CHAPTER 3

Investigation of Cellular Immune Responses after 2009 H1N1 Influenza A Vaccination in HIV-infected Adults

3.1 Introduction

During the spring of 2009, a novel swine-origin influenza A/H1N1 virus was identified as a significant cause of febrile respiratory illnesses in Mexico and the United States [234]. The disease spread globally causing a major threat to public health, prompting the World Health Organization (WHO) to declare a pandemic in June of that year [235, 236]. Vaccination is a cost-effective approach to prevent influenza disease. The Thailand Ministry of Public Health acquired two million doses of non-adjuvanted, inactivated, split-virion, monovalent influenza vaccine, which comprised 2009 H1N1 influenza A virus-like strain, for Thai populations at high risk for severe influenza illness, including HIV+ individuals. Resistance to infection with influenza virus correlates directly with both serum haemagglutination inhibition (HAI) and neutralizing antibody levels [199]. Lower titres and shorter durability of antibody after monovalent formulation vaccination of 2009 H1N1 influenza A have been widely reported in people living with HIV, compared to healthy persons [6-9].

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Infection with HIV of cells in the immune system such as CD4+ T cells, macrophages and DCs results in impairment of cell functions in immune system compared to healthy individuals [237-240]. As CD4+ helper T cells are necessary to activate both humoral and cellular immune responses, continuing loss of quantity and/or function of such T cells in HIV+ individuals eventually leads to immunodeficiency status.

The knowledge of cellular immunity in HIV+ individuals is still limited, particularly of their memory responses to influenza vaccination [220, 241-243]. In this study, we investigated cellular immune responses, *in vitro*, to 2009 H1N1 influenza A vaccination. The responses studied included cytokine production, phenotypes of

memory T cell subpopulations, and the expression of activation markers and chemokine receptors. The results from this study may provide fundamental information addressing important issues for influenza vaccine development, especially for HIV+ populations.

3.2 Methods

3.2.1. Vaccination and blood collection

The PANENZA vaccine (Panenza®, Sanofi Pasteur, Val de Reuil, Lyon, France), which is non-adjuvanted, inactivated, split virion of A/California/7/2009 (H1N1) virus-like strain (NYMC X-179A) containing 15 μg of haemagglutinin (HA) was used in this study. The vaccine was provided by Thai Ministry of Public Health as part of a routine campaign to vaccinate Thai citizens who were at risk of severe H1N1 disease. All participants were vaccinated intramuscularly in the deltoid with one dose (0.5mL) of PANENZA vaccine. Sixteen mL of venous blood were collected from all participants at baseline before vaccination on day 0 (D0) and at 1 month (M1) and 3 months (M3) after vaccination.

3.2.2. Study population

Participants in this study were a part of the study on seroconversion and seroprotection rate to 2009 H1N1 vaccine to in HIV-infected adults [7]. In our study, 40 female, 21 male HIV+ adults and 11 female, 9 male HIV negative (HIV−) adults, aged between 18 and 60 years were enrolled from January 2010 to March 2010 at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University (CMU) in Chiang Mai, Thailand. Characteristic at the time of vaccination and vaccine response are shown in table 3.1. The mean age of the HIV+ subjects was 42.1 ± 6.1, and of the HIV− controls was 32.4 ± 6.3 years. Ninety-eight percentage of the HIV+ participants were on combination antiretroviral therapy (ART) and 91% had absolute number of CD4+ cells > 200 cells/mm^3. Their mean absolute CD4+ cell counts was 466 ± 206 cells/mm3, compared to 762 ± 283 cells/mm^3 among the 20 HIV− healthy volunteers. The seroconversion and seroprotection rates after a single influenza vaccination in the HIV+ group were 32% and 33%, respectively, and in the healthy HIV− participants were 35% and 35%, respectively [7]. Seroconversion was defined as 2 indications: 1) pre-vaccination HI

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titre < 1:10 and post-vaccination HI titre ≥ 1:40 or 2) pre-vaccination HI titre ≥ 1:10 and a minimum of 4-fold rise in post-vaccination HI titre. For seroprotection, the indication was defined as a post-vaccination HI titre ≥ 1:40.

Table 3.1 Base line characteristic and vaccine response rate

Characteristic	HIV+	HIV−
Total participants	**61**	**20**
Female	40	11
Mean age	42.1 ± 6.1	32.4 ± 6.3
Receiving cART	98%	
Mean CD4+ cell counts	466 ± 206	762 ± 283
Humoral immune responses		
Seroconversion	32%	35%
Seroprotection	33%	35%

3.2.3. Isolation of PBMCs by gradient centrifugation

As described in chapter 2 section 2.3

3.2.4. Cell stimulation

Cryopreserved PBMCs were thawed and rested overnight as described in chapter 2 section 2.4. Resting PBMCs were stimulated with complete RPMI containing 5 μg/ml of phytohaemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, USA), 20 μL of dialyzed PANENZA vaccine, or complete media (unstimulated control). Cells were cultured in 37°C 5% CO_2 incubator.

3.2.5. Determination of cytokine-secreting T cells and granzyme-producing T cells (CD107a+) in response to specific antigen by intracellular cytokine staining (ICS) technique

As described in chapter 2 section 2.5

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3.2.6. Characterization of antigen-specific T cell phenotypes and subpopulations using cell surface staining technique

After 5 days stimulation, stimulated PBMCs were washed with FACS buffer and incubated with blocking buffer at 4 °C for 30 min. Then, cell surface markers were determined by staining cells with panels of monoclonal antibodies containing anti-CD3-Krome Orange, anti-CD8-PE Alexa Fluor 610, anti-CD4-APC/Cy7, anti-CD45RO-Pacific Blue, anti-CTLA4-PE or anti-CCR5-PE, anti-CXCR3-PerCP/Cy5.5 or anti-CD28-PE/Cy5, anti-CD69-PE/Cy7 or anti-CD62L-PE/Cy7 and anti-PD1-APC (Biolegend, San Diego, CA, USA). After incubation for 30 min at 4 °C, cells were washed with FACS buffer and resuspended with 1% paraformaldehyde in PBS. Cell surface markers of T cells subpopulation were analysed by flow cytometry analysis.

3.3 Results

3.3.1. Cytokine production and CD107a expression of CD4 and CD8 T cells

Cytokine-producing and CD107a-expressing CD4+ or CD8+ T cells were identified as described in chapter 2 section 2.6 (Figure 2.1A). Results are presented as fold increases of the TNF-α, IFN-γ, IL-2 and IL-10 production or expression of CD107a in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation over medium alone. There were no statistical differences of cytokine production or CD107a expression between CD4+ (Figure 3.1A) or CD 8+ (Figure 3.1B) T cells from HIV+ and HIV− donors. This was the case for all three time points studied: day 0 before *in vivo* vaccination, and at 1 month and 3 months afterwards. The values of the median and interquartile range of each analysis comparing between HIV+ and HIV− study groups, and among three visits are summarised in Table 3.1. Strong cytokine responses when PBMCs were stimulated with PHA as positive controls were observed, with median fold-rises of 3 to 25 or higher (Figure 3.2).

There were no differences in these responses within each group at 1 month and 3 months, nor between the HIV+ and HIV− study groups at comparable times (Table 3.1). When evaluated with regard to their absolute CD4+ T cell counts of $\leq 350/mm^3$ versus $>350/mm^3$, Anova analysis of cytokine-producing and CD107a-

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expressing CD4+ or CD8+ T cells did not reveal statistically significant differences (Figures 3.3A and B, respectively), or when compared among groups with absolute CD4 counts ≤ 200 cells/mm^3, 201-500 cells/mm^3 and > 500 cells/mm^3 (Figures 3.4A and B, respectively).

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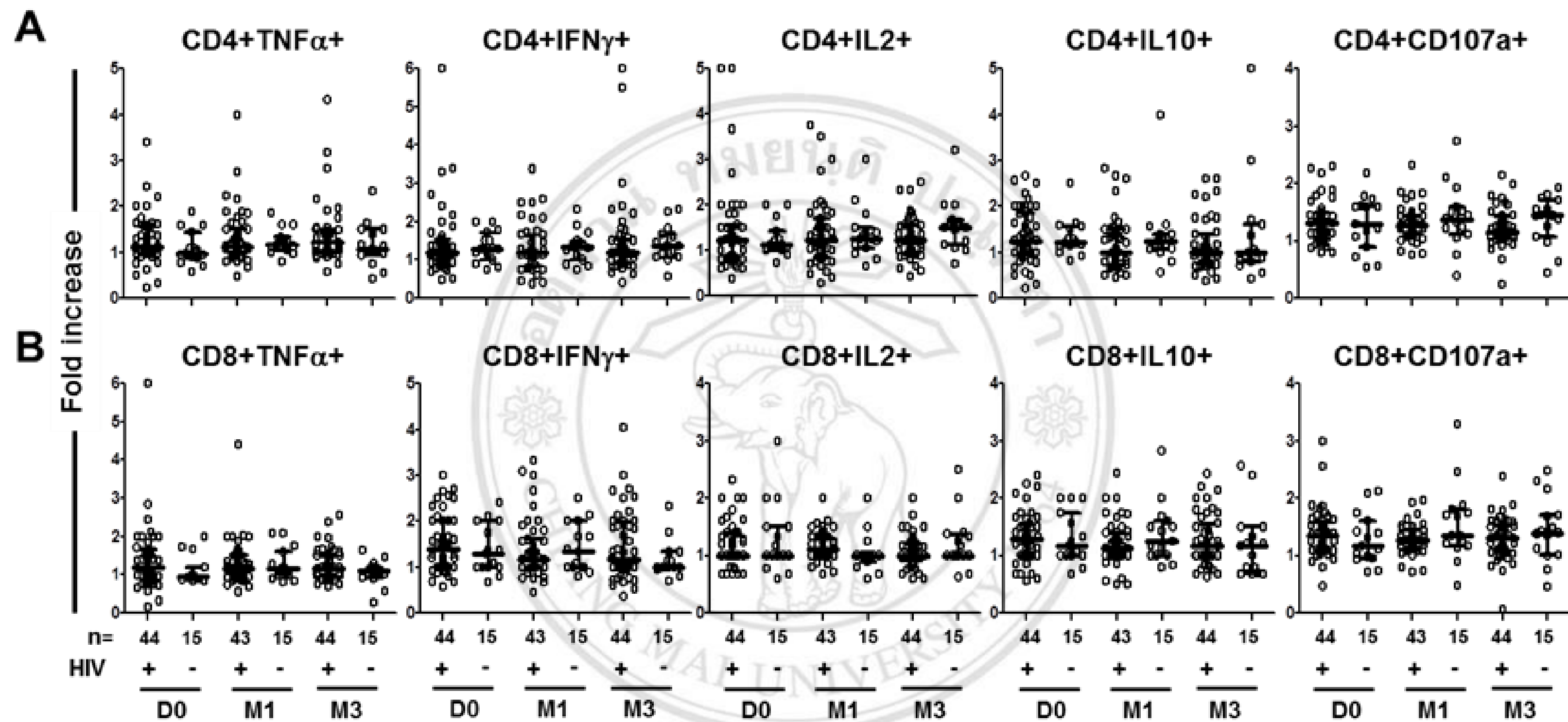

