CHAPTER 4

Investigation of Conserved Epitopes-Specific Memory CD8 T cells Responses in HIV+ Children after 2009 H1N1 Influenza A Vaccination

4.1 Introduction

Children are predominantly susceptible to influenza infection, have the highest age-related mortality rates, and are the primary reservoir and source of virus for spreading to other age groups [267]. According to the study in high HIV prevalence country, children under 5 years of age who living with HIV were at greater risk of death related to influenza, compared with HIV− healthy children [268]. Influenza vaccination competent of inducing immune responses is an efficient approach to reduce severity of illness and to protect viral infection. Immunization of children with influenza vaccine conferred a protection against influenza among population who did not receive the vaccine [269]. Induction of immune responses against conserved epitopes of any influenza virus serotypes is believed to be a concept for designing a cross-protective universal vaccine which does not necessitate yearly vaccination [270]. Memory CD8+ T cells are given consideration to have contributed in protective immune response against influenza virus in healthy human [110, 271]. However, their roles against influenza vaccination in HIV+ children are less well understood.

In this study, we investigated CD8+ T cell immune response against conserved epitopes of influenza virus stimulations *in vitro* in HIV-infected children following 2009 H1N1 influenza vaccination.

4.2 Methods

4.2.1. Vaccination and blood collection

All children received intramuscularly two doses of Panenza®, monovalent 2009 H1N1 influenza A vaccine 4 weeks apart. Venous bloods were collected at baseline before vaccination (Day 0) and at 28 days (D28) and 56 days (D56) after first dose vaccination.

4.2.2. Study populations

Children in this study were part of the study on immunogenicity and safety of monovalent 2009 H1N1 influenza A in HIV-infected Thai children [9]. In this study, 60 HIV+ children between 6 months to 18 years old who had been followed at Maharaj Nakorn Chiang Mai hospital, CMU, Thailand were enrolled during the second wave of 2009 H1N1 influenza A outbreak in Thailand, from February 16, 2010 to May 25, 2010 with consent from them and their parents. Eligible children without severe allergy to eggs, history of hypersensitivity to seasonal influenza vaccination, acute illness, history of Guillain–Barre syndrome were enrolled. Children who received other licensed live vaccines within 4 weeks or inactivated vaccines within 2 weeks of study entry and children who receiving immunosuppressive therapy for the previous 6 months or blood products in the previous 3 months were excluded from the study. Baseline characteristics and vaccine response are shown in table 4.1. All children were receiving ART. Their mean age was 10.9 ± 3.5 years and mean absolute number CD4 cells was 779.7 ± 397.9 cells/mm^3. Fifty-one children (51/60, 85.0%) had HAI antibody titres ≥1:40 to 2009 H1N1 influenza. The seroconversions to 2009 H1N1 influenza A were 51.7% and 70% after first and second doses, respectively. On the day of enrolment, 48 (80.0%) had HIV RNA level < 40 copies/mL, and 53 (88.3%) had < 1000 copies/mL.

Table 4.1 Baseline characteristic and vaccine response

Characteristic	Participants
Total	**60**
Female	28
Mean age (year)	10.9 ± 3.5
Mean CD4+ cell counts (cells/mm^3)	779.7 ± 397.9
Receiving cART	60/60(100%)
HIV RNA level <40 copies/mL	48/60 (80.0%)
HAI antibody titres ≥1:40 after vaccination	51/60 (85.0%)
Seroconversions at a month after vaccination	
First dose	31/60 (51.7%)
Second dose	42/60 (70.0%)

4.2.3. Isolation of PBMCs by gradient centrifugation

As described in chapter 2 section 2.3

4.2.4. Cell stimulations

Cryopreserved PBMCs were thawed and rested overnight as described in Chapter 2, section 2.4. Recovered PBMCs were stimulated with complete RPMI containing 5 μg/mL of PHA, 20 μg/mL of pooled H1N1 peptides (Life Technologies, Grand Island, N.Y., USA) or complete media (unstimulated control). The amino acid sequences of the H1N1 peptides obtained from the Immune Epitope Database and Analysis Resource (IEDB) website (www.iedb.org) are shown in table 4.2. These peptides correspond to predicted CD8+ T cell epitopes which bind to HLA-A2, HLA-A11 and HLA-A24, covering approximately 80% of HLA class I in Thai population [272-275]. Cells were cultured in 37°C 5% CO_2 incubator for 16-18 hours. Cytokine-producing and CD107a-expressing CD8+ T cells were assessed by ICS assay.

