responses against peptides in each individual from each group, comprising HIV-seronegative HLA-A11-positive individuals, HIV-seropositive HLA-A11-negative patients and HLA-A11-positive HIV-infected patients from 1st, 2nd and 3rd time points, were shown in figure 7. There are significant difference between the control groups (HIV-seronegative HLA-A11-positive individuals and HIV-seropositive HLA-A11-negative patients) and HLA-A11-positive HIV-infected patients analysed by Mann-Whitney test.

The numbers of peptides recognised per individuals in each time point were difference (Figure 8). Only one patient (PN) showed consistent pattern of T cell recognition to the same set of peptides throughout this longitudinal study (AIFQSSMTK, QIYAGIKVK, and QVPLRPMTYK peptides) (Figure 9). On the other hand, HIV-specific T cell responses of most patients had fluctuation of responses and direct against different epitopes over time. For example, patient CT had QIYA-specific T cell responses only in the first time point (Figure 10) and patient PS had QVPL-specific response only in third time point.

There were 5 peptides which were not recognised by any patients at 1st time point but their responses were detected by ELISpot at 2nd or 3rd time point. These peptides were GIPHPAGLKK, FVNTPPLVK, TQMNWPNLWK and ISLWDQSLK peptides (Table 8-10). A total of 16 out of 17 peptides were recognised by one or more HLA-A11-positive patients in this longitudinal study. The number of peptides recognised per subject ranged from one to eight. The broadest HIV-specific T cell responses in this longitudinal study were identified in the patient UP in all 3 time points. She recognised 8 out of 17 peptides in the 1st time point and 7 and 6 out of 17 peptides in 2nd and 3rd time points, respectively.

In addition, the Nef-specific T cell responses in most of patients were persistent T cell recognition to the same peptides throughout longitudinal study. For Gag-specific responses, the results showed the persistent T cell recognition but lower than Nef, whilst Pol-and Env-specific responses had fluctuation of responses (Figure 11).

Peptides		1 PC	2 PN	3 CT	4 RM	5 NI	6 PS	7 KL	8 OK	9 JL	10 SL	11 UP	12 KP	13 JN	14 PY	15 WY	16 SR	17 MW	18 AT	Total patients	Median	Min	Max
Gag epitope																					**713**	**266**	**870**
1	IATL					750	676	870				266								4	**713**	266	870
Pol epitope																					**585**	**146**	**5497**
2	GIPH																			0	**0**	0	0
3	SVPL	752										3360								2	**2056**	752	3360
4	AIFQ		438	310		1542	492			252	1184	2744	404	1202						9	**492**	252	2744
5	QIIE								146			5497							332	3	**332**	146	5497
6	QIYA		384	278	606	2134	612	554		2270	2144	210	522			330			564	12	**559**	210	2270
7	QIYQ					642	680				700		446	822	256	468				7	**642**	256	822
8	FVNT																			0	**0**	0	0
9	AVFI											616								1	**616**	616	616
Env epitope																					**307**	**116**	**448**
10	TQMN																			0	**0**	0	0
11	VTVY			448										392		116				3	**392**	116	448
12	ISLW																			0	**0**	0	0
13	ITVG				400							222								2	**311**	222	400
14	RVLK																			0	**0**	0	0
15	SLCL					208														1	**208**	208	208
Nef epitope																					**828**	**152**	**2568**
16	QVPL	752	262	494	152	2006		1020	466	1526	892	944	952	992	218		550	362	764	16	**758**	152	2006
17	GAFD	392		654	1956	220	1164	432		966	1604		2568	1778	1544	2516	518	450		14	**1065**	220	2568
Total peptides		3	3	5	4	7	5	4	2	4	5	8	5	5	3	4	2	2	3		609	116	5497

Table 8 HLA-A11-restricted HIV-specific CD8+ T cell responses in 1st time point.

Peptides		1 PC	2 PN	3 CT	4 RM	5 NI	6 PS	7 KL	8 OK	9 JL	10 SL	11 UP	12 KP	13 JN	14 PY	15 WY	16 SR	17 MW	18 AT	Total patients	Median	Min	Max
Gag epitope																					**832**	**152**	**1330**
1	IATL					1330	152	832												3	**832**	152	1330
Pol epitope																					**458**	**96**	**3416**
2	GIPH											96								1	**96**	96	96
3	SVPL	790										1322				98	360			4	**575**	98	1322
4	AIFQ		206	150		2050	606			256	638	944	220	1272						9	**606**	150	2050
5	QIIE											3416							314	2	**1865**	314	3416
6	QIYA		228		1228	2498	630	242		844	1086	264				490		162	714	11	**630**	162	2498
7	QIYQ	200				2058	458	246	122	144	196		889	984	736	1366	166			12	**352**	122	2058
8	FVNT																120			1	**120**	120	120
9	AVFI											350								1	**350**	350	350
Env epitope																					**384**	**271**	**3516**
10	TQMN																			0	**0**	0	0
11	VTVY			426							342			271						3	**342**	271	426
12	ISLW																			0	**0**	0	0
13	ITVG				3516															1	**3516**	3516	3516
14	RVLK																			0	**0**	0	0
15	SLCL																			0	**0**	0	0
Nef epitope																					**550**	**148**	**4652**
16	QVPL	594	148	312	456	2818		1486	348	1556	430	448	1448	1608	488		428	392	774	16	**472**	148	2818
17	GAFD	470		518	4652	506	1798	582		468	294		3084	2152	1904	2718	432	800		14	**691**	294	4652
Total peptides		4	3	4	4	6	5	5	2	5	6	7	4	5	3	4	5	3	3		498	96	4652

Table 9 HLA-A11-restricted HIV-specific CD8+ T cell responses in 2nd time point.

Peptides		1 PC	2 PN	3 CT	4 RM	5 NI	6 PS	7 KL	8 OK	9 JL	10 SL	11 UP	12 KP	13 JN	14 PY	15 WY	16 SR	17 MW	18 AT	Total patients	Median	Min	Max
Gag epitope																				**4**	**1155**	**578**	**1482**
1	IATL				1482	1354	578	956												4	**1155**	578	1482
Pol epitope																					**481**	**126**	**1970**
2	GIPH																			0	**0**	0	0
3	SVPL	330			126							716		228	144					5	**228**	126	716
4	AIFQ	458	482	186		1186	666			136	674	1500	216	1132			388			11	**482**	136	1500
5	QIIE											1600								1	**1600**	1600	1600
6	QIYA		406		1970	428	164			854	546						416			7	**428**	164	1970
7	QIYQ					1090	602				622		480	476	648	658				7	**622**	476	1090
8	FVNT																			0	**0**	0	0
9	AVFI											284								1	**284**	284	284
Env epitope																					**304**	**108**	**2520**
10	TQMN											304								1	**304**	304	304
11	VTVY			422										284						2	**353**	284	422
12	ISLW				108															1	**108**	108	108
13	ITVG				2520															1	**2520**	2520	2520
14	RVLK																			0	**0**	0	0
15	SLCL					814								296						2	**555**	296	814
Nef epitope																					**638**	**114**	**4198**
16	QVPL	258	466	398	478	1346	342	*46*	194	1150	618	376	1120	2174	448		1028	114	172	16	**457**	114	2174
17	GAFD	516		402	4198	1002	1802	892		422	658		2728	1844	1640	1570	756	396		14	**947**	396	4198
Total peptides		4	3	4	7	7	6	3	1	4	5	6	4	7	4	2	4	2	1		546	108	4198

Table 10 HLA-A11-restricted HIV-specific CD8+ T cell responses in 3rd time point.

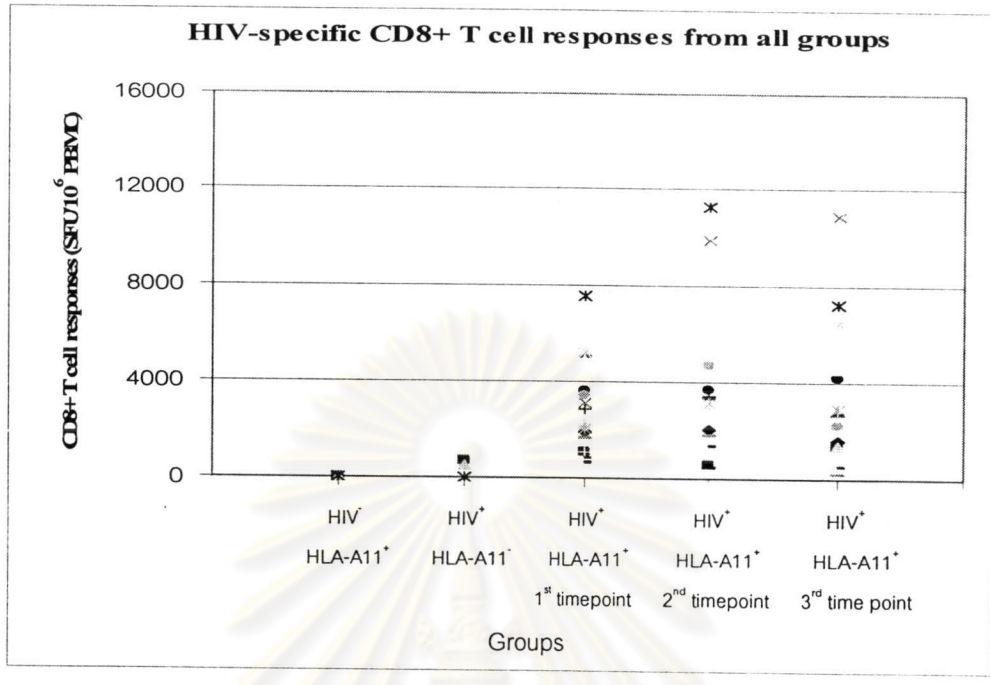

Figure 7 HLA-A11-restricted HIV-specific CD8+ T cell responses from all groups

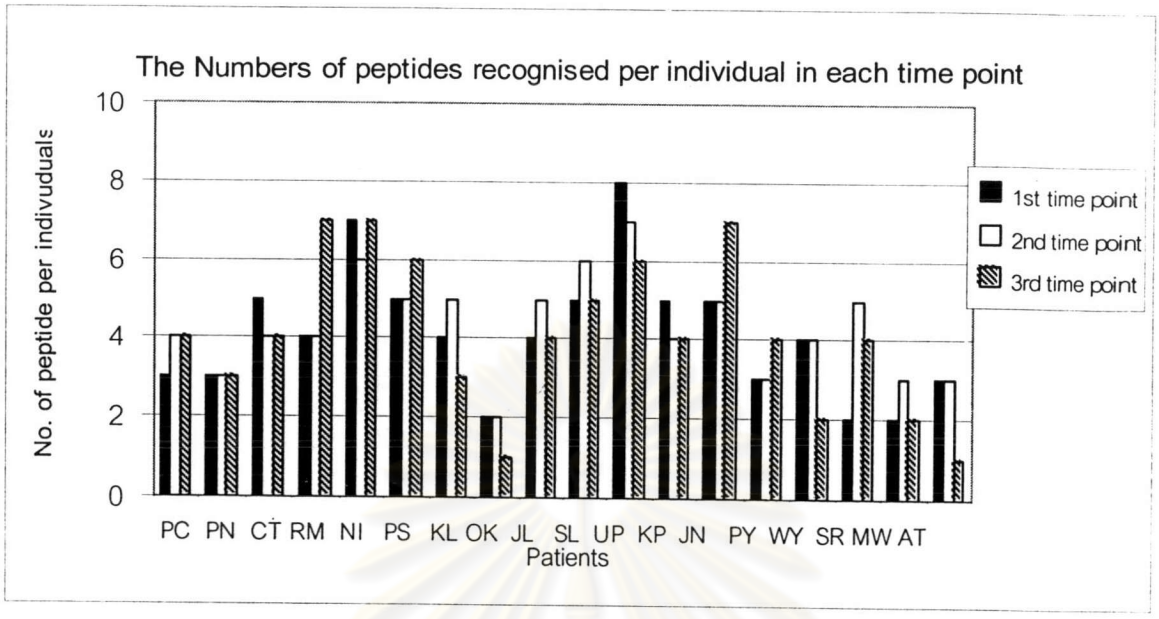

Figure 8 The number of peptides recognised per individual in each time point comprising 1st time point (dark bars), 2nd time point (open bars), and 3rd time point (hatched bars).