Figure 3.1. Fold increase of cytokine-producing and CD107a-expressing T cells. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by CD4+ (A) and CD8+ (B) T cells in response to vaccine antigen, by HIV-infected (HIV+) and healthy (HIV−) individuals, compared before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars.

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Table 3.2 Fold increase of cellular immune response in HIV-infected and healthy individuals after 2009 H1N1 influenza vaccine antigen stimulation *in vitro*

		Median with interquartile range of fold increase after pH1N1 influenza vaccine antigen stimulation *in vitro*[a]									*p* value[b]					
		D0			M1			M3			HIV+			HIV-		
		HIV+	HIV-	*p*-value	HIV+	HIV-	*p*-value	HIV+	HIV-	*p*-value	D0vsM1	D0vsM3	M1vsM3	D0vsM1	D0vsM3	M1vsM3
Cytokine-producing and CD107a-expressing cells in response to H1N1 antigen stimulation																
CD4+	TNFα+	1.12 (0.97-1.57)	0.97 (0.86-1.44)	ns	1.11 (1.00-1.5)	1.17 (1.05-1.33)	ns	1.21 (1.00-1.49)	1.07 (0.93-1.50)	ns	ns	ns	ns	ns	ns	ns
	IFNγ+	1.18 (1.00-1.54)	1.29 (1.00-1.73)	ns	1.19 (0.86-1.67)	1.33 (1.00-1.50)	ns	1.19 (0.92-1.55)	1.38 (1.07-1.67)	ns	ns	ns	ns	ns	ns	ns
	IL2+	1.23 (0.84-1.55)	1.14 (1.00-1.42)	ns	1.24 (0.86-1.70)	1.27 (1.07-1.50)	ns	1.23 (0.94-1.55)	1.50 (1.13-1.67)	ns	ns	ns	ns	ns	ns	ns
	IL10+	1.23 (0.93-1.86)	1.22 (1.14-1.57)	ns	1.00 (0.71-1.50)	1.25 (1.06-1.40)	ns	1.00 (0.75-1.38)	1.00 (0.80-1.60)	ns	ns	ns	ns	ns	ns	ns
	CD107a+	1.31 (1.03-1.48)	1.30 (0.90-1.61)	ns	1.28 (1.12-1.43)	1.38 (1.11-1.59)	ns	1.15 (1.04-1.44)	1.44 (1.07-1.70)	ns	ns	ns	ns	ns	ns	ns
CD8+	TNFα+	1.19 (0.86-1.65)	0.94 (0.90-1.19)	ns	1.16 (0.93-1.53)	1.15 (1.00-1.59)	ns	1.16 (0.98-1.50)	1.11 (0.92-1.20)	ns	ns	ns	ns	ns	ns	ns
	IFNγ+	1.39 (1.00-2.00)	1.29 (1.00-2.00)	ns	1.17 (1.00-1.60)	1.33 (1.00-2.00)	ns	1.17 (1.00-1.97)	1.00 (1.00-1.33)	ns	ns	ns	ns	ns	ns	ns
	IL2+	1.00 (1.00-1.40)	1.00 (1.00-1.50)	ns	1.11 (1.00-1.33)	1.00 (0.90-1.00)	ns	1.00 (0.90-1.21)	1.00 (1.00-1.38)	ns	ns	ns	ns	ns	ns	ns
	IL10+	1.29 (1.00-1.55)	1.18 (1.00-1.75)	ns	1.14 (1.00-1.40)	1.25 (1.00-1.60)	ns	1.17 (1.00-1.55)	1.17 (0.70-1.50)	ns	ns	ns	ns	ns	ns	ns
	CD107a+	1.36 (1.09-1.59)	1.18 (0.95-1.60)	ns	1.27 (1.13-1.44)	1.35 (1.18-1.81)	ns	1.31 (1.06-1.54)	1.40 (1.02-1.71)	ns	ns	ns	ns	ns	ns	ns
CD4+ CD45RO+	TNFα+	1.09 (0.94-1.38)	0.95 (0.78-1.36)	ns	1.08 (0.92-1.50)	1.11 (1.07-1.45)	ns	1.15 (0.94-1.51)	1.09 (0.88-1.43)	ns	ns	ns	ns	ns	ns	ns
	IFNγ+	1.24 (0.94-1.76)	1.22 (1.10-1.59)	ns	1.20 (0.91-1.69)	1.30 (1.12-1.57)	ns	1.28 (1.00-1.65)	1.36 (1.06-1.89)	ns	ns	ns	ns	ns	ns	ns
	IL2+	1.08 (0.87-1.81)	1.33 (1.00-1.80)	ns	1.33 (0.86-2.17)	1.46 (1.15-2.00)	ns	1.23 (1.01-1.71)	1.53 (1.33-1.81)	ns	ns	ns	ns	ns	ns	ns
	IL10+	1.13 (0.83-1.75)	1.25 (1.10-1.57)	ns	1.14 (0.73-1.33)	1.11 (0.96-1.33)	ns	1.00 (0.79-1.59)	1.00 (0.70-1.36)	ns	ns	ns	ns	ns	ns	ns
	CD107a+	1.26 (1.00-1.40)	1.25 (0.87-1.68)	ns	1.24 (1.11-1.44)	1.43 (1.03-1.72)	ns	1.13 (1.00-1.38)	1.49 (1.04-1.67)	ns	ns	ns	ns	ns	ns	ns
CD8+ CD45RO+	TNFα+	1.12 (0.77-1.41)	0.91 (0.87-1.24)	ns	1.05 (0.83-1.35)	1.02 (0.96-1.30)	ns	1.04 (0.86-1.33)	1.06 (0.85-1.13)	ns	ns	ns	ns	ns	ns	ns
	IFNγ+	1.38 (0.85-2.10)	1.18 (0.75-2.00)	ns	1.20 (0.97-1.66)	1.47 (1.00-1.75)	ns	1.28 (0.87-1.93)	0.97 (0.80-1.18)	ns	ns	ns	ns	ns	ns	ns
	IL2+	1.00(0.83-1.50)	1.29 (1.00-1.80)	ns	1.14 (1.00-1.38)	1.00 (0.89-1.50)	ns	1.00 (0.87-1.37)	1.08 (1.00-1.50)	ns	ns	ns	ns	ns	ns	ns
	IL10+	1.16 (0.87-1.50)	1.33 (1.14-1.80)	ns	1.14 (0.82-1.50)	1.33 (0.86-1.57)	ns	1.25 (1.00-1.60)	0.80 (0.56-1.58)	ns	ns	ns	ns	ns	ns	ns
	CD107a+	1.23 (1.02-1.46)	1.07 (0.90-1.58)	ns	1.20 (1.01-1.37)	1.23 (1.05-1.99)	ns	1.17 (1.04-1.42)	1.35 (1.00-1.67)	ns	ns	ns	ns	ns	ns	ns
Cytokine-producing and CD107a-expressing cells in response PHA stimulation																
CD4+ PHA+	TNFα+	20.09 (4.83-38.69)	25.78 (3.95-52.60)	ns	25.14 (4.59-42.67)	20.63 (3.56-53.62)	ns	17.71 (4.73-41.86)	20.88 (8.24-54.85)	ns	ns	ns	ns	ns	ns	ns
	IFNγ+	5.96 (2.44-12.18)	9.6 (4.83-19.80)	ns	5.32 (2.33-15.11)	6.39 (4.47-15.36)	ns	6.47 (1.82-12.38)	7.66 (3.70-13.00)	ns	ns	ns	ns	ns	ns	ns
	IL2+	26.32 (9.49-59.28)	22.85 (11.78-51.67)	ns	22.55 (10.25-42.83)	31.20 (12.96-49.00)	ns	17.41 (8.56-32.69)	27.00 (15.31-48.00)	ns	ns	ns	ns	ns	ns	ns
	IL10+	3.23 (1.94-6.05)	4.00 (2.13-5.7)	ns	2.67 (1.72-5.83)	3.50 (2.17-6.40)	ns	2.45 (1.40-3.88)	3.50 (2.00-6.33)	ns	ns	ns	ns	ns	ns	ns
	CD107a+	3.21 (2.12-4.48)	4.75 (3.21-6.54)	ns	3.37 (2.24-4.87)	3.98 (2.86-7.09)	ns	3.55 (2.11-5.78)	4.65 (2.74-6.83)	ns	ns	ns	ns	ns	ns	ns
Activation of memory T cell subpopulations																
CD4+	CM	0.94 (0.87-0.97)	1.01 (0.99-1.03)	ns	0.96 (0.91-0.99)	1.02 (0.93-1.04)	ns	0.95 (0.92-1.00)	1.05 (0.97-1.13)	0.0008	ns	ns	ns	ns	ns	ns
	EM	1.27 (1.16-1.35)	1.20 (1.16-1.31)	ns	1.26 (1.13-1.34)	1.23 (1.16-1.30)	ns	1.20 (1.10-1.32)	1.25 (1.22-1.38)	0.036	ns	ns	ns	ns	ns	ns
CD8+	CM	0.92 (0.85-1.00)	1.06 (0.94-1.13)	0.0002	0.99 (0.91-1.06)	1.07 (0.99-1.22)	0.0037	0.97 (0.93-1.01)	1.11 (1.05-1.38)	<0.0001	ns	ns	ns	ns	ns	ns
	EM	1.14 (1.07-1.23)	1.16 (1.07-1.22)	ns	1.14 (1.05-1.20)	1.18 (1.13-1.26)	ns	1.14 (1.05-1.22)	1.36 (1.13-1.45)	0.0003	ns	ns	ns	ns	0.01	0.03