Table 4.2 HLA-restricted amino acid sequences of H1N1 influenza A epitopes obtained from the immune epitope database and analysis resource (IEDB) website (www.iedb.org).

H1N1 antigens	HLA isotypes	Amino acid sequences
Matrix protein 1	A2	GILGFVFTL
	A11	SIIPSGPLK
	A12	LYRKLKREITF
Non structural protein	A13	AIMDKNIIL

4.2.5. Determination of cytokine-secreting T cells in response to specific antigen by ICS technique

As described in chapter 2 section 2.5

4.3 Results

In order to investigate the correlation of cellular and humoral immune responses, participants were classified into three groups based on their serologic responses as previously reported [9]. After accomplishment of 2 doses Panenza®, monovalent 2009 H1N1 influenza A vaccine vaccination, 18 children who did not respond to the vaccine were classified as negative group (Neg). Thirty-one and 11 children had seroconversion after 1 dose (1do) and 2 doses (2do) of vaccination, respectively were also classified.

Gating strategy of flow-cytometric analysis of cytokine-producing and CD107a-expressing CD8+ T cells are described in chapter 2 section 2.6 (Fig. 2.2). The median fold increases of the percentages of cytokine-producing and CD107a-expressing memory CD8+ T cells (Figure 4.1A and table 4.3) in response to the pooled H1N1 influenza A peptide stimulation *in vitro* over medium alone between groups and between visits were compared. Statistical analysis by Kruskal-Wallis test between groups in any visits did not indicate statistical differences of memory CD8+ T cells that produced TNF-α (p = 0.87), IFN-γ (p = 0.20), IL-2 (p = 0.89) or expressed CD107a (p = 0.14). There were also no statistically significant differences in the median fold increase

of the percentages of all cytokine-producing and CD107a-expressing total CD8+ T cells between groups in any visits (TNF-α; p = 0.85, IFN-γ; p = 0.43, IL-2 p = 0.60 and CD107a; p = 0.43).

The production of cytokine and expression of CD107a in total CD8+ T cells were further analysed by flow-cytometric analysis as described in chapter 2 section 2.6 (Figure 2.1). Comparable median fold increases of the percentages of cytokine-producing and CD107a-expressing total CD8+ T cells (Figure 4.1B and table 4.3) in response to the pooled H1N1 influenza A peptide stimulation *in vitro* over medium alone between groups and between visits were observed. All samples showed the increases of cytokine production and CD107a expression after the PHA stimulation. The fold increases in cytokine production and CD107a expression on day 56 after vaccination in response to PHA stimulation are demonstrated in Figure 4.2.

Table 4.3 The median and interquartile range of % cytokine producing CD8+ memory T cells in HIV+ children after pooled peptide stimulation *in vitro*.