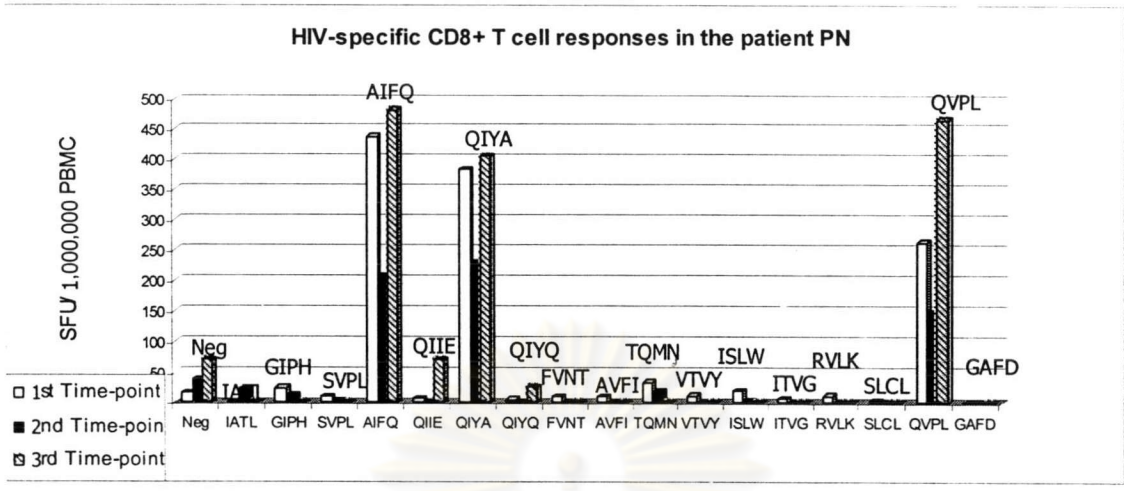

Figure 9 HIV-specific CD8+ T cell responses of patient PN in longitudinal analysis. Results are presented as a total numbers of SFU (mean of duplicate wells) for 1st time point (open bars), 2nd time point (dark bars), and 3rd time point (hatched bars). The numbers of SFU were calculated by subtracting the negative control value from the established SFU count. Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive.

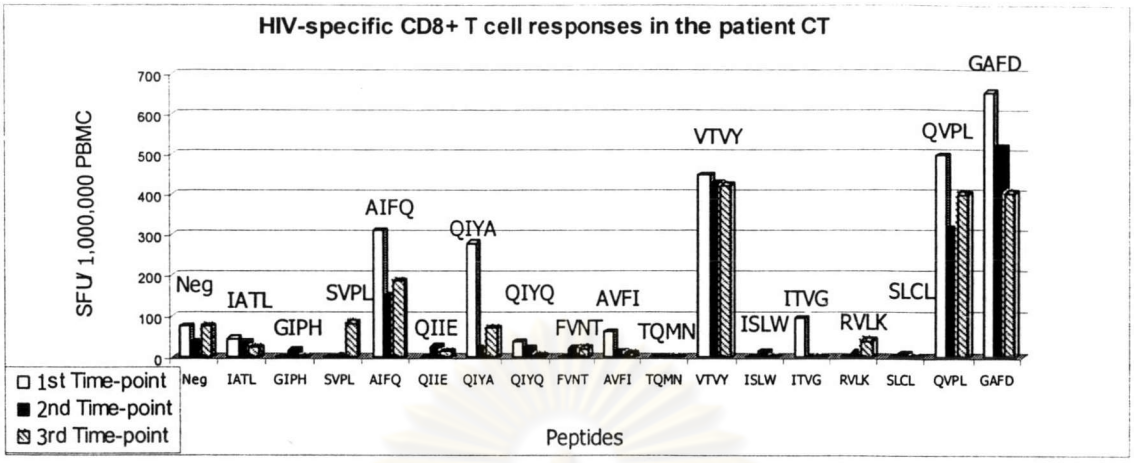

Figure 10 HIV-specific CD8+ T cell responses of patient CT in longitudinal analysis. Results are presented as a total numbers of SFU (mean of duplicate wells) for 1st time point (open bars), 2nd time point (dark bars) and 3rd time point (hatched bar). The numbers of SFU were calculated by subtracting the negative control value from the established SFU count. Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive.

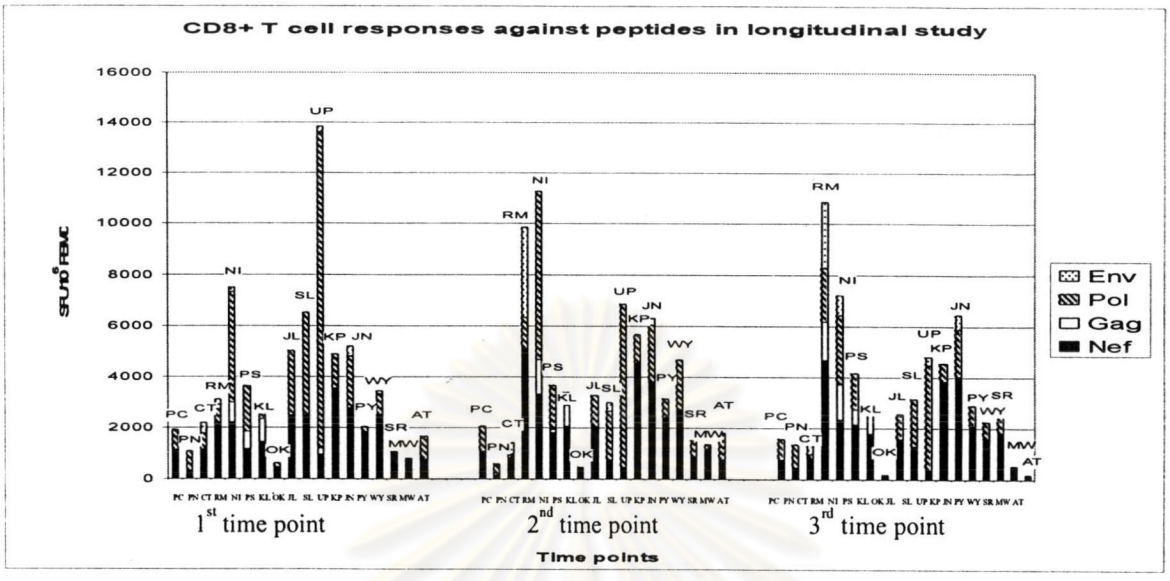

Figure 11 HIV-specific CD8+ T cell responses of each patient in longitudinal analysis. Results are presented as CD8+ T cell responses against Env (doted bars), Pol (hatched bars), Gag (dark bars), Nef (open bars).

2.2.4 HIV-specific T cells responses following CD8+ T cells depletion

In our study, ELISpot assay was used to enumerate HIV-specific CD8+ T cells secreting IFN-γ upon stimulation. However, IFN-γ could also be secreted from CD4+ T cells and NK cells. We therefore depleted CD8+ T cells by Immunomagnitic beads (Dynal®beads) to prove that these responses were mediated solely or mainly by CD8+ T cells. We selected three HLA-A11-restricted HIV-infected patients who had T cell responses against HIV-specific peptides by ELISpot assays. The selected patients were the patients MW, KL and JL. After CD8+ T cell depletion, we found that the magnitude of responses was significantly reduced. Indeed, the fact that more than 80% of spots were abrogated by CD8+ depletion confirms these responses were mediated by CD8+ T cells (Table 11).

Patients	Peptides	Magnitude of HIV-specific T cell responses (SFU/10^6 PBMC)		% reduction
		Pre depletion	Post depletion	
MW	QVPLRPMTYK	114	12	89.47
	GAFDLSFFLK	396	46	88.38
JL	AIFQSSMTK	136	0	100
	QIYAGIKVK	854	0	100
	QVPLRPMTYK	1150	0	100
	GAFDLSFFLK	422	0	100
KL	IATLWCVHQR	956	0	100
	QVPLRPMTYK	1224	0	100
	GAFDLSFFLK	892	0	100

Table 11 HLA-A11-restricted HIV-specific T cell responses after depletion of CD8+ T cells by immunomagnetic beads of the patients MW, JL, and KL. The numbers of SFU were calculated by subtracting the negative control value from the established SFU count. Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive.

2.3 Correlation between CD8+ T cell responses and viral load

Nef plays an important role in enhancement of pathogenicity of HIV infection by interfering with immunity against infected cells. Investigation into Nef-specific immune responses may therefore provide useful information for HIV/AIDS pathogenesis. In this study, HIV-specific CD8+ T cell responses against QVPLRPMTYK and GAFDLSFFLK peptides within Nef protein seemed to be two most immunodominant epitopes. We wanted to see whether these two immunodominant epitopes might mediate protective HIV-specific immunity. We then tried to establish the correlation between CD8+ T cell responses against Nef peptides detected by ELISpot assay and plasma HIV RNA. We determined the correlation both in cross-sectional and longitudinal study using Pearson Correlation. For a cross-sectional study, we analysed the correlation between CD8+ T cell responses and viral load from all patients in three time points, on the other hand, we determined this correlation in each patient for longitudinal study.

In cross-sectional study, we demonstrated that there was no correlation between total HIV-or Nef-specific CD8+ T cell responses and plasma RNA viral load (Figure 12-13). In addition, when we analysed the correlation between viral load and Nef-specific CD8+ T cell responses in each epitope (QVPL-or GAFD-specific CD8+ T cell responses), the results showed that there were no correlations (data not shown).

Moreover, we then looked for a relationship between CD8+ T cell response and viral load in each patient in longitudinal study. We failed to show the correlation between the QVPL-specific CD8+ T cell responses and plasma HIV-1 RNA (9/13) in most patients including PN, SR, RM, NI, KL SL, JN, PY, and MW (Figure 14-15). Whereas a positive correlation was found in 2 out of 13 patients (UP and OK) (Figure 16), 4 out of 13 patients had inverse correlation between T cell responses and viral load (PC, CT, KP, and JL) (Figure 17).

We also failed to demonstrate the correlation between GAFD-specific CD8+ T cell responses and plasma HIV RNA in most patients (8/12) including RM, SR, CT, PS, KL, JN, WY, and SL (Figure 18). On the contrary, 4 of 12 patients had positive correlation (PC, NI (correlation is significant at the 0.01 level), PY, and MW) (Figure 19) and 2 out of 12 patients (JL and KP) had inverse correlation between CD8+ T cell response and viral load (Figure 20).

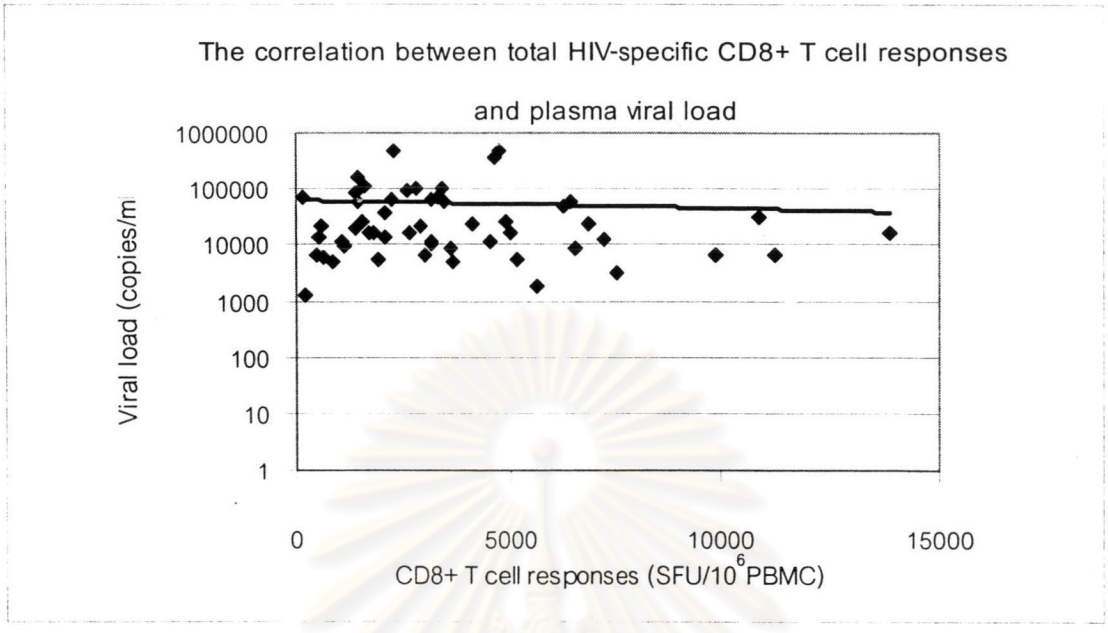

Figure 12 The correlation between total HIV-specific CD8+ T cell responses and viral load in cross-sectional study.