Expression of co-stimulation molecules and activation molecule on T cells																
CD4+	CD69+	1.15 (1.06-1.34)	1.24 (1.01-1.32)	ns	1.19 (1.04-1.33)	1.15 (1.05-1.26)	ns	1.20 (1.05-1.04)	1.23 (1.14-1.36)	ns	ns	ns	ns	ns	ns	ns
	CTLA4+	1.64 (1.17-2.20)	1.66 (0.81-2.00)	ns	1.52 (1.00-2.29)	1.77 (0.94-2.21)	ns	1.63 (1.00-2.34)	1.66 (0.94-1.95)	ns	ns	ns	ns	ns	ns	ns
CD8+	CD69+	1.09 (1.02-1.17)	1.20 (1.06-1.35)	0.0357	1.12 (1.01-1.19)	1.26 (1.17-1.53)	0.0009	1.16 (1.04-1.26)	1.30 (1.21-1.56)	0.0029	ns	ns	ns	ns	0.0464	ns
	CTLA4+	1.56 (1.25-2.63)	1.87 (1.00-2.50)	ns	1.33 (0.71-2.00)	1.00 (1.00-1.46)	ns	1.29 (0.88-1.09)	1.00 (1.00-1.88)	v	ns	ns	ns	ns	ns	ns
CD8+ CD45RO+	CD69+	1.08 (0.99-1.17)	1.11 (1.05-1.25)	ns	1.07 (1.01-1.19)	1.27 (1.17-1.43)	0.0027	1.13 (1.04-1.26)	1.33 (1.15-1.59)	0.0099	ns	ns	ns	ns	0.0223	ns
CD4+ CD45RO+	CD69+	1.16 (0.99-1.34)	1.18 (0.95-1.30)	ns	1.17 (1.03-1.28)	1.10 (1.02-1.26)	ns	1.13 (1.02-1.34)	1.20 (1.08-1.27)	ns	ns	ns	ns	ns	ns	ns
Expression of T cell trafficking molecules																
CD4+	CCR5+	1.70 (1.27-1.05)	1.29 (0.99-1.65)	ns	1.51 (1.26-1.80)	1.31 (1.10-1.49)	ns	1.42 (1.19-1.74)	1.70 (1.18-2.21)	ns	ns	ns	ns	ns	ns	ns
	CXCR3+	1.52 (1.36-1.76)	1.71 (1.27-2.16)	ns	1.55 (1.37-1.77)	1.70 (1.36-1.85)	ns	1.54 (1.29-1.88)	1.93 (1.52-2.18)	0.0074	ns	ns	ns	ns	ns	ns
	CCR5+ CXCR3+	2.00 (1.53-2.40)	1.96 (1.46-2.87)	ns	1.86 (1.38-2.45)	1.83 (1.44-2.45)	ns	1.79 (1.24-2.16)	2.48 (1.85-3.26)	0.0008	ns	ns	ns	ns	ns	ns
CD8+	CCR5+	1.52 (1.08-1.89)	1.06 (0.96-1.28)	ns	1.35 (1.05-1.63)	0.88 (0.72-1.08)	ns	1.11 (0.74-1.08)	1.33 (0.95-2.29)	ns	ns	ns	ns	ns	ns	ns
	CXCR3+	0.98 (0.92-1.04)	1.05 (1.01-1.10)	ns	1.01 (0.95-1.12)	1.12 (0.97-1.20)	ns	1.01 (0.95-1.09)	1.13 (1.05-1.23)	0.0284	ns	ns	ns	ns	ns	ns
	CCR5+ CXCR3+	1.50 (0.92-1.75)	1.00 (0.95-1.25)	ns	1.31 (0.87-1.72)	1.01 (0.85-1.41)	ns	1.10 (0.78-1.46)	1.16 (0.85-1.84)	ns	ns	ns	ns	ns	ns	ns
CD4+EM	CCR5+	1.39 (1.13-1.58)	1.18 (0.89-1.39)	ns	1.45 (1.15-1.73)	1.13 (1.00-1.25)	ns	1.31 (1.04-1.59)	1.36 (1.14-2.17)	ns	ns	ns	ns	ns	ns	ns
	CXCR3+	1.41 (1.21-1.58)	1.59 (1.33-2.25)	ns	1.45 (1.18-1.76)	1.51 (1.37-1.83)	ns	1.43 (1.24-1.72)	1.67 (1.46-2.40)	0.0152	ns	ns	ns	ns	ns	ns
	CCR5+ CXCR3+	1.82 (1.36-2.21)	1.94 (1.47-2.70)	ns	1.75 (1.37-1.99)	1.87 (1.32-2.22)	ns	1.61 (1.20-2.19)	2.17 (1.63-3.27)	0.0118	ns	ns	ns	ns	ns	ns
CD8+EM	CCR5+	1.09 (0.86-1.54)	0.94 (0.82-1.19)	ns	1.10 (0.84-1.40)	0.70 (0.53-0.99)	ns	0.93 (0.53-1.44)	0.97 (0.56-2.33)	ns	ns	ns	ns	ns	ns	ns
	CXCR3+	0.99 (0.91-1.04)	1.02 (0.96-1.09)	ns	1.01 (0.96-1.07)	1.05 (1.02-1.10)	ns	1.00 (0.93-1.08)	1.05 (0.98-1.09)	ns	ns	ns	ns	ns	ns	ns
	CCR5+ CXCR3+	1.13 (0.82-1.42)	1.06 (0.80-1.27)	ns	1.07 (0.85-1.76	0.94 (0.72-1.20)	ns	0.95 (0.64-1.42)	1.02 (0.58-2.10)	ns	ns	ns	ns	ns	ns	ns

[a] Comparison of fold increase between HIV-infected (HIV+) and healthy (HIV−) individuals at before vaccination baseline (D0), 1 month (M1) and 3 months (M3) after vaccination

[b] Comparison of fold increase between blood collected at different time point in each study groups

Statistical analysis significance is defined as a *p*-value (*p*) < 0.05.

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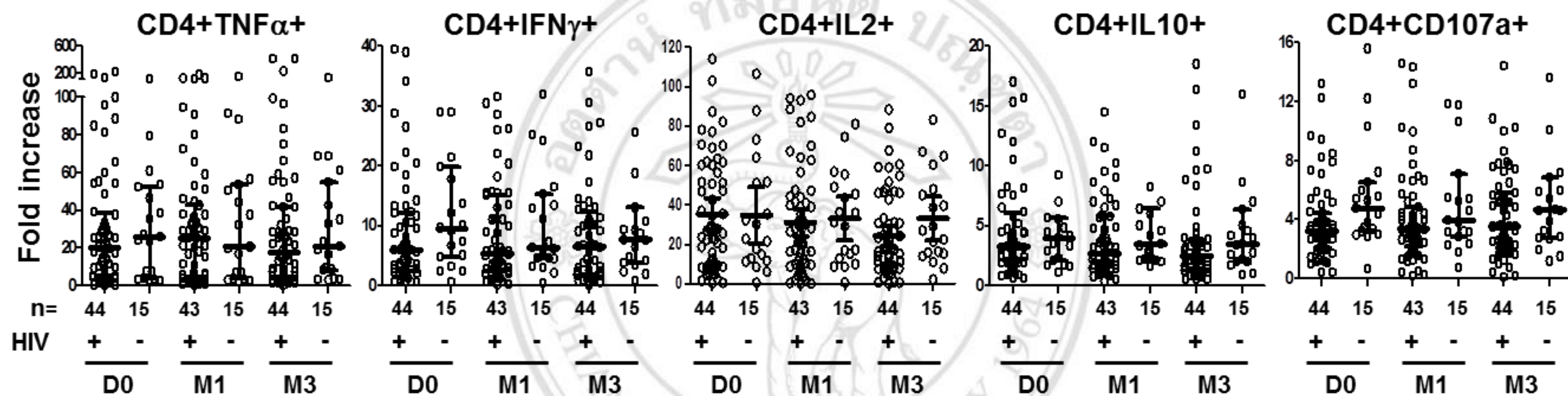

Figure 3.2. Fold increase of cytokine-producing and CD107a-expressing T cells in response to PHA stimulation. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by CD4+ T cells in response to PHA stimulation by HIV-infected (HIV+) and healthy (HIV−) individuals, compared at before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars

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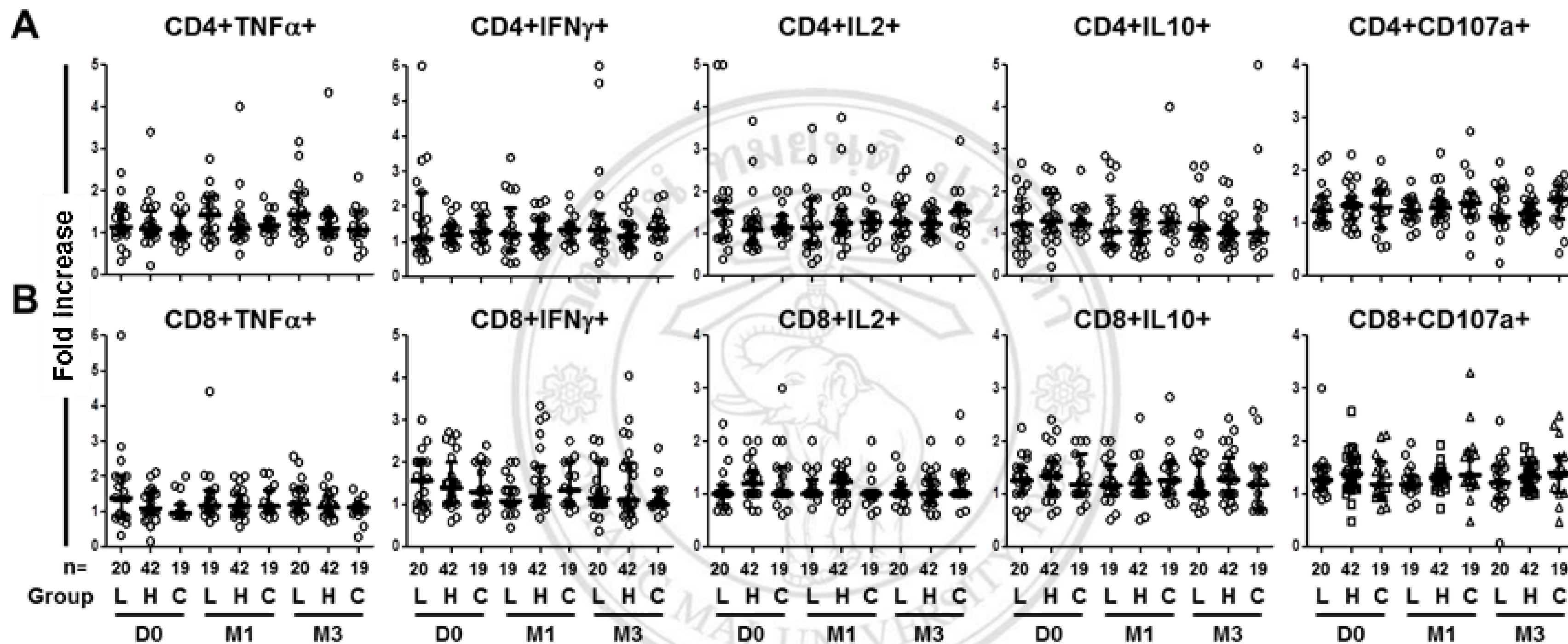

Figure 3.3. Fold increase of cytokine-producing and CD107a-expressing T cells between HIV+ with regard to absolute CD4+ T cell counts of 350 cells/mm^3. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by CD4+ (A) and CD8+ (B) T cells in response to vaccine antigen, by HIV+ groups with regard to their absolute CD4+ T cell counts of ≤ 350 cells/mm^3(L) versus > 350 cells/mm^3 (H), and healthy (C) individuals, compared before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars.

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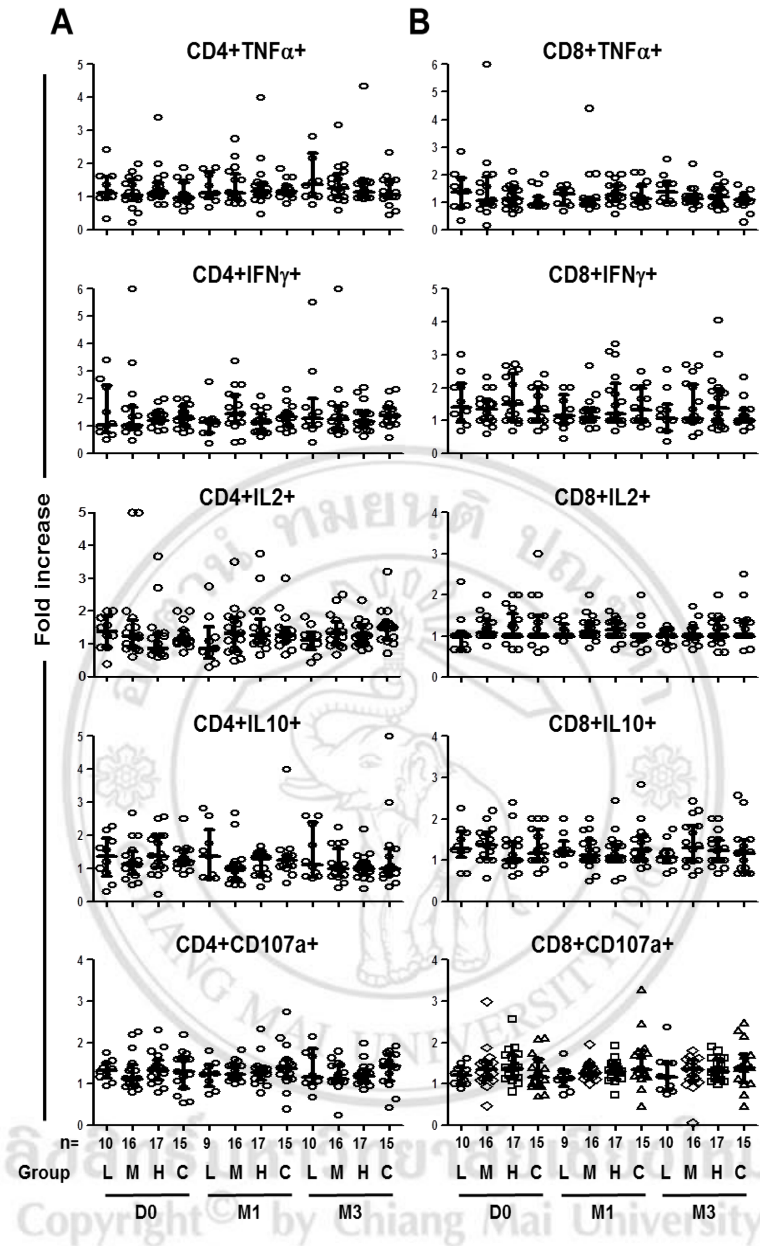

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Figure 3.4. Fold increase of cytokine-producing and CD107a-expressing T cells between HIV+ with regard to absolute CD4+ T cell counts of ≤ 200, 201-500 and > 500 cells/mm^3. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by CD4+ (A) and CD8+ (B) T cells in response to vaccine antigen, by HIV+ groups with regard to their absolute CD4+ T cell counts of ≤ 200 cells/mm^3 (L), 201-500 cells/mm^3 (M) and > 500 cells/mm^3 (H), and healthy (C) individuals, compared before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars.

3.3.2. Cytokine production and CD107a-expression of memory CD4 and CD8 T cells

Cytokine-producing CD4+ or CD8+ memory T cells were identified as described in chapter 2 section 2.6 (Figure 2.2). The median fold increases of cytokine-producing and CD107a-expressing memory CD4+ (Figure 3.5A) or CD8+ T cells (Figure 3.5B) in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation over medium alone within each study group did not differ significantly between baseline, 1 month, and 3 months after vaccination. Nor were there any differences between HIV+ and HIV− subjects at any time point of the study.

When analysed with regard to their absolute CD4+ T cell counts of ≤ 350 cells/mm^3 versus > 350 cells/mm^3, Anova analysis of cytokine-producing and CD107a-expressing CD4+ or CD8+ memory T cells did not reveal statistically significant differences (Figures 3.6A and B, respectively), or when compared among groups with CD4 counts ≤ 200 cells/mm^3, 201-500 cells/mm^3 and > 500 cells/mm^3 (Figures 3.7A and B, respectively).

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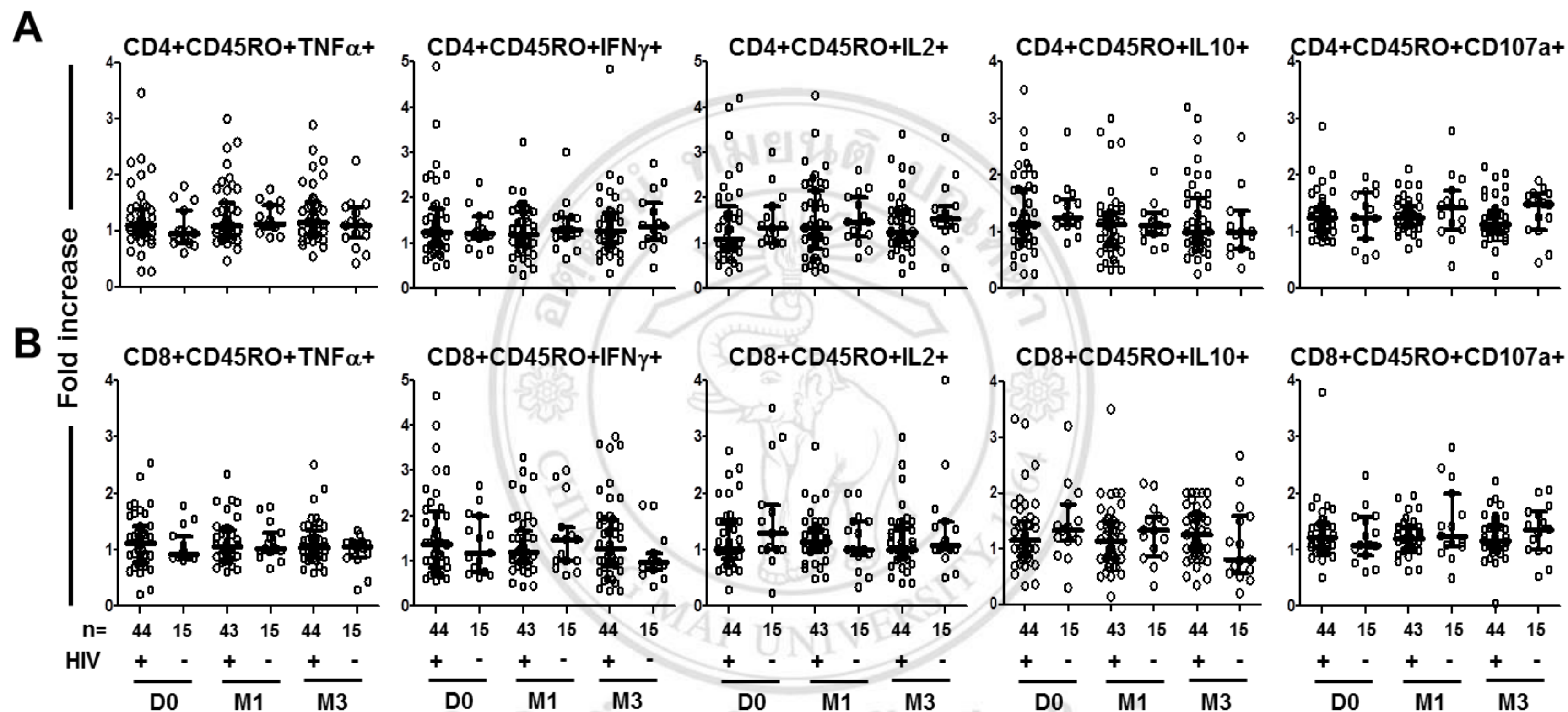