Visit	Group	Antigen/ Fold increase	% Cytokine-producing and CD107a-expressing CD8+ memeory Tcells			
			TNF-α	IFN-γ	IL-2	CD107a
Day 0	Negative (n=18)	Unstimulated	0.09 (0.07-0.20)	0.23 (0.13-0.31)	0.08 (0.06-0.12)	0.49 (0.33-1.02)
		Pooled peptides	0.15 (0.08-0.23)	0.22 (0.08-0.33)	0.07 (0.04-0.10)	0.47 (0.34-1.03)
		Fold increase	1.12 (0.88-1.44)	0.64 (0.53-1.19)	0.87 (0.67-1.00)	0.95 (0.75-1.13)
	2 doses (n=11)	Unstimulated	0.10 (0.08-2.28)	0.35 (0.18-0.48)	0.11 (0.06-0.17)	0.77 (0.37-4.35)
		Pooled peptides	0.18 (0.08-2.20)	0.23 (0.15-0.37)	0.08 (0.06-0.12)	0.76 (0.34-5.48)
		Fold increase	1.13 (0.89-1.80)	0.63 (0.58-0.88)	0.90 (0.57-1.36)	0.94 (0.99-1.26)
	1 dose (n=31)	Unstimulated	0.11 (0.07-0.38)	0.21 (0.09-0.51)	0.06 (0.05-0.13)	0.36(0.28-1.01)
		Pooled peptides	0.14 (0.09-0.38)	0.23 (0.13-0.44)	0.06 (0.04-0.15)	0.38 (0.22-0.91)
		Fold increase	1.28 (0.91-1.83)	1.00 (0.80-1.20)	1.00 (0.69-1.48)	0.90 (0.76-1.11)
Day 28	Negative (n=18)	Unstimulated	0.07 (0.04-0.16)	0.15 (0.08-0.24)	0.06 (0.05-0.11)	0.24 (0.14-0.47)
		Pooled peptides	0.11 (0.08-0.13)	0.14 (0.08-0.22)	0.07 (0.02-0.09)	0.24 (0.16-0.49)
		Fold increase	1.42 (0.88-2.19)	0.84 (0.54-1.52)	0.83 (0.64-1.00)	1.01 (0.94-1.38)
	2 doses (n=11)	Unstimulated	0.08 (0.05-0.16)	0.33 (0.22-0.47)	0.09 (0.07-0.14)	0.27 (0.24-0.34)
		Pooled peptides	0.10 (0.08-0.17)	0.31 (0.19-0.40)	0.07 (0.04-0.13)	0.28 (0.18-0.34)
		Fold increase	1.20 (1.06-2.00)	0.85 (0.81-1.38)	1.00 (0.64-1.13)	1.00 (0.65-1.22)
	1 dose (n=31)	Unstimulated	0.08 (0.06-0.11)	0.14 (0.07-0.29)	0.05 (0.03-0.09)	0.17 (0.12-0.26)
		Pooled peptides	0.12 (0.07-0.16)	0.16 (0.07-0.30)	0.04 (0.02-0.09)	0.23 (0.13-0.27)
		Fold increase	1.23 (1.00-2.00)	0.93 (0.67-1.24)	1.00 (0.73-1.25)	1.15 (0.96-1.43)
Day 56	Negative (n=18)	Unstimulated	0.06 (0.05-0.12)	0.18 (0.07-0.23)	0.06 (0.03-0.08)	0.18 (0.12-0.36)
		Pooled peptides	0.07 (0.05-0.15)	0.11 (0.08-0.26)	0.06 (0.04-0.11)	0.18 (0.15-0.36)
		Fold increase	1.24 (0.95-1.65)	0.78 (0.50-1.35)	0.92 (0.73-1.37)	0.98 (0.67-1.25)
	2 doses (n=11)	Unstimulated	0.11(0.06-0.14)	0.28 (0.14-0.35)	0.08 (0.06-0.11)	0.24 (0.15-0.49)
		Pooled peptides	0.11 (0.07-0.21)	0.21 (0.17-0.26)	0.06 (0.05-0.12)	0.24 (0.19-0.36)
		Fold increase	1.07 (0.85-1.57)	0.89 (0.71-1.21)	0.88 (0.75-1.09)	1.04 (0.77-1.43)
	1 dose (n=31)	Unstimulated	0.09 (0.04-0.13)	0.14 (0.08-0.29)	0.05 (0.04-0.10)	0.18 (0.11-0.32)
		Pooled peptides	0.10(0.07-0.19)	0.16 (0.07-0.29)	0.04 (0.03-0.08)	0.18 (0.12-0.32)
		Fold increase	1.33 (1.00-1.60)	1.00 (0.67-1.4)	1.00 (0.70-1.22)	0.98 (0.83-1.07)