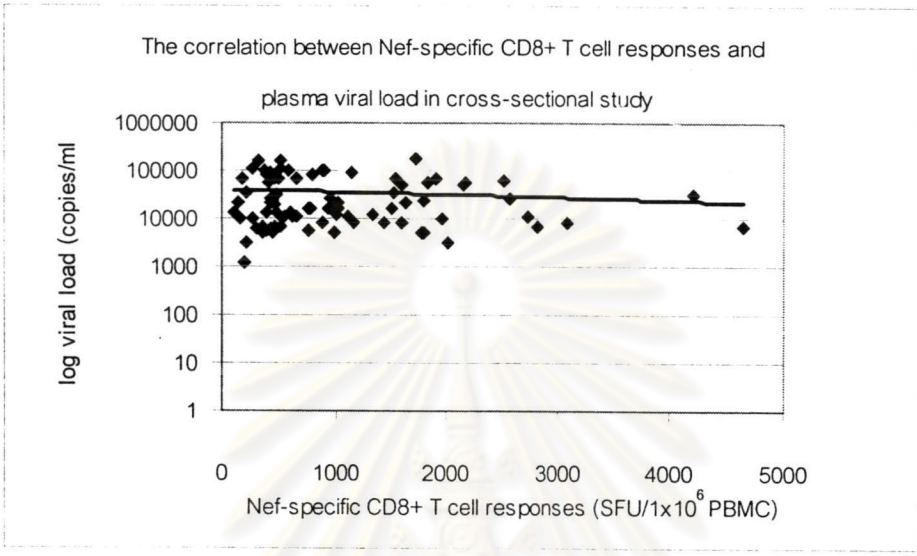

Figure 13 The correlation between Nef-specific CD8+ T cell responses and viral load in cross-sectional study.

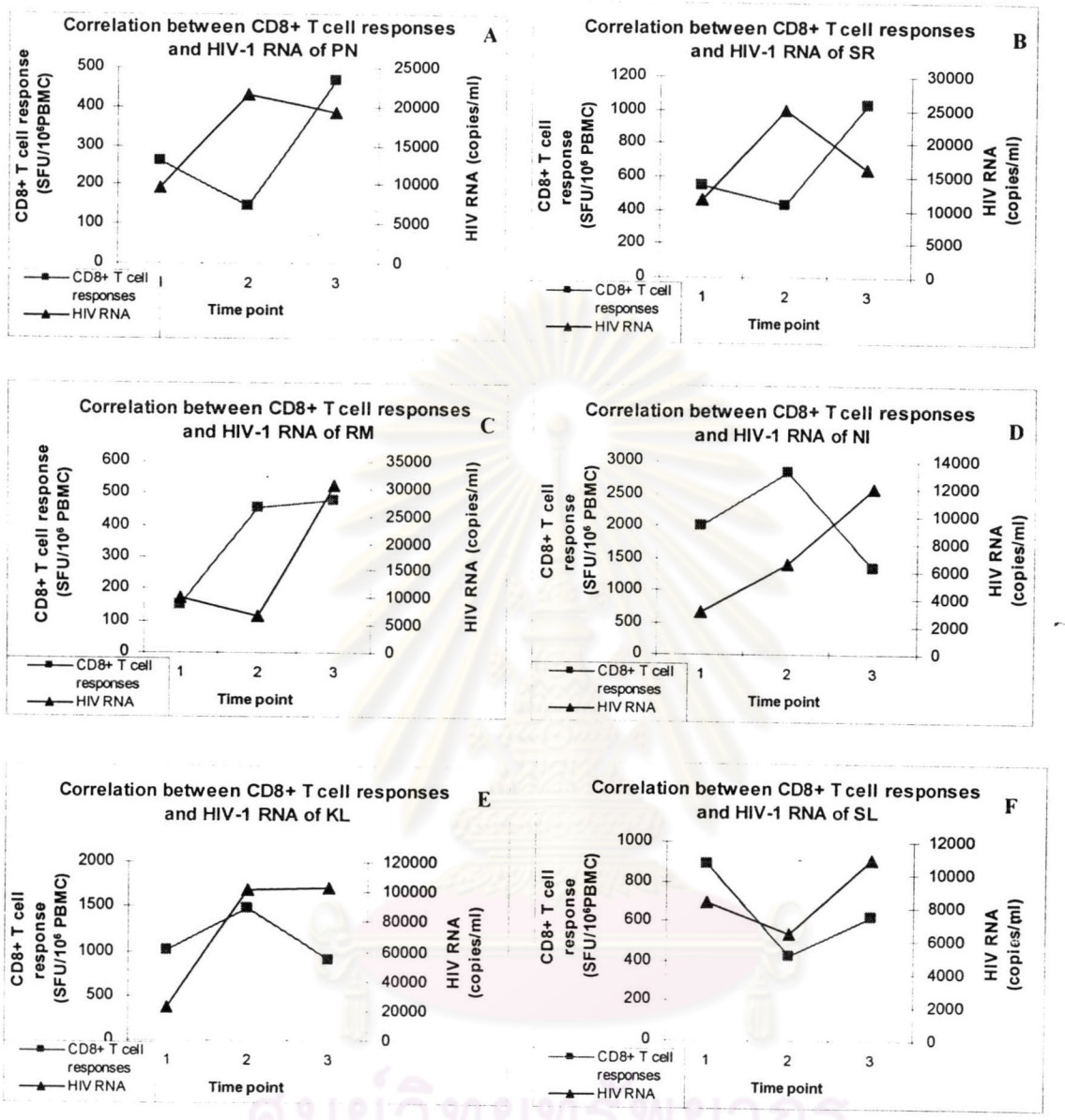

Figure 14 The no correlations between QVPL-specific CD8+ T cell responses and HIV RNA of the patients PN (A), SR (B), RM (C), NI (D), KL (E) and SL (F). A square line is CD8+ T cell response (SFU/10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

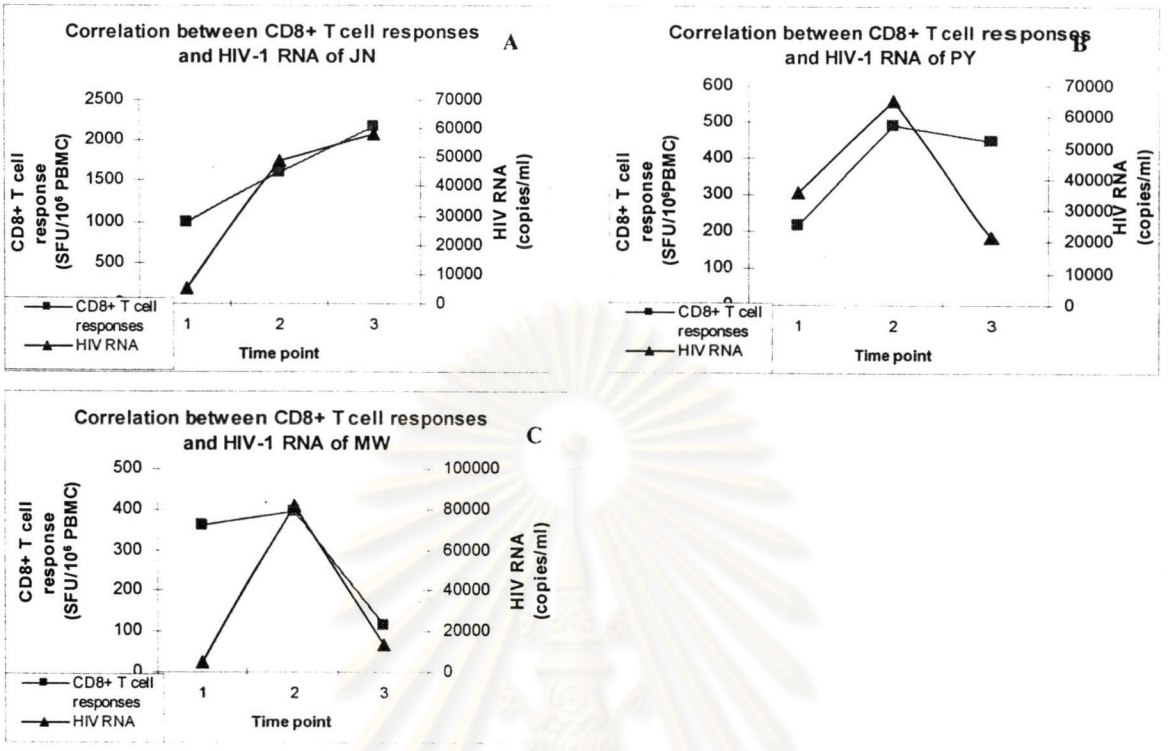

Figure 15 The no correlations between QVPL-specific CD8+ T cell responses and HIV RNA of the patients JN (A), PY (B), and MW (C). A square line is CD8+ T cell response (SFU/10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

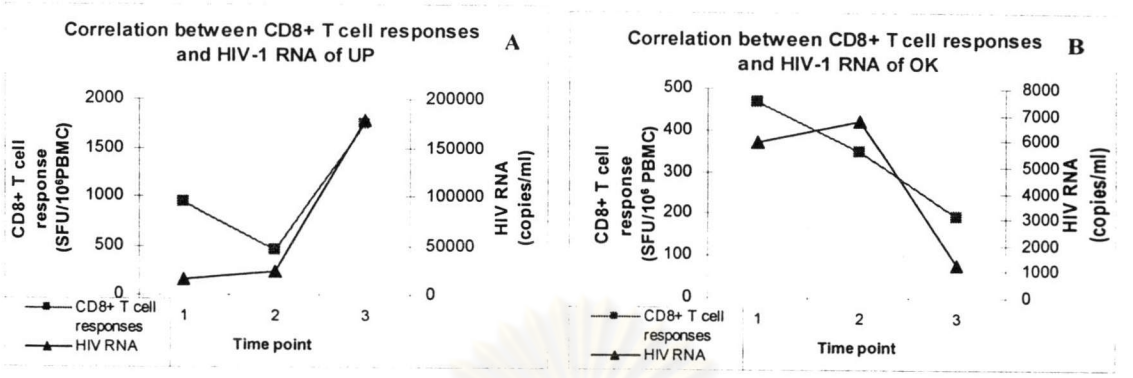

Figure 16 The positive correlations between QVPL-specific CD8+ T cell responses and HIV RNA of the patients UP (A) and OK (B). A square line is CD8+ T cell response (SFU/10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

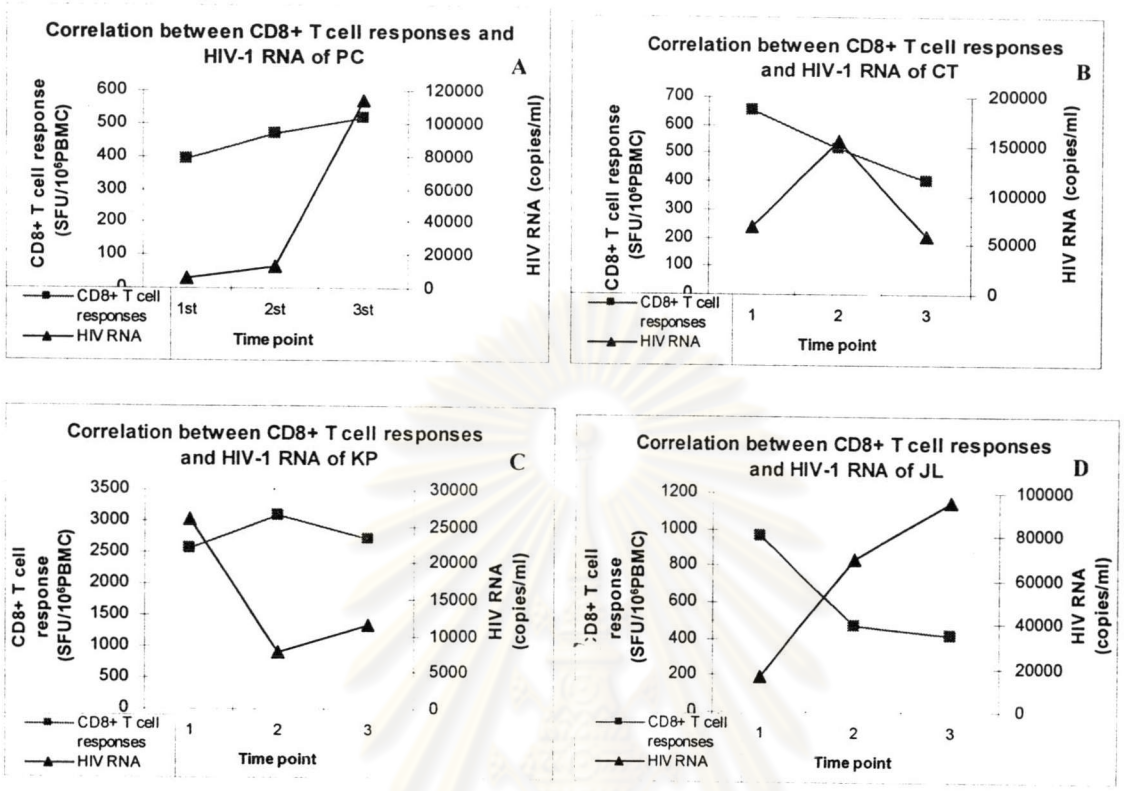

Figure 17 The inverse correlations between QVPL-specific CD8+ T cell responses and HIV RNA of the patients PC (A), CT (B), KP (C), and JL (D). A square line is CD8+ T cell response (SFU/10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