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Figure 3.5. Flow cytometry of cytokine producing memory T cells. Fold increase of memory CD4+ (D) or CD8+ (E) T cells were compared between HIV-infected (HIV+) and healthy (HIV−) individuals after stimulation at before (D0), one month (M1) and 3 months (M3) after vaccination. Statistical analysis was performed. p-value < 0.05 were considered statistically significant. Solid lines show the medians with interquartile ranges of each group.

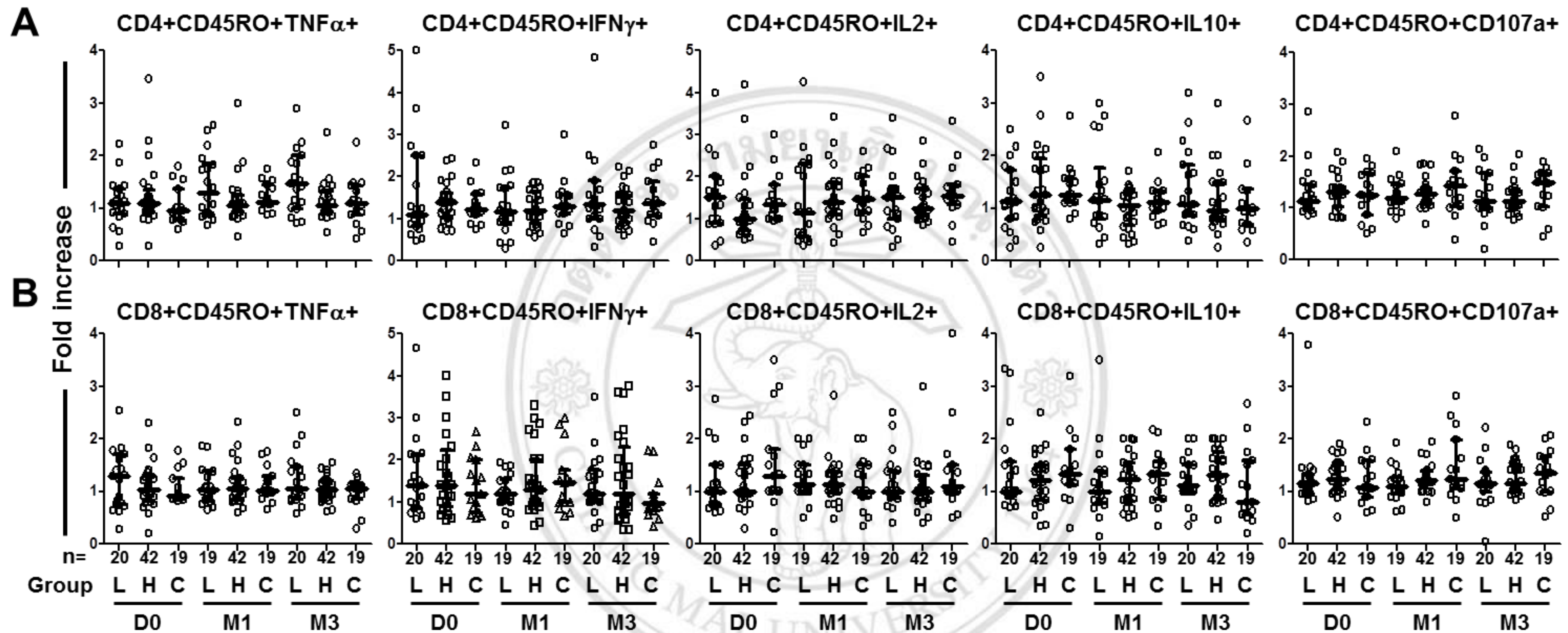

Figure 3.6. Fold increase of cytokine-producing and CD107a-expressing memory T cells between HIV+ groups with regard to absolute CD4+ T cell counts of 350 cells/mm^3. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by memory CD4+ (A) and CD8+ (B) T cells in response to vaccine antigen, by HIV+ with regard to their absolute CD4+ T cell counts of ≤ 350 cells/mm^3(L) versus > 350 cells/mm^3 (H), and healthy (C) individuals, compared before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars.

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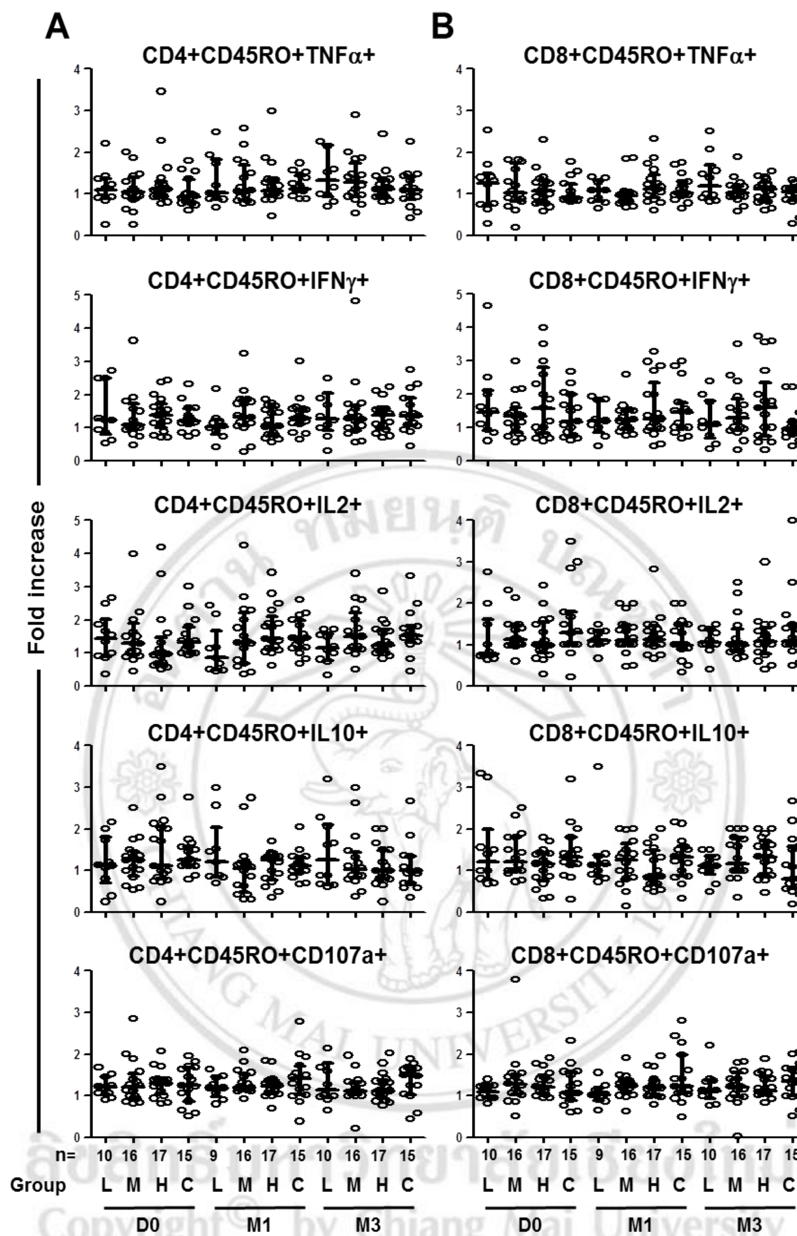

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Figure 3.7. Fold increase of cytokine-producing and CD107a-expressing memory T cells between HIV+ groups with regard to absolute CD4+ T cell counts of ≤ 200, 201-500 and > 500 cells/mm^3. Fold increases of TNF-α, IFN-γ, IL-2, and IL-10 cytokine production, and CD107a–expression, by memory CD4+ (A) and CD8+ (B) T cells in response to vaccine antigen, by HIV+ groups with regard to their absolute CD4+ T cell counts of ≤ 200 cells/mm^3 (L), 201-500 cells/mm^3 (M) and > 500 cells/mm^3 (H), and healthy (C) individuals, compared before *in vivo* vaccination on day zero (D0), and at one month (M1) and 3 months (M3) after vaccination. Medians represented by thick, wide horizontal bars, and 25%-75% interquartile ranges by thin, narrow bars.