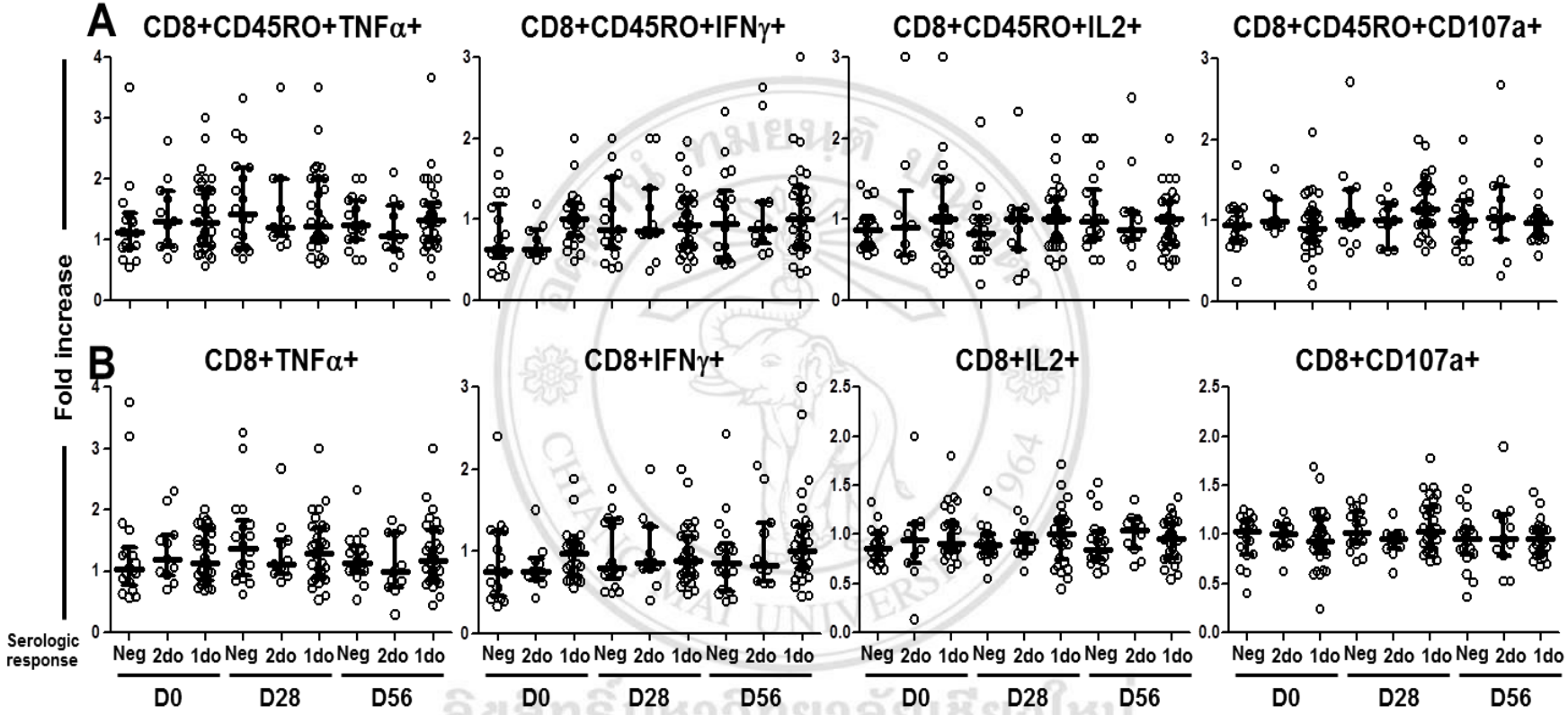

Figure 4.1. Cytokine production and CD107a expression of memory CD8+ T cells. The fold increase in the cytokine-producing and CD107a-expressing memory (A) and total (B) CD8+ T cells in response to the pooled peptide stimulation by HIV+ children who had no antibody response after two doses of vaccination (Neg), who had antibody response after two doses (2do) and a single dose (1do), compared before *in vivo* vaccination on day zero (D0), and at 28 days (D28) and 56 days (D56) after vaccination were analysed.

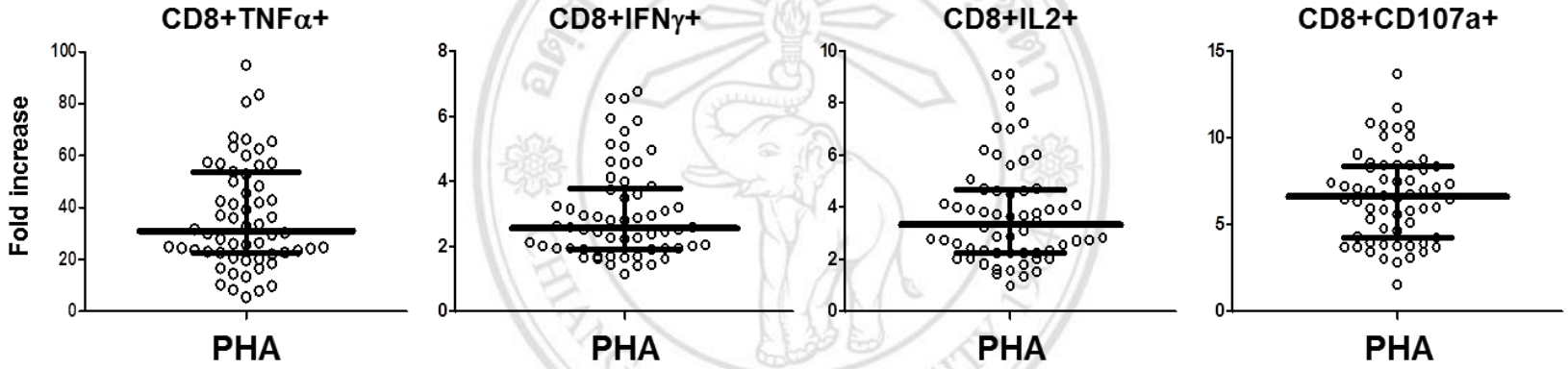

Figure 4.2. Cytokine production and CD107a expression of total CD8+ T cells at D56.The fold increase in the cytokine–producing and CD107a-expressing CD8+ T cells after the PHA stimulation in all participants have been shown. The solid lines show the medians with the interquartile ranges.