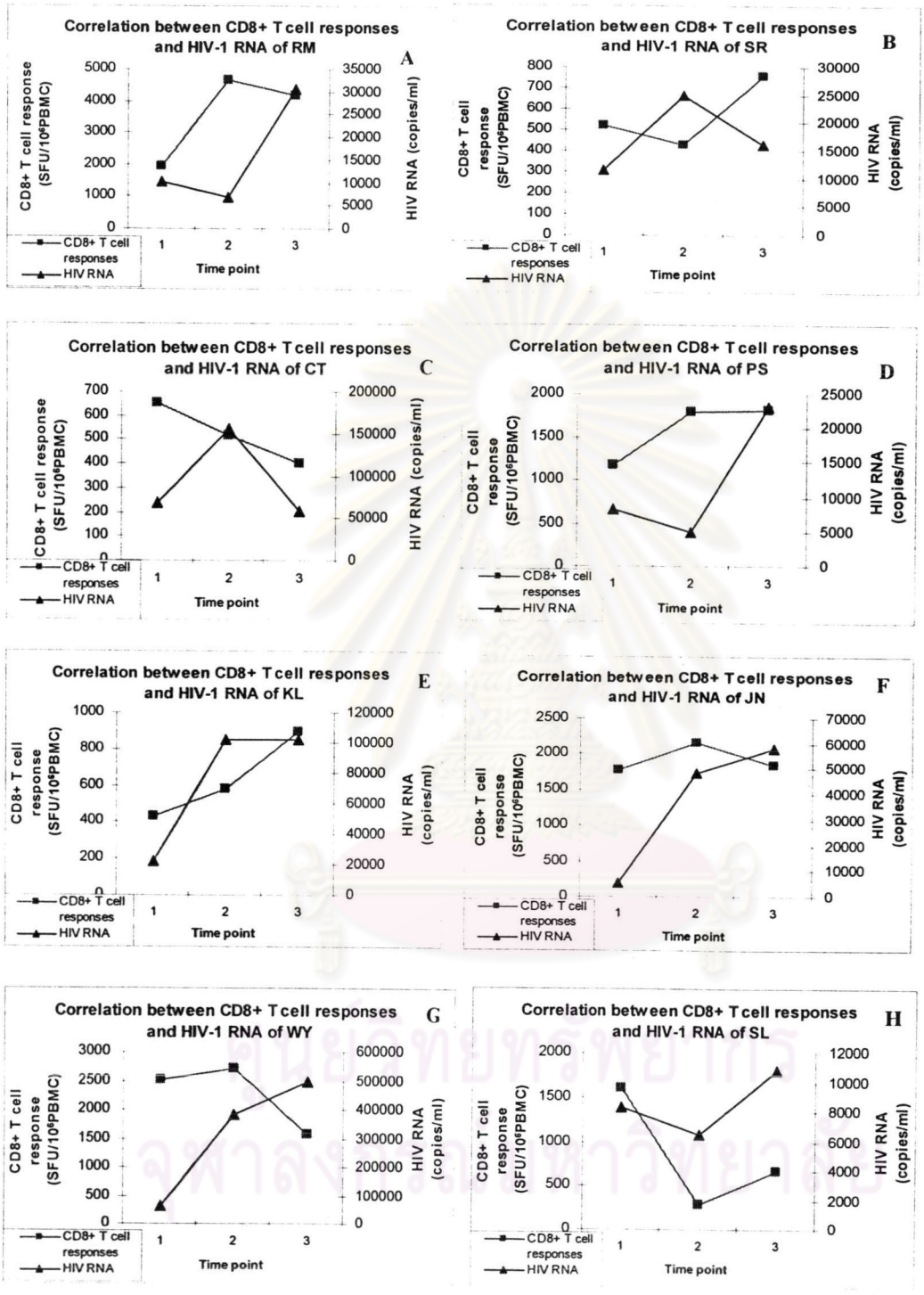

Figure 18 The no correlations between GAFD-specific CD8+ T cell responses and HIV RNA of the patients RM (A), SR (B), CT (C), PS (D), KL (E), JN (F), WY (G), and SL (H). A square line is CD8+ T cell response (SFU/10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

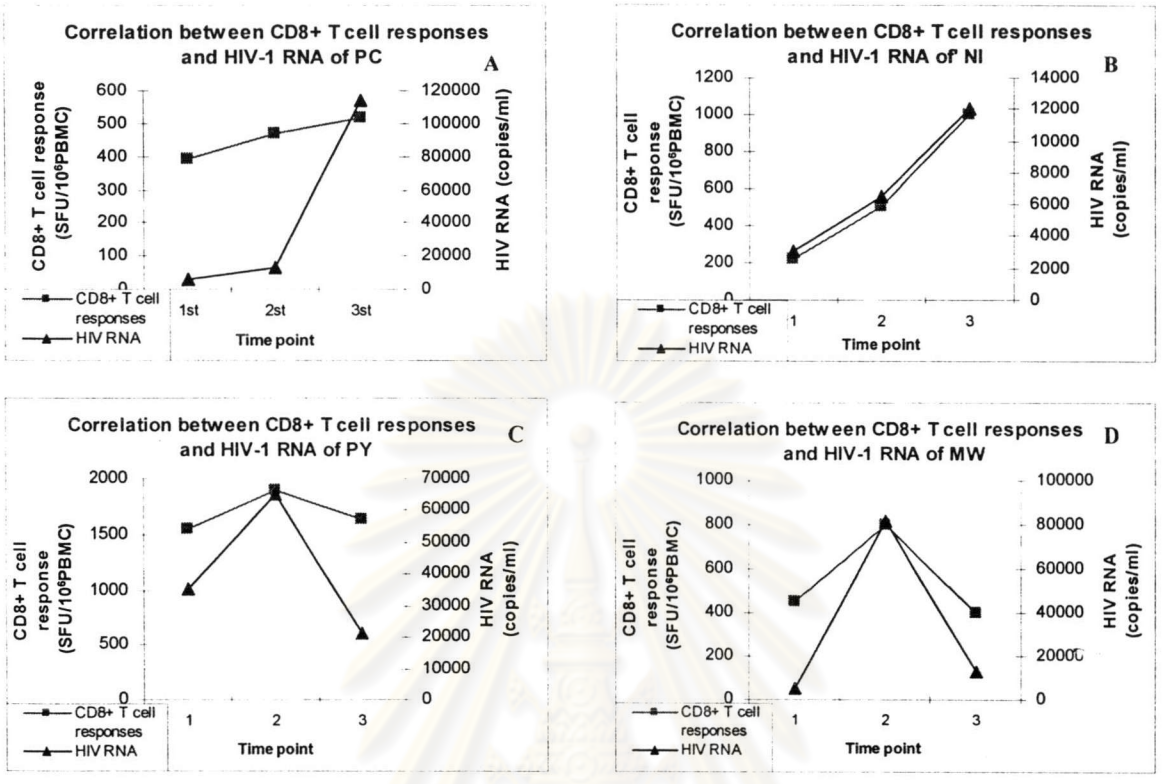

Figure 19 The positive correlations between GAFD-specific CD8+ T cell responses and HIV RNA of the patients PC (A), NI (B), PY (C), and MW (D). A square line is CD8+ T cell response (SFU/1x10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

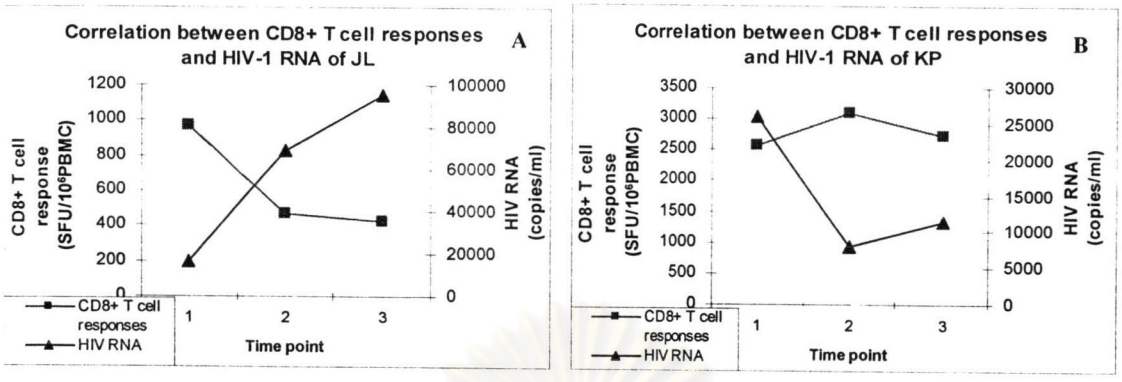

Figure 20 The inverse correlations between GAFD-specific CD8+ T cell responses and HIV RNA of JL (A) and KP (B). A square line is CD8+ T cell response (SFU/1x10^6 PBMC) by ELISpot assay. A triangle line is HIV RNA (copies/ml).

2.4 Cytotoxicity of immunodominant epitopes

The ELISpot assay measures the ability of T cells to produce IFN-γ but not the ability to kill target cells. Five of seventeen peptides which showed strong CD8+ T cell responses in this study were selected to determine cytotoxicity of CTL against cognate peptides (QVPLRPMTYK, GAFDLSFFLK, AIFQSSMTK, QIYAGIKVK, and QIYQEPFKNLK). The cytotoxicity function of CTL lines against the target cells were tested at an effector: target (E: T) ratio of 50: 1, 25: 1, and 12.5: 1. The patients who had high response to these peptides by ELISpot assay were subjected to this analysis. CTL lines that were stimulated by peptides comprising QIYA-and AIFQ-specific CTL lines of PN, GAFD-specific CTL line of WY, QIYQ-specific CTL line of PS, and QVPL-specific CTL line of AT.

Patient PN QIYA-and AIFQ-specific CTL lines were tested with QIYA-and AIFQ-pulsed autologous BLCL. These CTL line showed CTL activity against their target cells (Figure 21-22). These activities were consistent with the results of T cell response detected by ELISpot assay.

Patient WY This patient could recognise GAFDLSFFLK peptide detected by ELISpot assay. When GAFD-specific CTL line of WY was tested with GAFD-pulsed HLA-A11-matched BLCL, the CTL line showed a high level of specific CTL killing activity (Figure 23) which was consistent with high level of T cell responses observed by ELISpot assay.

Patient PS QIYQ-specific CTL line was tested with QIYQ-pulsed HLA-A11-matched BLCL. This CTL line recognised target cell and showed CTL specific killing activity (Figure 24) which was consistent with positive T cell responses by ELISpot analysis.

Patient AT QVPL-pulsed autologous BLCL was used as target cell against QVPL-specific CTL line. This CTL line showed CTL activity against target cells which was consistent with positive T cell responses by ELISpot analysis. This CTL line had CTL activity as high as at E: T ratio of 50: 1 (Figure 25).

All 5 patients showed CTL activity against autologous-transformed BLCL or HLA-A11-matched targeted cell-lines pulsed peptides investigated by cytotoxicity assay. These could be concluded that cytotoxicity activity of these CTL lines were consistent with T cell responses detected by ELISpot assay.

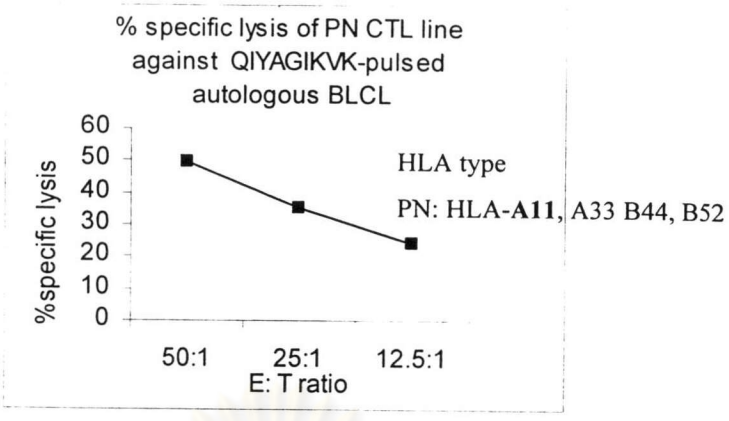

Figure 21 The QIYA-specific CTL responses of patient PN determined by cytotoxicity assay. The CTL assay shown were perform on day 10-14 in culture, by co-culturing the CTL with autologous BLCL pulsed with QIYAGIKVK peptide at an effector: target (E: T) ratio 50: 1, 25: 1, and 12.5: 1. The specific lysis was calculated by subtracting from the negative control. Results were regarded as more than 10% above that of the negative control and spontaneous release less than 30%.