3.3.3. Activation of 2009 H1N1 influenza A-specific memory T cell sub-populations

T cells were identified as CD3+ population from CD3-versus-SSC dot plot (Figure 3.8A). Single-positive CD4 or CD8 T cells were then selected from a CD4-versus-CD8 dot plot (Figure 3.8B). CD4+ or CD8+ T cells were then analysed for central memory (T_{CM}; CD45RO+ CD62L+) or effector memory (T_{EM}; CD45RO+ CD62L−) populations from CD45RO-versus-CD62L dot plot (Figure 3.8C). There were no significant changes in the median fold increases of the percentages of CD4+ T_{CM} cells in responses to the vaccine antigen over medium alone neither in the HIV+ individuals nor in healthy controls at baseline and 1 month after vaccination (Figure 3.8D [D0, M1]). However, at 3 months after vaccination, the median fold increase of CD4+ T_{CM} cells in response to the vaccine antigen in HIV+ individuals (Figure 3.8D [M3]) was significantly lower than that of healthy controls (p = 0.0008). The median fold increase of CD4+ T_{CM} cells in response to the vaccine antigen stimulation at 3 months in healthy controls was slightly higher than those at day 0 and 1 month but this was not statistically significant (Table 3.1).

The median fold increase of CD8+ T_{CM} cells in response to the vaccine antigen stimulation *in vitro* in healthy individuals increased slightly after vaccination, but the differences between PBMC collections were not statistically significant (Figure 3.8E). A lower-fold increase of CD8+ T_{CM} cells in response to *in vitro* 2009 H1N1 influenza vaccine antigen stimulation was observed in HIV+ individuals compared to controls at day 0, 1 month and 3 months (Figure 3.8E and Table 3.1, p = 0.0002, 0.0037 and <0.0001, respectively).

A statistically significant lower fold increase of CD4+ T_{EM} cells in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation was found in the HIV+ individuals compared to HIV− healthy controls at 3 months after vaccination (p = 0.036, Figure 3.8F). For CD8+ T_{EM} cells, the fold increase in HIV+ individuals was also significantly lower at 3 months (p = 0.0003, Figure 3.8G). In healthy individuals, the median fold increase of CD8+ T_{CM} cells in response to 2009 H1N1 influenza vaccine antigen stimulation at 3 months after vaccination were statistically significant increased

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compared to day 0 (p = 0.01) and 1 month (p = 0.03). There were no changes of fold increase of CD8+ T_{EM} cells over time in HIV+ individuals.

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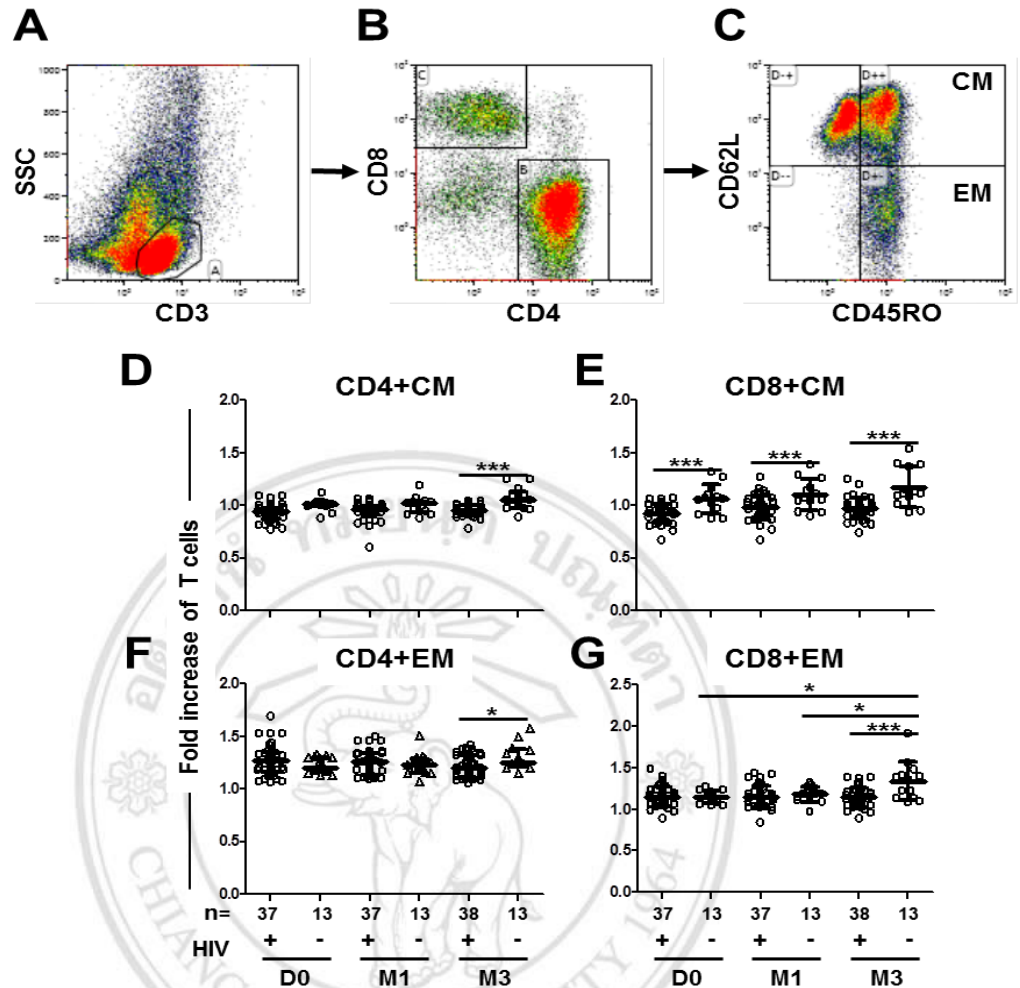

Figure 3.8. Increases of memory T cell subpopulations in response to 2009 H1N1 influenza A vaccine antigen. (A) T cell populations were identified as CD3+ population from CD3 versus side scatter (SSC) dot plot. (B) Single positive of CD4 or CD8 population were gated from CD4 versus CD8 dot plot. (C) Central memory (CM; CD45RO+ CD62L+) and effector memory (EM; CD45RO+ CD62L−) T cells were then evaluated from CD45RO versus CD62L dot plot. The increase of antigen-specific CD4+ T_{CM} (D) and CD8+ T_{CM} (E) and CD4+ T_{EM} (F) and CD8+ T_{EM} (G) cells were compared between HIV-infected (HIV+) and healthy (HIV−) individuals after stimulation at before (D0), one month (M1) and 3 months (M3) after vaccination. Statistical analysis was performed. *p*-value < 0.05 were considered statistically significant. * and *** show $p < 0.05$ and 0.001, respectively. Solid lines show the medians with interquartile ranges of each group.

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3.3.4. Expression of co-stimulatory molecules CD28 and activation marker CD69 on T cells

Single-positive CD4 and CD8 T cells were identified from dot plots of CD3-versus-SSC (Figure 3.9A) and CD4-versus-CD8 (Figure 3.9B), respectively. CD4+ or CD8+ memory T cells were identified as CD45RO+CD4+ or CD45RO+CD8+ from CD4 or CD8-versus-CD45RO dot plots, respectively. Figure 3.9C shows an example of how memory CD4+ T cells were identified and the expression of CD28 and CD69 of memory CD4+ T cells were evaluated (Figure 3.9D). There were no statistically significant differences in the median fold increase of the numbers of CD4+ or CD8+ T cells or CD4+ or CD8+ memory T cells that express CD28 between HIV+ and healthy groups (Figure 3.9E-H). No significant differences of the median fold increase of activated (CD69+) CD4+ (Figure 3.9I) or memory CD4+ T cells (Figure 3.9K) were determined between HIV+ and HIV− healthy individuals after *in vitro* stimulation by the vaccine antigen, and there were no changes within each study group between PBMCs collected at three time points during the study. Among the HIV− healthy controls, the fold increase of activated CD8+ (Figure 3.9J) and memory CD8+ T cells (Figure 3.9L) in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation increased significantly from day 0 to 3 months (p = 0.04 and 0.02, respectively). Among the HIV+, increases of activated CD8+ T cells were significantly lower compared to HIV− healthy controls at all-time points of study (day 0: $p = 0.035$; 1 month: $p = 0.0009$; 3 months: $p = 0.0029$) (Figure 3.9J). The increase of activated memory CD8+ T cells of HIV+ were also significantly lower than controls at 1 month and 3 months after vaccination ($p = 0.0027$ and 0.0099, respectively) (Figure 3.9L).

The changes of inhibitory molecules, CTLA-4 and PD-1, after 2009 H1N1 influenza A vaccine antigen stimulation were also investigated using the same gating strategy as CD28 and CD69. There were no statistically significant differences of fold increase of CD4+ or CD8+ T cells expressing CTLA-4 (Figures 3.10A and B, respectively) or PD-1 (Figures 3.10E and F, respectively) between cells collected at baseline and 1 and 3 months, nor between HIV+ and HIV− healthy individuals. Comparable results were also found in both memory CD4+ and CD8+ T cells responses (Figures 3.10C, G and D, H).