4.4 Discussion

In Thailand, influenza virus infection is a cause of respiratory morbidity and mortality in children [276]. Immune responses induced by influenza vaccine are the gold approach for reducing disease severity and preventing infection in children. Previous study demonstrated that the cellular immunity elicited through vaccination contributes to defence against influenza infection in young children [246]. Our study found no statistical differences in the frequencies of both total and memory CD8+ T cells that produced cytokines or expressed CD107a in response to the pooled H1N1 influenza A peptides stimulation *in vitro* after vaccinations between groups with different serological responses. This was not due to defect of T cells function or technical performing assay since T cells from all HIV+ children in this study produced cytokines normally in response to PHA stimulation, demonstrating functional activity of T cells in our study population. Memory CD8+ T cells responses to other specific antigens such as cytomegalovirus antigen (CMV) was not evaluated in this study due to the limitation of samples but needs to be assessed in the future in order to confirm that there was no defect of T cell functions.

The cellular immune response to influenza vaccine in HIV+ children is still controversial. Previous study demonstrated that the levels of IFN-γ in culture supernatant of PBMCs from influenza-vaccinated HIV+ children were significantly increased after *in vitro* stimulation with vaccine antigens [277]. However, the lower magnitude and longevity of influenza specific IFN-γ- and IL-2- responses in HIV+ children compared to HIV− healthy control have been reported [246]. The study presented here found no significant differences in the percentages of cytokine-producing or CD107a-expressing CD8+ T cells between HIV+ children with different serological response. Although this study cannot conclude that cellular immunity induced by the vaccine may be not correlated with antibody response, due to poor responses to the pooled peptides, studies in both healthy children [278] and adults [279] reported no evidence of a correlation between the change in IFN-γ-producing T cells and the change of HAI titre. In further investigation, the precise correlation between cellular and humoral immune responses against influenza vaccination needs to be elucidated in either HIV+ or HIV− individuals.

A goal of influenza vaccine development is to induce cross-reactive T cell immune responses against conserved T cell epitopes of heterosubtypic influenza viruses [270]. Cytotoxic T cell responses are correlated with viral clearance and alleviate illness severity [110, 114]. This study utilized IEDB website to acquire T cell epitope sequences against influenza A viruses that most frequently recognized by the most common HLA in Thai populations. In previous study, cytotoxic T cells recognizing M158-66 epitope (same sequence as one of the pooled peptides used in this study) from healthy adults have strong cytolytic activity capable of killing either seasonal or 2009 H1N1 influenza A virus-infected cells [253]. Low cytokines-producing and CD107a-expressing CD8+ T cell in response to the pooled conserved-epitope peptides stimulation *in vitro* observed in our study could be owing to poor immunogenicity of the Panenza® vaccine as reflected by low antibody response rates to the same vaccine using in this study as reported previously [7, 9].

Some limitation in this report should be taken into consideration. The Panenza® vaccines used in this study were provided by the Thai MOPH and were prioritized to persons at risk of more severe manifestations of the influenza disease including HIV+ children, consequently HIV− healthy children could not be included in this study. Whether the vaccine would induce conserved epitope-specific CD8+ T cell responses in HIV− children remains to be investigated. Another limitation was the unavailability of the influenza vaccine antigens to be used as antigen specific control for *in vitro* stimulation assay. In addition, the peptides used in this study might not match HLA of children in our study. Characterization of HLA in the study population prior to *in vitro* stimulation with HLA-specific peptides or peptide tetramer assay would prove our observation. However, these assays were not performed in this study due to several limitations. The strategy of vaccination to induce immunological memory responses to conserved-epitopes of influenza viruses in either HIV+ or HIV− healthy population warrants further investigation.

In conclusion, this study suggests that vaccination with 2009 H1N1 influenza A Panenza® vaccine could not induce conserved-epitope-specific memory CD8+ T cells responses in HIV+ northern Thai children. This information may provide valuable perception for future vaccine design to improve immune responses to influenza vaccination for these populations.