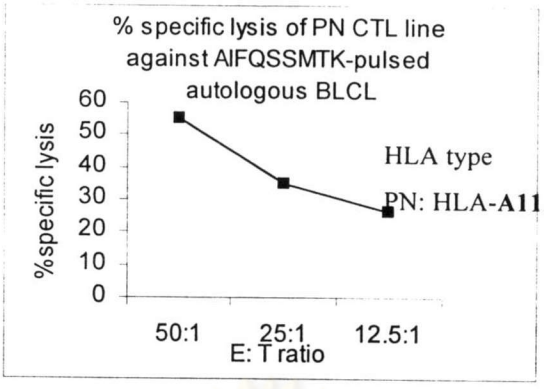

Figure 22 The AIFQ-specific CTL responses of patient PN determined by cytotoxicity assay. The CTL assay shown were perform on day 10-14 in culture, by co-culturing the CTL with autologous BLCL pulsed with AIFQSSMTK peptide at an effector: target (E: T) ratio 50: 1, 25: 1, and 12.5: 1. The specific lysis was calculated by subtracting from the negative control. Results were considered positive of % specific lysis was more than 10% above that of the negative control and spontaneous release less than 30%.

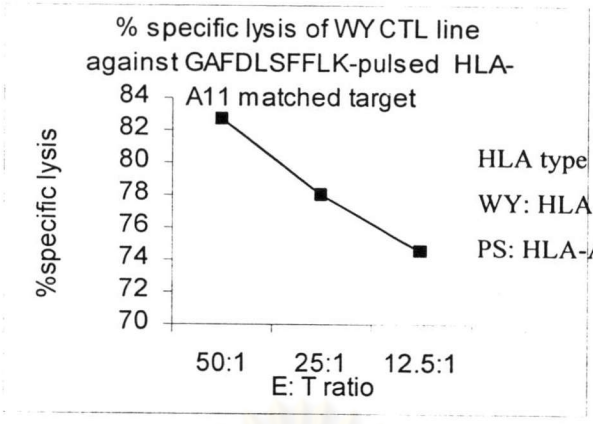

Figure 23 The GAFD-specific CTL responses of patient WY determined by cytotoxicity assay. The CTL assay shown were perform on day 10-14 in culture, by co-culturing the CTL with a target cell line matched only at HLA-A11 pulsed with GAFDLSFFLK peptide at an effector: target (E: T) ratio 50: 1, 25: 1, and 12.5: 1. The specific lysis was calculated by subtracting from the negative control. Results were considered positive of % specific lysis was more than 10% above that of the negative control and spontaneous release less than 30%.

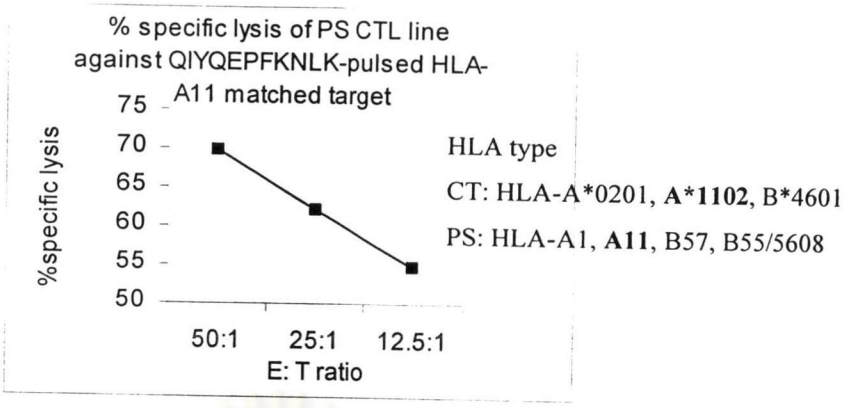

Figure 24 The QIYQ-specific CTL responses of patient PS determined by cytotoxicity assay. The CTL assay shown were perform on day 10-14 in culture, by co-culturing the CTL with a target cell line matched only at HLA-A11 pulsed with QIYQEPFKNLK peptide at an effector: target (E: T) ratio 50: 1, 25: 1, and 12.5: 1. The specific lysis was calculated by subtracting from the negative control. Results were considered positive of % specific lysis was more than 10% above that of the negative control and spontaneous release less than 30%.

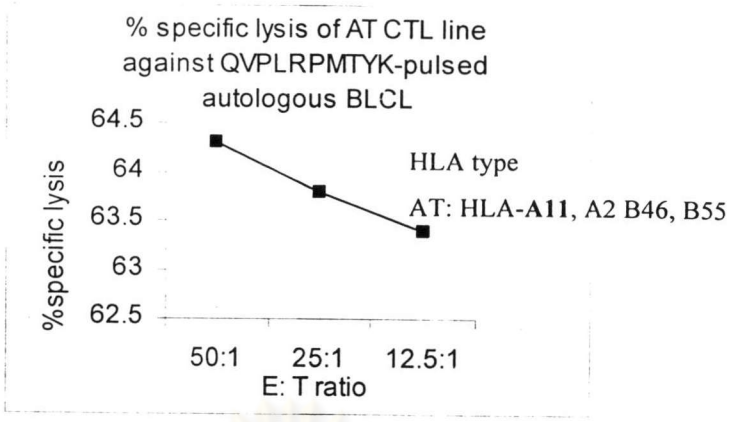

Figure 25 The QVPL-specific CTL responses of patient AT determined by cytotoxicity assay. The CTL assay shown were perform on day 10-14 in culture, by co-culturing the CTL with autologous BLCL pulsed with QVPLRPMTYK peptide at an effector: target (E: T) ratio 50: 1, 25: 1, and 12.5: 1. The specific lysis was calculated by subtracting from the negative control. Results were considered positive of % specific lysis was more than 10% above that of the negative control and spontaneous release less than 30%.

3. Cloning and sequencing

In this study, Nef seemed to be most immunodominant epitopes recognised by HLA-A11 positive HIV-infected patients. However, some patients had fluctuation or absence of responses against these immunodominant epitopes. We hypothesised that the unusual responses observed in this study was due to escape mutation in the patients. In order to prove this hypothesis, we analyses the *nef* sequences of the HIV quasispecies by DNA cloning and sequencing.

3.1 Primer design for *nef* amplification

To amplify *nef* we used nested PCR method to enhance both sensitivity and specificity of the PCR. The most common of subtype of HIV in HIV-1-infected Thai patients is subtype A/E. Therefore, the consensus sequence of subtype A/E of HIV-1 (CM240: U54771) was selected for primers design. Our primers were designed using Oligos 9.1 by Ruslan Kalendar institute of Helsinki, Finland.

The information of primers was used for *nef* amplification.

Primer combinations: Inner primers

Inner forward primer: 5' CCTAGAAGAATCAGACAGGGCTTAG 3'

Position: 8328 Tm = 52.58 Length: 25

Inner reverse primer: 5' TCCCCTGGAAAGTCCCCAGC 3'

Position: 9011 Tm = 52.80 Length: 20

Length of PCR product = 703

Primer combinations: Outer primers

Outer forward primer: 5' GGTGGAACTTCTGGGACACAGC 3'

Position: 8120 Tm = 53.45 Length: 22

Outer reverse primer: 5' GGGTTAGCTACTCCCCAACTCC 3'

Position: 9045 Tm = 53.45 Length: 22

Length of PCR product = 947

The amplified *nef* product was 703bp. The outer and inner forward primers are located at *env*, whilst the inner and outer reverse primers are located at *LTR* (Figure 26). The primers were then blasted to study the specificity of our primers at http://www.ncbi.nlm.nih.gov/BLAST using Nucleotide-nucleotide BLAST (blastn). In doing so, we were able see whether our primers bind to other *human genes*, and hence amplifying unwanted *human gene* products. The result demonstrated that our primer sets were able to amplify *human gene*, but this amplified product was far larger (>2000bp) than amplified *nef* product. On the other hand, our primers were analysed to identify their false priming sites (non-specific binding site) in HIV-1 genome. The result showed that our primer sets were able to amplify HIV-1 gene but the false amplified HIV-1 products were far larger (>2000) than the amplified *nef* product (Table 12).

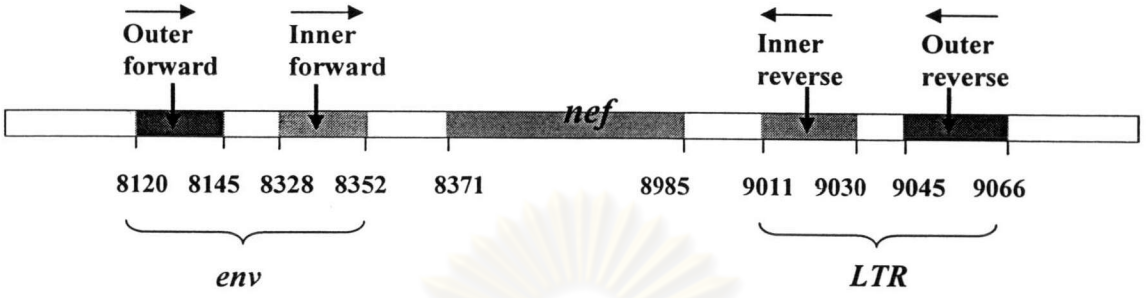

Figure 26 The positions of outer and inner primers in HIV-1 subtype A/E reference strain (U54771). The arrow represents the position of the outer forward, inner forward, inner reverse, and outer reverse primers used to amplify *nef* in this study.

Primers	The specific binding site of primers	Non-specific binding site of primers
Outer forward	8120	289, 703
Outer reverse	9043	No regions found
Inner forward	8328	5253, 5527
Inner reverse	9011	2755

Table 12 The representative non-specific binding site of primers. HIV-1 subtype A/E reference strain (U54771) was selected to use as template in predicting of the non-specific binding site of the primers.

3.2 Sensitivity of PCR assay

To determine sensitivity of PCR assay, *nef* from the patient PN was diluted in 10-fold dilutions and amplified. The result showed that PCR assay could detect *nef* at equal to 10 ng of DNA (Figure 27).

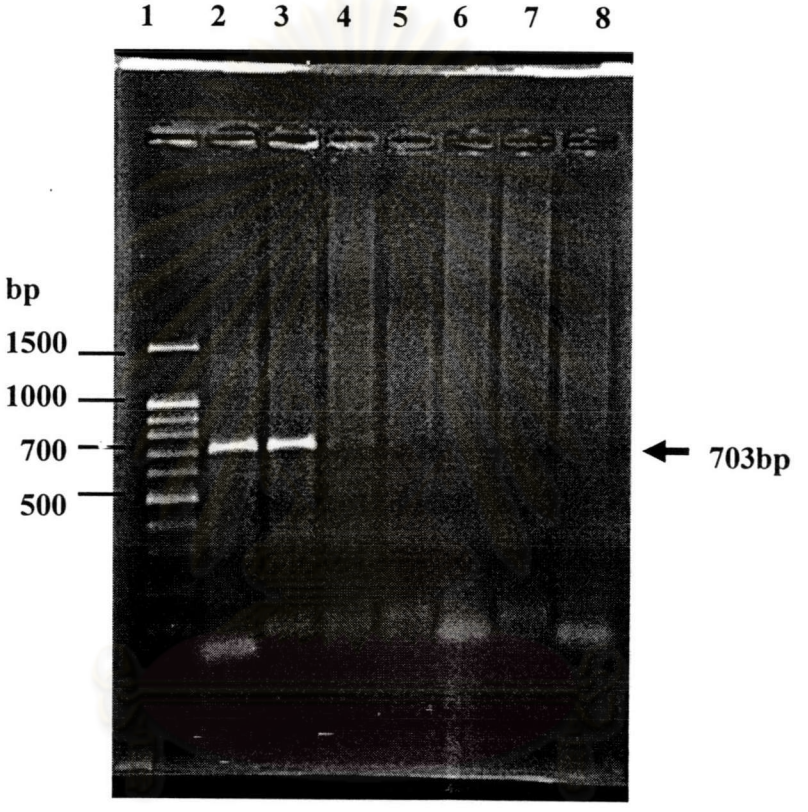

Figure 27 Sensitivity of PCR assay. *nef* was amplified from proviral DNA of patient PN. Lane 1: 100bp molecular marker, lane 2: 290ng, lane 3: 100ng, lane 4: 10ng, lane 5: 1ng, lane 6: 100pg, lane 7: 10pg, lane 8: negative control (distilled water). The amplified *nef* product was 703bp.

3.3 Amplification of *nef*

In our study, HIV-specific CD8+ T cell responses against QVPLRPMTYK and GAFDLSFFLK peptides within Nef seemed to be two most immunodominant epitopes. However, some of patients who had no QVPL-and GAFD specific responses or had fluctuation of T cell response against immunodominant epitopes. The absence of these two dominant responses might be resulted from escape mutation. We therefore analysed the *nef* sequence from our donors (n=6) who had unusual response either epitope (Table 13).