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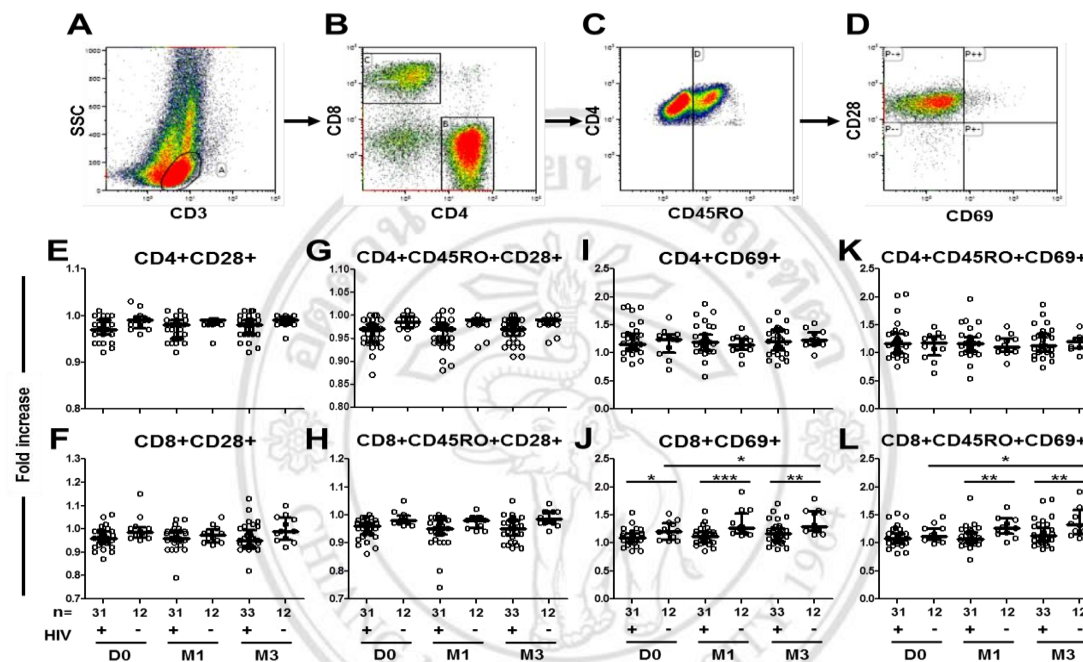

Figure 3.9. Expression of activation markers of T cells after stimulation with 2009 H1N1 influenza A vaccine antigen. Gating of CD3+ T cells (A) was identified for CD4 or CD8 single positive populations from CD4 versus CD8 dot plot (B). Memory T cells were identified by expression of CD45RO (C) and the expressions of CD28 and CD69 (D) were then evaluated. The increase of expressions of CD28 and CD69 on total CD4+ (E and I, respectively) and CD8+ (F and J, respectively) and memory CD4+ (G and K, respectively) and CD8+ (H and L, respectively) T cells were compared between HIV-infected (HIV+) and healthy (HIV−) individuals after stimulation at before (D0), one month (M1) and 3 months (M3) after vaccination. *p*-value < 0.05 were considered statistically significant. *, ** and *** show $p < 0.05$, 0.01 and 0.001, respectively. Solid lines show the medians with interquartile ranges of each group.

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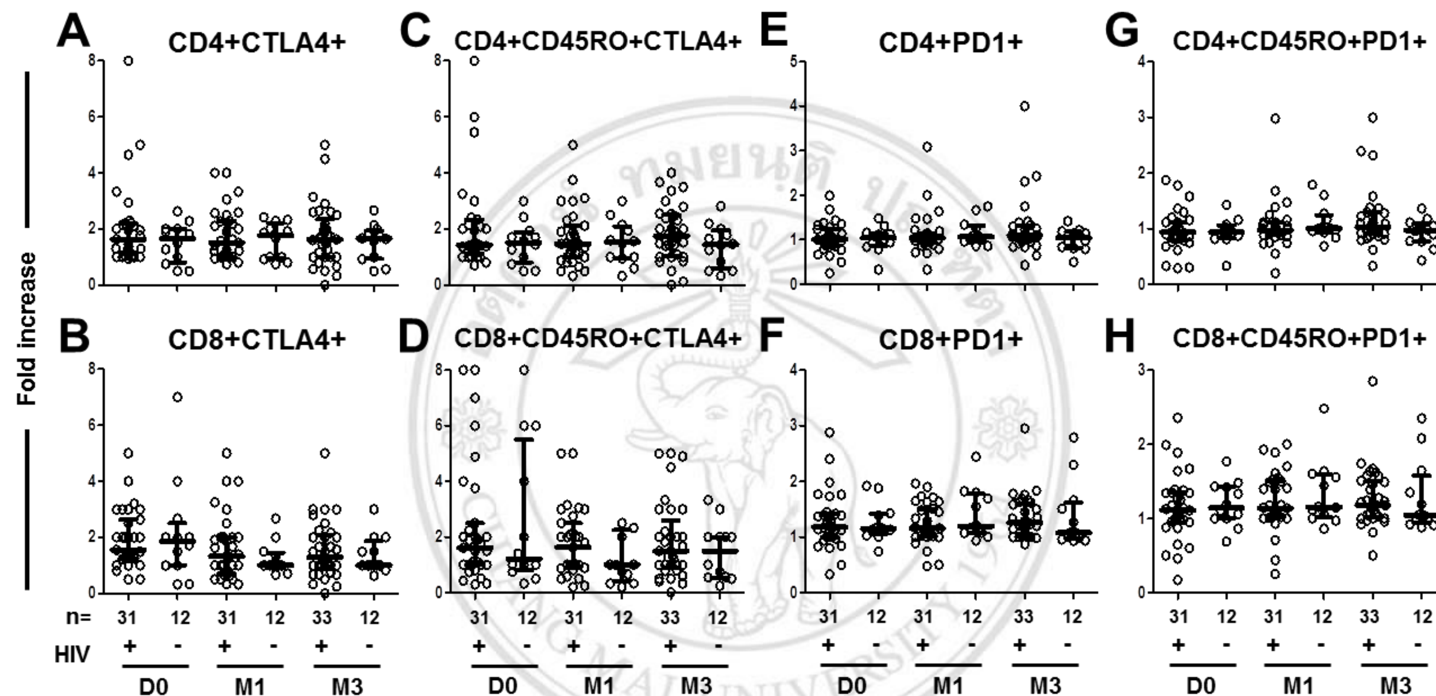

Figure 3.10. Fold increase of inhibitory molecule-expressing total and memory T cells. The changes of inhibitory molecules, CTLA-4 and PD-1, after 2009 H1N1 influenza A vaccine antigen stimulation were also investigated using the same gating strategy as CD28 and CD69.The increase of expressions of CTLA4 and PD1 on total CD4+ (A and E, respectively) and CD8+ (B and F, respectively) and memory CD4+ (C and G, respectively) and CD8+ (D and H, respectively) T cells were compared between HIV-infected (HIV+) and healthy (HIV−) individuals after stimulation at before (D0), one month (M1) and 3 months (M3) after vaccination. Statistical analysis was performed. p-value < 0.05 were considered statistically significant. Solid lines show the medians with interquartile ranges of each group.

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3.3.5. Expression of T-cell trafficking molecules

The expression of molecules involved in the migration of T_{EM} cells to the inflammatory tissues was then investigated. T_{EM} (CD45RO+ CD62L−) cells were determined as described in section 3.3.3 (Fig. 3.8A-C) and the expressions of CXCR3 and CCR5 in response to *in vitro* H1N1 vaccine antigen stimulation were then evaluated. The frequencies of CD4+ (Figure 3.11A), CD8+ (Figure 3.11B), memory CD4+ (Figure 3.11C) or memory CD8+ (Figure 3.11D) T cells that expressed CCR5 in response to the H1N1 vaccine stimulation within both study groups did not change after vaccination and there were no differences between HIV+ and HIV− healthy individuals. The fold increase of CXCR3+CD4+ T cells (Figure 3.11E) and CXCR3+CD4+ T_{EM} (Figure 3.11G) cells in response to 2009 H1N1 influenza A vaccine antigen stimulation of HIV+ subjects at 3 months after vaccination were significantly lower than in HIV− healthy controls (p = 0.0074 and 0.0152, respectively).

The frequencies of CD8+ T cells expressing CXCR3 in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation within the two study groups did not change significantly over time, and there were no differences between the two groups at day 0 and 1 month after vaccination (Figure3.11F). At 3 months, a significantly lower fold increase of CXCR3+CD8+ T cells in HIV+ individuals compared to HIV− healthy controls was observed. There were no differences in the frequencies of CXCR3+CD8+ T_{EM} cells over the time course of study within neither study groups, nor differences between HIV+ and healthy individuals (Figure 3.11H).

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Co-expressions of CCR5 and CXCR3 on T cell subpopulations in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation were further analysed. There were no differences of the frequencies of CCR5+CXCR3+CD4+ T cells (Figure 3.11I) and CCR5+CXCR3+CD4+ T_{EM} cells (Figure 3.11K) at day 0 and 1 month after vaccination within the study groups, nor differences between the two groups. The fold increases of CCR5+CXCR3+CD4+ T cells and CCR5+CXCR3+CD4+ T_{EM} cells in HIV+ individuals at 3 months after vaccination were significantly lower than HIV− healthy controls (p = 0.0008 and 0.011, respectively). There were no differences of CCR5+CXCR3+CD8+ T cells and CCR5+CXCR3+CD8+ T_{EM} cells between the two study groups (Figure 3. 11J and L).