1. GAFD-non responders

   There were 4 patients PN, OK, UP, and AT who had no GAFD-specific T cell responses.

2. QVPL-non responder

   There was only one patient (WY) who had no QVPL-specific T cell response.

3. The patient had fluctuation of T cell response

   There was one patient (PS) who had fluctuation of T cell response against QVPLRPMTYK. PS did not have QVPL-specific T cell response at 1st and 2nd time points, but he had the T cell response at 3rd time point (Figure 28). Therefore, *nef* from 2nd and 3rd time points was amplified, sequenced and compared.

nef of the patients PN, PY, UP, WY, and PS at 2nd and 3rd time point were successfully amplified (Figure 29 and 30). *nef* of the patients OK and AT, on the other hand, could not be amplified (Figure 30).

Patients	Peptide	
	QVPLRPMTYK	GAFDLSFFLK
PN	466	NR
UP	1740	NR
OK	348	NR
AT	774	NR
WY	NR	1570
PS	NR	1798

Table 13 The selected patients for nucleotide and amino acid sequences analysis. The patients PN, OK, UP, and AT could not recognise GAFDLSFFLK peptide, whilst WY and PS could not recognise QVPLRPMTYK peptide. NR indicated no T cell responses.

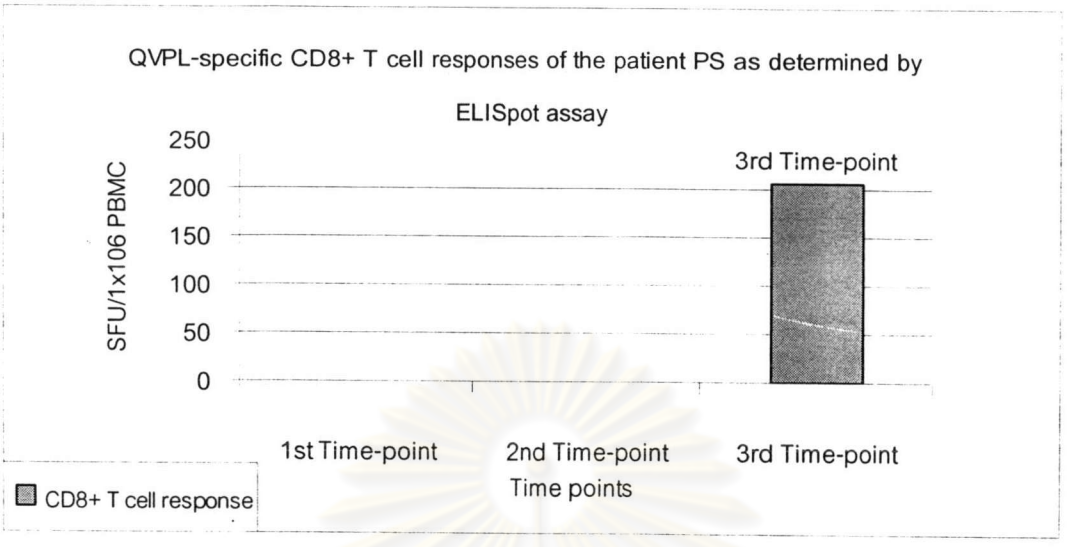

Figure 28 HIV-specific CD8+ T cell responses of patient PS against QVPLRPMTYK peptide as determined by ELISpot assay in longitudinal analysis. Results are presented as SFU of HIV-specific CD8+ T cell responses subtracting with cut off in each time point.

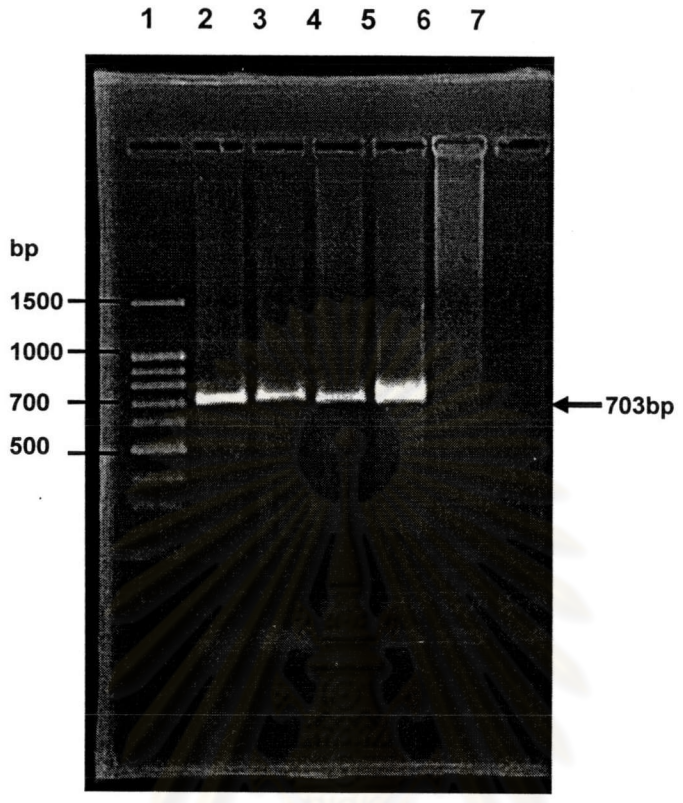

Figure 29 The representative amplified product of *nef* from the patients PN, PY, UP, and WY. Lane 1: 100bp molecular marker, lane 2: PN, lane 3: PY, lane 4: UP, lane 5: WY, lane 6: PBMC of healthy individual (negative control), lane 7: distilled water (negative control). The amplified *nef* product was 703bp.

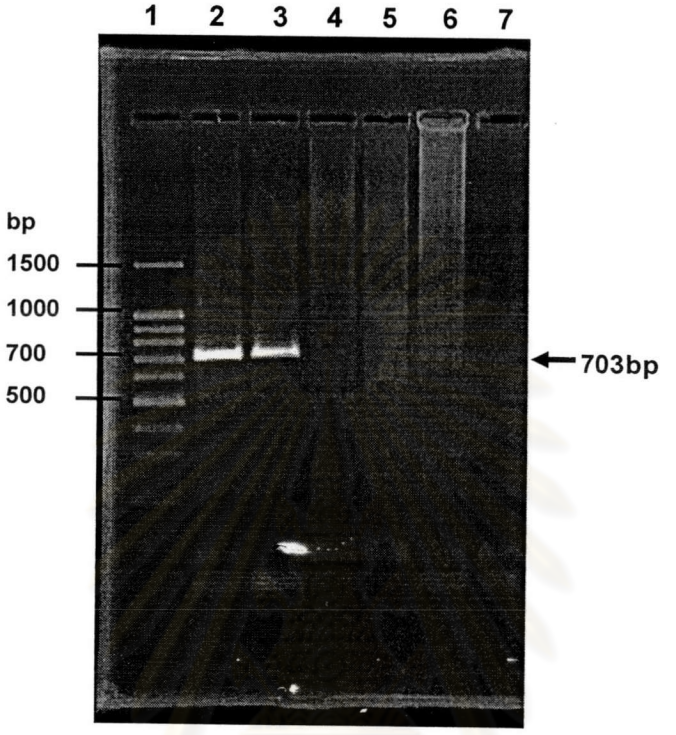

Figure 30 The representative amplified product of *nef* from the patients PS at 2nd and 3rd time point, OK, and AT. Lane 1: 100bp molecular marker, lane 2: PS at 1st time point, lane 3: PS at 2nd time point, lane 4: OK, lane 5: AT, lane 6: PBMC of healthy individual (negative control), lane 7: distilled water (negative control). The amplified *nef* product was 703bp.

There were two patients (OK and AT) the *nef* of which could not be amplified. To determine whether absence of the amplified product in these patients was due to insufficient amount of DNA or DNA damage, we amplified *β-globulin* in parallel to amplification of *nef* (Figure 31). In this assay, we amplified PY, OK and AT using both *nef*-specific primers and *β-globulin*-specific primers. The results showed that only *nef* from the patient PY could be amplified, whilst amplified *β-globulin* product was seen in all patients. This experiment confirmed that the unsuccessful amplification of *nef* from the patients OK and AT was not due to the amount or the quality of the DNA.

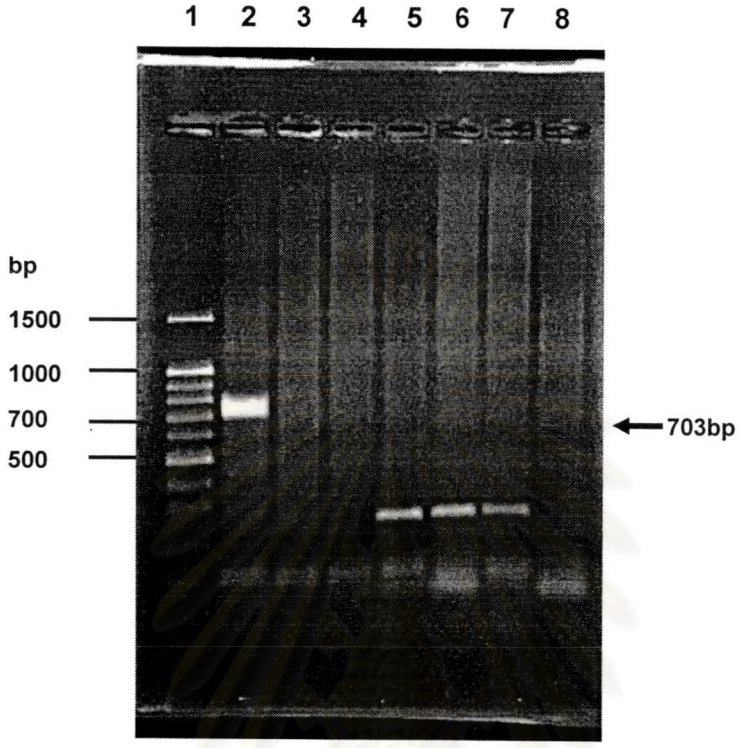

Figure 31 The representative amplified product of *nef* and *β-globulin* from the patients OK, AT, and PY. Lane 1: 100bp molecular marker, lane 2 to 4: the PCR products of PY, OK, and AT which were amplified by *nef*-specific primers, lane 5 to 7: the PCR product of PY, OK, and AT which were amplified by *β-globulin*-specific primers, lane 8: distilled water (negative control). The amplified *nef* product was 703bp.

The question still remained at this point as to why we could not amplify *nef* from OK and AT. Since our primers were designed based on DNA sequences from HIV-1 subtype A/E reference strain (U54771), the primers might not be able to amplify *nef* from the other subtype such as HIV-1 subtype B. Indeed, the patients OK and AT was previously shown to be infected with subtype B (data not shown). We therefore analysed the primers to determine the difference of nucleotide sequences at the primer binding sites between subtype A/E and Asian subtype B.

The outer and inner forward primer binding sites of subtype A/E reference strain (U54771) were compared with *env* of HIV-1 subtype B. The result of comparison of outer forward primer binding site showed the differences of nucleotide sequences at several positions which were G1T (36/36), A18G (22/36), A18T (14/36) and C22G (36/36) (Figure 32), whereas the differences of inner forward primer binding sites were G5C (18/36), C12A (33/36), and G14A (20/36) (Figure 33).

For inner and outer reverse primer binding sites, these primer binding sites of subtype A/E were compared with nucleotide sequences in *LTR* of HIV-1 subtype B. In a case of inner reverse primer binding site, the amino acid deletion was found at position 19 in all isolates of subtype B (Figure 34), whilst outer reverse primer had mismatch nucleotides including A3G (6/7), G4A (7/7), T5C (7/7), A14G (7/7), T17G (6/7), and A19G (7/7) (Figure 35).

This primer binding sites discrepancies might help to explain as to why our primers could not amplify *nef* of the patients OK and AT whom were previously infected with subtype B (Figure 32-35).