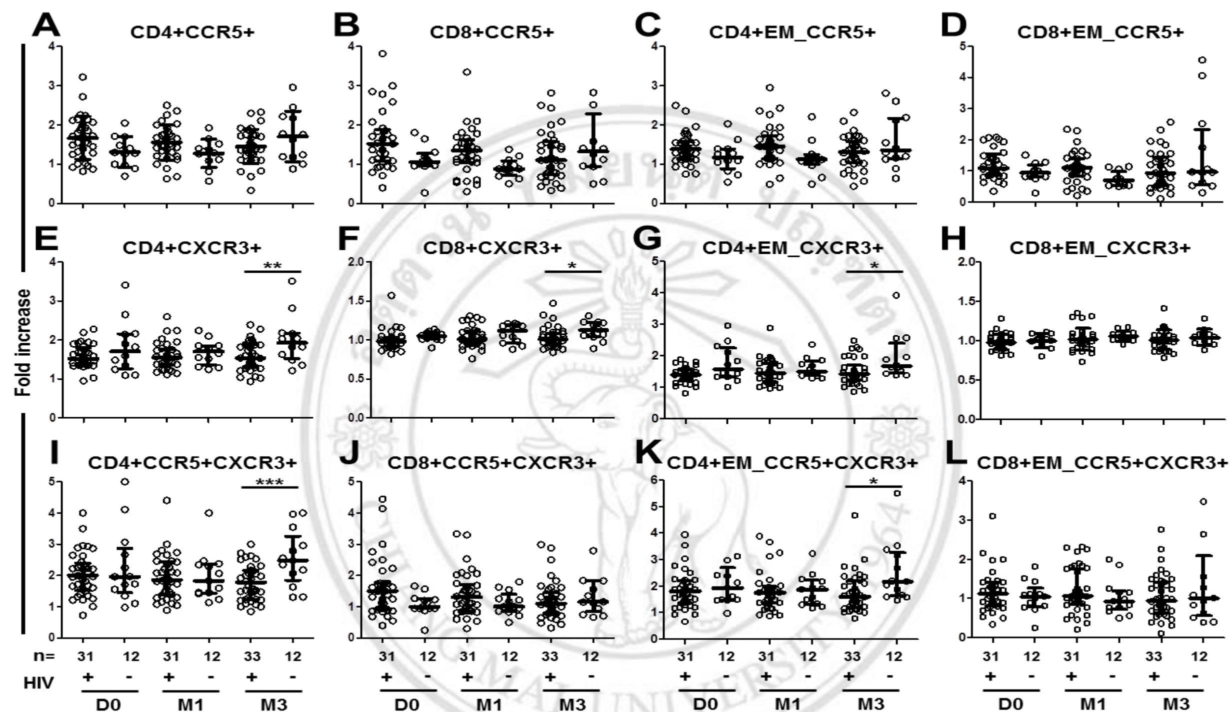

Figure 3.11. Expression of trafficking molecules on T cells after stimulation with 2009 H1N1 influenza A antigen. Expressions of CCR5, CXCR3 and both molecules on total CD4+ (A, E and I, respectively) or CD8+ (B, F and J, respectively) and effector memory CD4+ (C, G and K, respectively) or memory CD8+ (D, H and L, respectively) T cells were compared between HIV-infected (HIV+) and healthy (HIV−) individuals at before (D0), one month (M1) and 3 months (M3) after vaccination. Statistical analysis was performed. *p*-value < 0.05 were considered statistically significant. *, ** and *** show $p < 0.05$, 0.01 and 0.001, respectively. Solid lines show the medians with interquartile ranges of each group

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3.4 Discussion

Influenza virus infection can cause critical morbidity and mortality in immunocompromised persons, including those living with HIV, vaccination is recommended to prevent or reduce disease severity in these populations. Several studies have reported the efficacy of influenza vaccination in HIV-infected population, but most of these studies focused on the humoral immune response [6-9], while information of the cellular immunity to influenza remains limited. This study found no temporal changes in memory T cell cytokine production in response to *in vitro* influenza vaccine antigen during the three months of study in both HIV+ and HIV− healthy individuals. However, the vaccine induced more expansion of both CD4+ and CD8+ T_{CM} and T_{EM} cells after *in vitro* stimulation with the 2009 H1N1 influenza A vaccine antigen in HIV− healthy compared to HIV+ individuals at the end of three months of study. The increases of CD4+ and CD8+ T_{CM} and T_{EM} cells were accompanied by increases in expression of activation marker CD69 and chemokine receptors CCR5 and CXCR3 in HIV− healthy individuals, suggesting that memory T cells in the healthy may more efficiently migrate to the site of infection.

Several cytokines play an essential role in immunity to influenza A virus infection [7]. Ideally, immunization with vaccines should induce a robust cytokine response on re-encountering specific antigens. This study did not find significant changes in cytokine production in response to the 2009 H1N1 influenza A vaccine antigen *in vitro* during the three months of study in both HIV+ and HIV− individuals. This was not likely due to T cell defects, nor to technical problem performing the assays, since T cell from both groups produced cytokines in normal fashion in response to PHA, demonstrating functional activity of cryopreserved PBMCs. Additional data on cytokine ELISA or mRNA levels will strengthen the results. We could not obtain such data in this study due to limitation of cell numbers to perform the assays.

The investigation of humoral immune responses in these same study groups previously reported low seroconversion and seroprotection rates after monovalent 2009 H1N1 influenza A vaccination, and other measures of antibody response did not differ between the HIV+ individuals and HIV− healthy controls [7]. Their suboptimal

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antibody responses [7] and the low cytokine responses reported here could both be attributable to low immunogenicity of the vaccine itself, perhaps due to cold chain or vaccine management problems. Several studies also reported low to moderate immune responses after a single dose of monovalent 2009 H1N1 influenza A [8, 244, 245]. Recent studies suggested that even at the very low, non-toxic amounts of thimerosal preservative in the Panenza vaccine, there was suppression of the responses of *ex vivo* CD4+ T cells and dendritic cells, which may skew the immune response to the vaccine [241, 243]. Whether preservative amounts of thimerosal might affect the immune response *in vivo* after vaccination remains to be determined.

Although there were no significant elevations of cytokine productions after 2009 H1N1 influenza A vaccination, CD4+ and CD8+ T cells from HIV+ individuals produced cytokine levels comparable to that of cells from HIV− healthy individuals were observed, despite lower absolute CD4 cell counts in the HIV+ individuals. This study also observed comparable levels of multiple-cytokine-producing CD4+ and CD8+ T cells between the two study groups. Different levels of absolute CD4+ cell counts did not reveal differences in cytokine production in this study. Nevertheless, data on cytokine responses to influenza A vaccination in HIV+ and HIV− healthy individuals remains controversial. Using ELISPOT technique, Vigano et al. [246] and Fritz et al. [242] demonstrated lower responses after vaccination of influenza-specific IL-2-producing CD4+ T cells, IFN-γ-producing CD8+ T cells, and IFN-γ-producing CD4+ T cells in cART-treated HIV+ individuals. In contrast, Agrati et al. [241] showed no difference in influenza-specific IFN-γ-producing T cells between cART-treated HIV+ and HIV− healthy individuals. Further studies are needed in order to unravel the precise correlation of cytokines and protection against influenza in humans.

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Generation of memory T cells capable of rapidly recall responses upon reencounter with the antigen is a goal of vaccination. Memory T cells can be divided into two major subsets, T_{CM} (CD45RO+ CD62L+ CCR7+) and T_{EM} cells (CD45RO+ CD62L− CCR7−), depending on their homing capacity and effector function [247, 248]. T_{CM} cells have high potential to produce IL-2 and proliferate extensively, whereas T_{EM} cells are less proliferative and tend to produce effector cytokines such as IFN-γ [247, 248]. Memory T cells are thought to have contributed to protective immunity to

influenza by directly killing viral-infected cells and inhibiting viral replication [249]. Although only slightly fold changes were noted in this study, the results showed lower fold increases of both the percentages of T_{CM} and T_{EM} cells in response to *in vitro* 2009 H1N1 influenza A vaccine antigen stimulation in HIV+ patients compared to HIV− healthy controls.

In addition, the increases of memory CD4+ and CD8+ T cells expressing CD69, CCR5 and CXCR3 in response to the vaccine antigens stimulation *in vitro* were also more pronounced in HIV− healthy individuals after vaccination. Expressions of CD69, CCR5 and CXCR3 are associated with localization of memory CD4+ and CD8+ T cells in inflammatory tissues [250-252]. CCR5 and CXCR3-expressing cytotoxic memory CD8+ T cells are efficiently destroy cells infected by heterologous strains of H1N1 influenza A [253]. These cells produced IFN-γ and TNF-α and may play an important role in protection to influenza infection by promoting virus clearance and reducing illness severity. Lower expression of these molecules on both memory CD4+ and CD8+ T cells in responses *to in vitro* 2009 H1N1 influenza A antigen stimulation in vaccinated HIV+ individuals compared to HIV− healthy individuals in this study raises concern about the poor capability of effector memory cells to migrate to the site of infection.

Several previous studies have postulated that the impairment of expression of trafficking molecules on T cells in HIV+ individuals leads to compromised effector cells trafficking into the site of infection, and eventual defective immune responses [254-256]. The expression of CXCR3 and other inflammatory chemokines strongly correlated with advanced HIV disease progression and high viral load [254]. The expression of CXCR3 in patients with advanced HIV infection was lower than in HIV+ individuals with non-progressive chronic infection [254], their CD8+ T cells were capable to produce IFN-γ to the similar extent [257]. The consistency was observed in this study that although memory T cells from the HIV+ group were able to produce cytokines comparable to normal individuals, reduced CXCR3 expression in response to *in vitro* stimulation was demonstrated. These data may manifest heterogeneity of memory T cells subpopulations which are regulated independently [258-260].

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The mechanism underlying the down-regulation of chemokine receptors on memory T cells in HIV infection remains to be elucidated. The finding of lower expression of the CXCR3 molecule in the HIV+ group was unlikely to have resulted from the elevation of expression of the CXCL9 and CXCL10 molecules to which is its ligand, other findings of no significant differences of CXCL9 and CXCL10 levels between chronic and advanced infection [254]. Other studies have demonstrated down-regulation of several chemokine receptors on the surface of various cell types by the negative regulatory factor (Nef) protein encoded by HIV virus, perturbing receptor re-cycling which results in receptor degradation [261]. Approaches to induce expression of inflammatory chemokines and chemokine receptors on memory T cells capable of efficiently migrate to inflammatory tissues should be considered when designing a vaccine to be used in HIV+ population.

Until further research can solve the necessity better to protect the HIV+ from influenza infection, temporal public health strategies might be to increase the antigen mass of hemagglutinin, as already done to 60 μg of HA for each antigen in the elderly in high-dose formulations [262-264], or give two doses 3-to-4 weeks apart [265, 266]. Whether these strategies would improve the prevention or mitigation of illness by influenza in people living with HIV remains to be investigated. Better understanding of how to enhance cellular immune responses may lead to new influenza vaccines which result in better improve the protection in the HIV+ population.

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