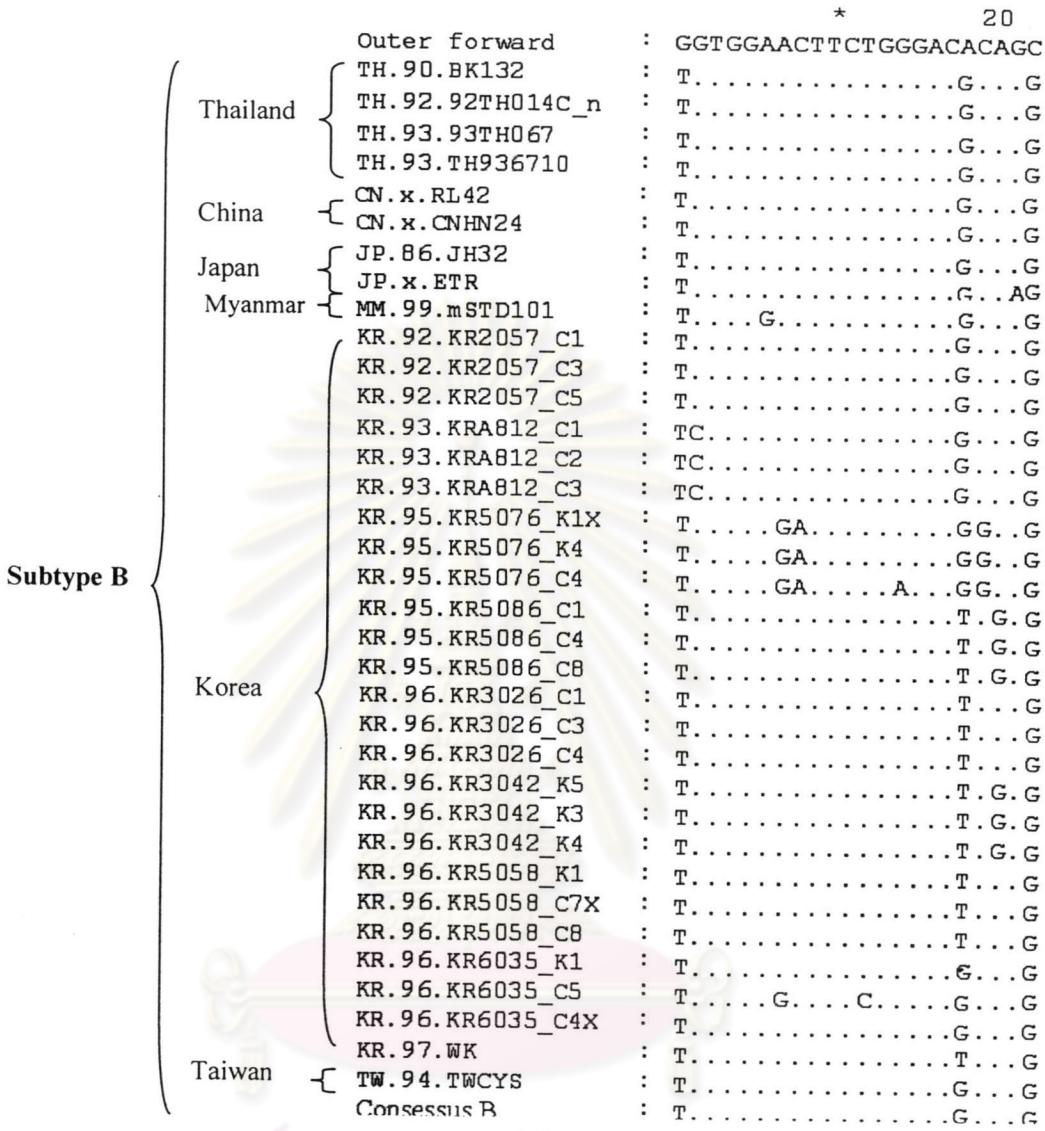

Figure 32 Alignment of nucleotide sequences of outer forward primer binding sites between subtype A/E and subtype B strains. Outer forward primer binding site was shown at the top. The first column indicates country, sampling year, and name of HIV-1 subtype B for example TH.90.BK132; TH indicates country (Thailand), 90 indicates sampling year (1990), BK132 indicates name of sequences derived from Los alamos database. The second column indicates the nucleotide sequences of primer binding sites.

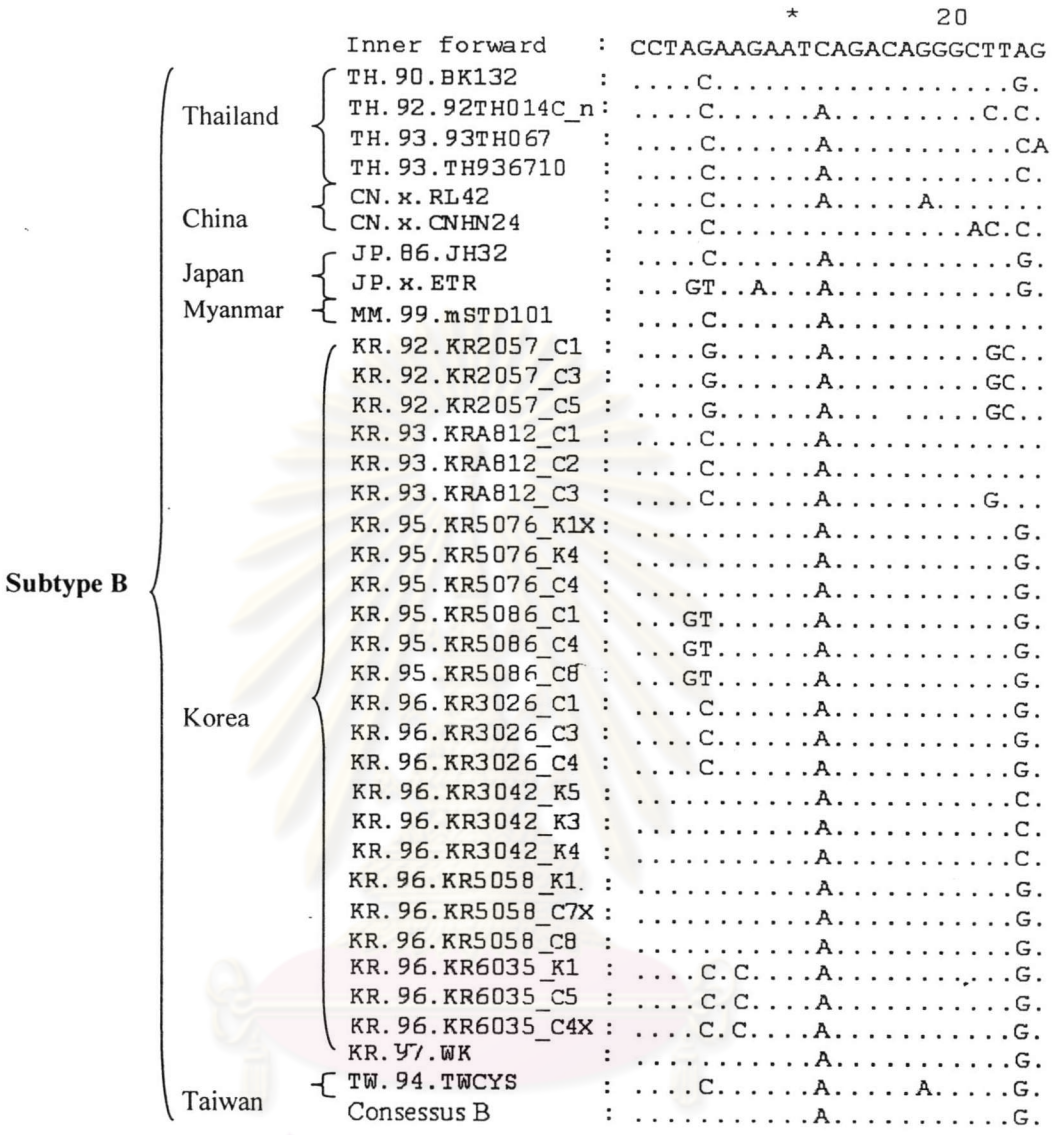

Figure 33 Alignment of nucleotidesequences of inner forward primer binding sites between subtype A/E and subtype B strains. Inner forward primer binding site was shown at the top. The first column indicates country, sampling year, and name of HIV-1 subtype B for example TH.90.BK132; TH indicates country (Thailand), 90 indicates sampling year (1990), BK132 indicates name of sequences derived from Los alamos database. The second column indicates the nucleotide sequences of primer binding sites.

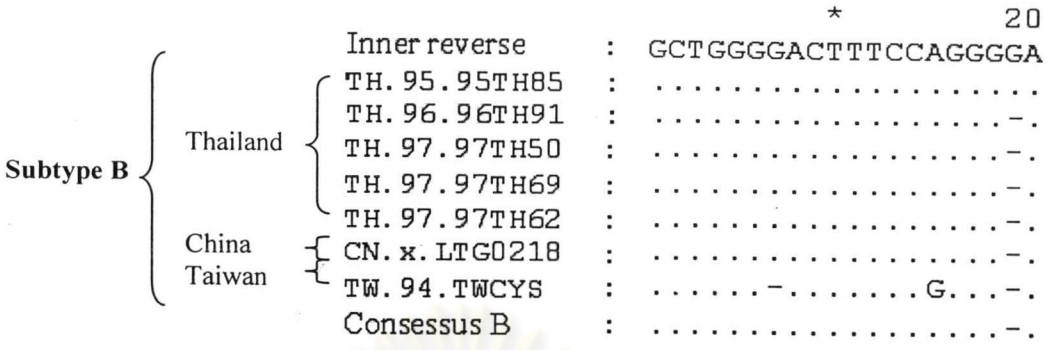

Figure 34 Alignment of nucleotide sequences of inner reverse primer binding sites between subtype A/E and subtype B strains. Inner reverse primer binding site was shown at the top. The first column indicates country (Thailand), 95 indicates sampling year (1995), 95TH85 indicates name of sequences derived from Los alamos database. The second column indicates the nucleotide sequences at primer binding sites.

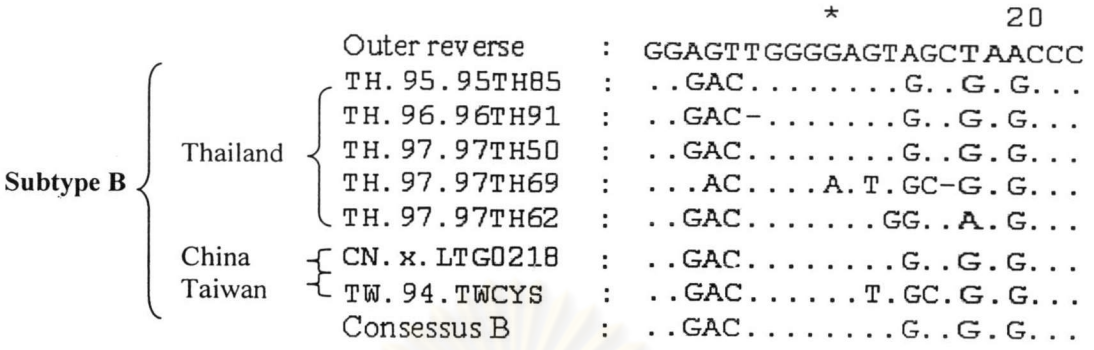

Figure 35 Alignment of nucleotide sequences at outer reverse primer binding sites between subtype A/E and subtype B strains. Outer reverse primer binding site was shown at the top. The first column indicates country, sampling year, and name of HIV-1 subtype B for example TH.95.95TH85; TH indicates country (Thailand), 95 indicates sampling year (1995), 95TH85 indicates name of sequences derived from Los alamos database. The second column indicates the nucleotide sequences at primer binding sites.

3.4 Cloning of *nef*

The high mutation rate of HIV can lead to emergence of quasispecies and escape mutations. Either epitope mutations or mutation at flanking region could account for abrogation of CTL recognition. Since the sequence acquired by direct sequencing method might represent only the most prevalent strain of HIV quasispecies, we decided to use cloning and sequencing method to acquire sequences of both major and minor strains. To determine whether the unusual response of patients PN, UP, PY, WY, and PS were due to epitope or flanking region mutation, we cloned and sequenced *nef* of these donors.

3.5 Detection of *nef* inserts

The *nef*-inserted plasmids were cloned to competent cells (*E. coli* strain DH5α). After cloning, *nef* of each clone was amplified to confirm that these clones had *nef* in the plasmid (Figure 36) whereby the amplified *nef* product was 703bp.

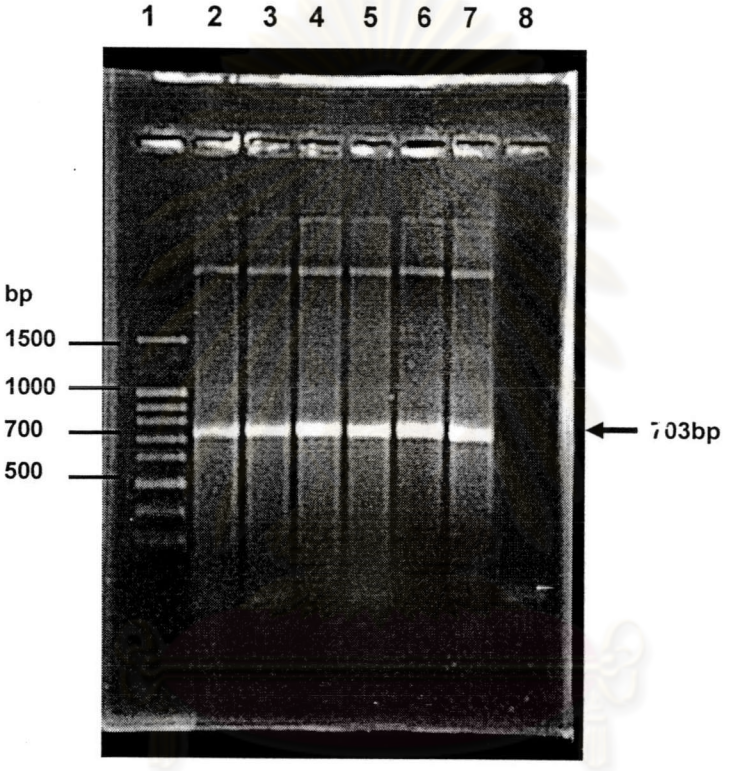

Figure 36 The representative amplified *nef* product from the *nef*-inserted clones analysed by PCR assay. This figure showed the amplified product from the patient PN. Lane 1: 100bp molecular marker. Lane 2 to 7: the amplified PCR product from 6 clones of the patient PN. Lane 8: negative control (distilled water). The amplified *nef* product was 703bp.

3.6 *nef* sequencing

Amino acid sequences within the epitope and the sequences of flanking regions from each patient were compared with subtype A/E reference strain (U54771). The five clones of the purified plasmids of PN, UP, PY, WY, and PS (at 2nd and 3rd time points) were selected to determine the nucleotide and amino acid sequences. We found consistent amino acid substitution of M69R in all patients and Y79F in 4 of 5 patients (Figure 37).

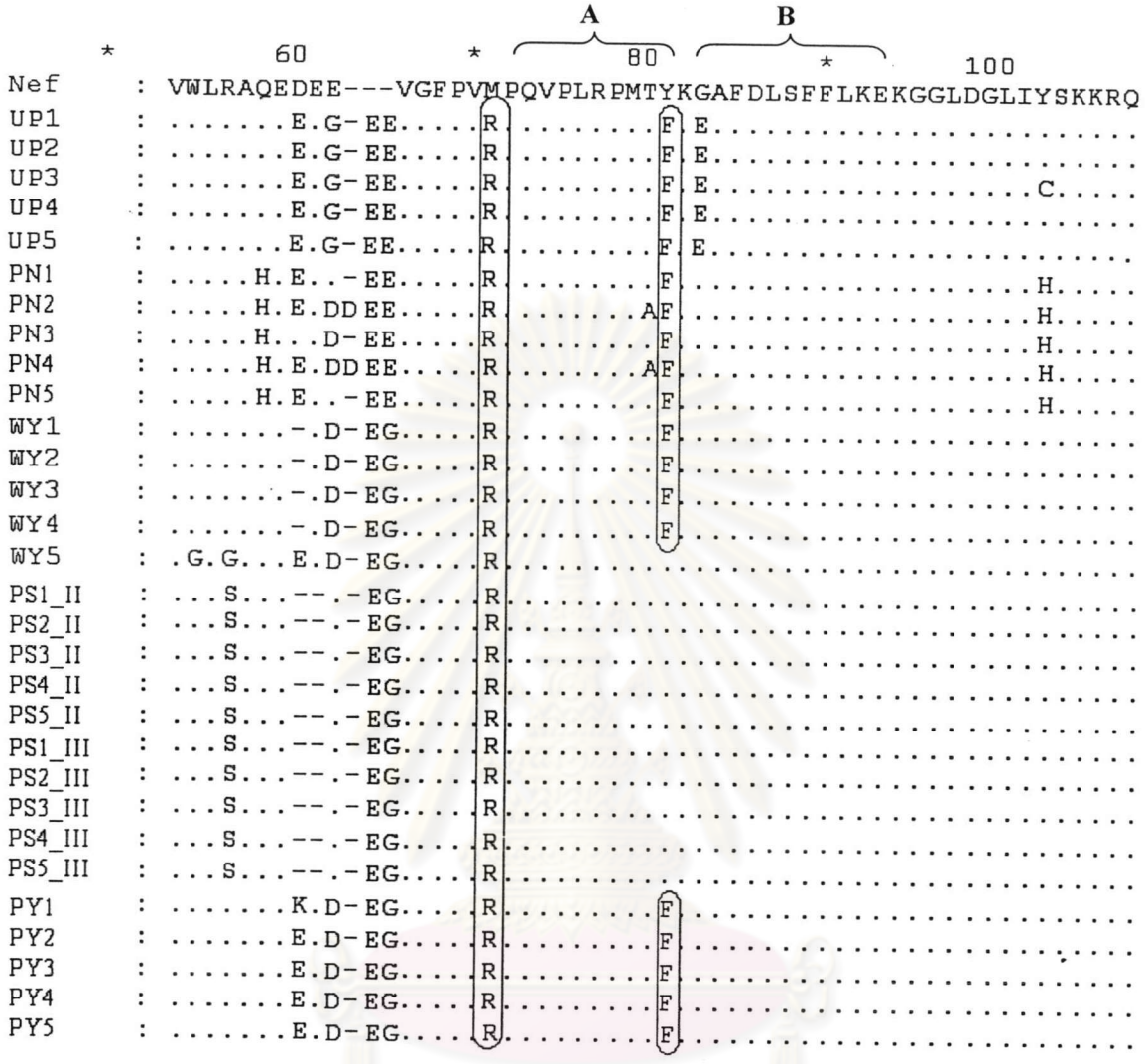

Figure 37 Alignment of Nef amino acid sequences. These aligned sequences were selected to demonstrate GAFDLSFFLK, QVPLRPMTYK epitopes and their flanking regions of 5 *nef* clones from each HIV-infected individual. A representative Nef amino acid sequence of subtype A/E reference strain (U54771) was shown at the top. The first column indicates 5 clones of each patient (PS_II; the amino acid sequences of PS at 2nd time point and PS_III; the amino acid sequences of PS at 3rd time point). The second column indicates the amino acid sequence of 5 clones in each patient. The A and B indicate the position of QVPLRPMTYK and GAFDLSFFLK epitopes. A rounded rectangle indicates the amino acid substitution of M69R in all patients and Y79F in 4 of 5 patients.

3.6.1 GAFD-non responder

Due to the absence of CTL recognition to GAFDLSFFLK peptide in the patients UP and PN, HIV from both patients were cloned and sequenced to study the amino acid and nucleotide sequences. For patient UP, all clones contained G83E mutation within this epitope (GAFDLSFFLK vs. EAFDLSFFLK) (Figure 38).

No. clones	Epitope (variants)		
	Nef	:	GAFDLSFFLK
5/5	UP5	:	E.........
	UP4	:	E.........
	UP3	:	E.........
	UP2	:	E.........
	UP1	:	E.........

Figure 38 The representative amino acid sequences of 5 clones from patient UP. A rectangle positions the mutated amino acid of GAFDLSFFLK epitope (G83E) in the patient UP.

In a case of PN, the amino acid residues within GAFDLSFFLK epitope were conserved but the flanking amino acids were mutated. Only the Y102H amino acid mutation which is located on 10th amino acid away from the C-terminal of the epitope was observed in 3/5 clones, while 2/5 clones have T80A amino acid substitution which is located on 3rd amino acid away from the N-terminal and Y102H on 10th amino acid away from the C-terminal of the epitope (Figure 39). For Y79F amino substitution, this mutation was observed in both GAFD-non responder and GAFD-responder. Thus, this point mutation might not affect GAFD-specific CD8+ T cell responses.

No. clones			* 100
	Nef	:	TYKGAFDLSFFLKEKGGLDGLIY
3/5	PN5	:	.F.....H
	PN3	:	.F.....H
	PN1	:	.F.....H
2/5	PN2	:	AF.....H
	PN4	:	AF.....H

Figure 39 The representative amino acid sequences of 5 clones from the patient PN. A rectangle positions the mutated amino acid in flanking region of GAFDLSFFLK epitope compared to consensus sequence (CM240; U54771).

3.6.2 QVPL-non responder

HIV-1 *nef* from WY who did not respond to QVPLRPMTYK epitope was analysed to determine sequence of this epitope and its flanking region. The *nef* sequences of WY were compared with QVPL-responder.

The amino acid residues within epitope were conserve, but the deletion mutation was observed in the flanking region. Whereas 4/5 clones from the patient PY had deletion mutation on 10^{th} amino acid away from the N-terminal, the other patients who were QVPL-non responder or QVPL-responder have not mutation in this position (Figure 39). This point mutation therefore might affect QVPL-specific CD8+ T cell responses.

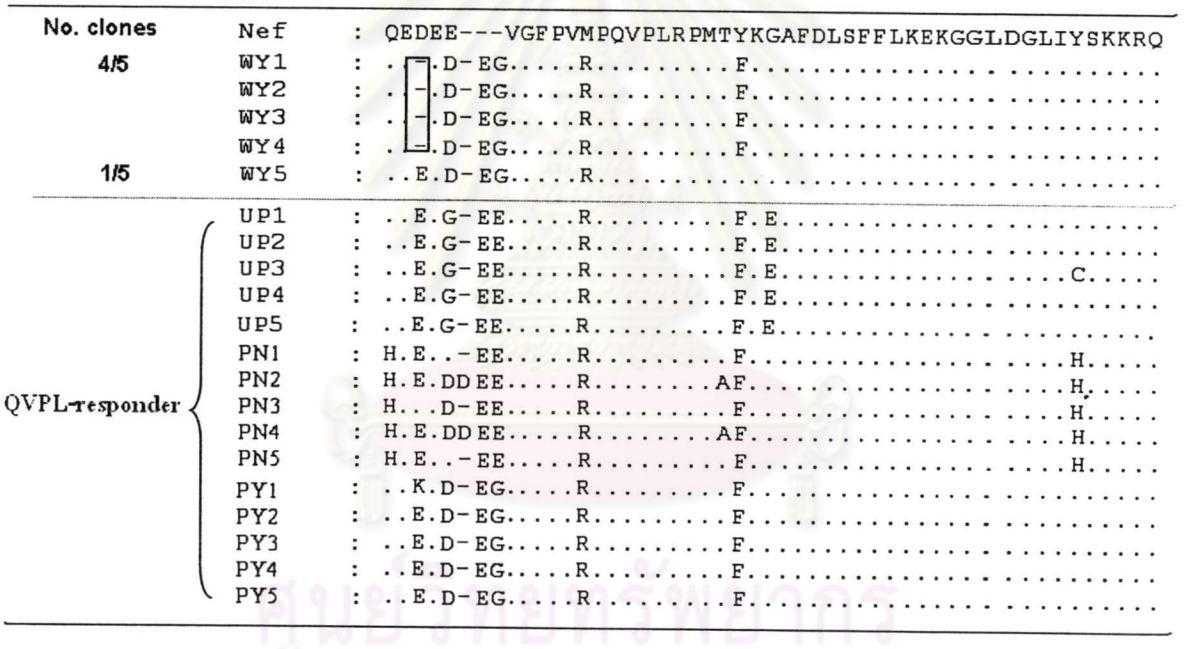

Figure 39 The representative amino acid sequences of the patients WY. A rectangle positions the deleted amino acid in flanking region of QVPLRPMTYK epitope compared with amino acid of QVPL-responder.

3.6.3 The patient had fluctuation of T cell response

In this part, we compared amino acid sequence of the patient PS at 2nd and 3rd time points to determine sequencing of the epitope and its flanking region. In a case of PS, this patient could not recognise at 1st and 2nd time points, but he could recognise QVPLRPMTYK peptide at 3rd time point. Proviral DNA of PS at 2nd and 3rd time point were therefore cloned and sequenced.

The amino acid sequences within QVPLRPMTYK epitope and flanking region were conserved in both 2nd and 3rd time point (Figure 40). In addition, when we compared the nucleotide sequences within epitope between 2nd and 3rd time point, the nucleotide sequences were also conserved. However, we found that the synonymous mutation was detected in this epitope region. In contrast to QVPL responders, Lysine (K) at position 10 (QVPLRPMTY**K**) of patient PS was translated from AAA codon, whilst this amino acid of the other patients was translated from AAG codon (data not shown).

No. clones	Nef	: LRAQEDEE---VGFPVMPQVPLRPMTYKGAFDLSFFLKEKGGL
5/5	PS1_II	: .S...--.-EG.....R.....
	PS2_II	: .S...--.-EG.....R.....
	PS3_II	: .S...--.-EG.....R.....
	PS4_II	: .S...--.-EG.....R.....
	PS5_II	: .S...--.-EG.....R.....
5/5	PS1_III	: .S...--.-EG.....R.....
	PS2_III	: .S...--.-EG.....R.....
	PS3_III	: .S...--.-EG.....R.....
	PS4_III	: .S...--.-EG.....R.....
	PS5_III	: .S...--.-EG.....R.....

Figure 40 The representative amino acid sequences of patient PS at 2nd and 3rd time points. PS_II indicated the amino acid sequences of PS at 2nd time point and PS_III indicates amino acid sequences of PS at 3rd time point.