CHAPTER 2

Study design, Materials and Methods

2.1 Participants and Sample collection

Heparinized blood samples (~10 mL) were obtained from 76 HIV infected patients who have been initiated combination antiretroviral therapy (cART) at Maeon Hospital in Chiang Mai, Thailand. The patients were selected based on the following criteria;

1) Between the ages of 18-60

2) Received ART drug (first line drug regimen; TDF, 3TC, EFV or NVP)

3) Infected with HIV for at least 1 year

4) Viral loads less than 50 copies/mL

5) Contain CD4 number at any levels

6) No history of autoimmune diseases

7) No history of opportunistic infections (OIs)

8) Not pregnant

In addition, Heparinized blood samples (~10 mL) were collected from 100 HIV-negative, aged between 18 and 60 years, no history of autoimmune disease donors from Maharaj Nakorn Chiang Mai Hospital. All participants had sign an inform consent before enrolling in this study and the ethical protocols were approved by the ethics committee at the Faculty of Medicine, Chiang Mai University (Study Code: NONE-2558-03290).

The plasma was separated from the collected Heparinized blood sample for antinuclear antibody assay. After ANA analysis, the samples were randomly separated into 4 groups based on HIV infection and ANA result for Th17 and Treg determination; 1) HIV negative with ANA negative (n=15), 2) HIV negative with ANA positive (n=11), 3) HIV positive with ANA negative (n=15), and 4) HIV positive with ANA positive (n=11).

To determine the percentages of Th17 and Treg cells, peripheral blood mononuclear cells (PBMCs) were separated from the remaining heparinized blood by ficoll-hypaque density gradient centrifugation technique and cultured in RPMI complete medium overnight in 5% CO_2 incubator before Th17 and Treg determination in the following day.

2.2 Antinuclear antibody (ANA) detection

For the detection of antinuclear antibodies, separated plasma from heparinized blood samples were subjected to the ANA analysis by indirect immunofluorescent assay using the commercial cultured HEp-20-10/Liver (Monkey) slides (EUROIMMUN, Lübeck, Germany) according to manufacturer's protocol. This assay was designed exclusively for the *in vitro* determination of human antibodies in serum or plasma. First, the sample to be investigated was diluted 1:80 in PBS-Tween (dilute 10 µl sample in 790 µl PBS-Tween and mix thoroughly). Then, 30 µl of diluted sample was applied to each reaction field of the reagent tray and started the reaction by fitting the tissue substrate slide into the corresponding recesses of the tray, incubated for 30 minutes at room temperature. After incubation, the slide was rinsed with a flush of PBS - Tween and immersed in a cuvette containing PBS-Tween for 5 minutes. Next, 25 µl of fluorescein labelled antihuman immunoglobulin was applied to each reaction field of a clean reagent tray and the slide was put into the recesses of the reagent tray, incubated for 30 minutes in the dark at room temperature. After incubation, the slide was rinsed with a flush of PBS-Tween and immersed in a cuvette containing PBS-Tween and Evans blue dye (for background staining) for 5 minutes. Then, the embedding medium was placed onto a cover glass and the slide was put onto the prepared cover glass and visualized ANA titer and pattern by a fluorescence

microscope. In positive sample, the plasma was diluted to 1:160, 1:320, 1:640 or 1:1280 depended on the fluorescence intensity (figure 2.1).

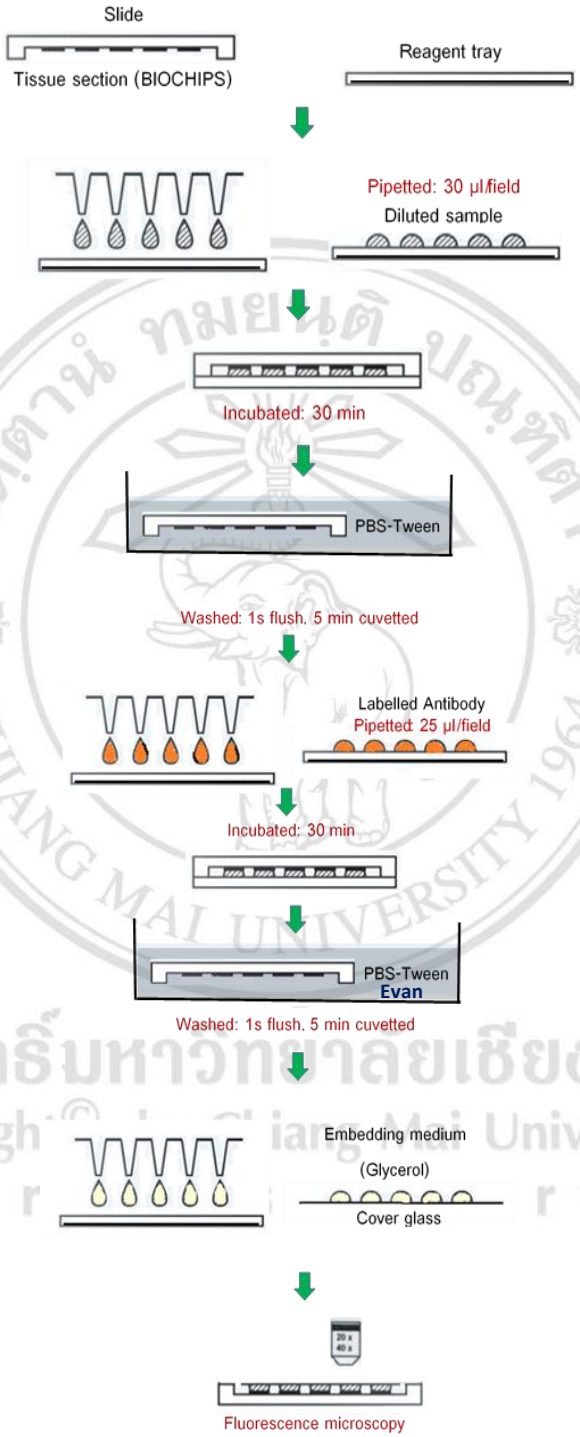

Figure 2.1 EUROIMMUN ANA test method (the figure modified from EUROIMMUN Immunofluorescence test instruction sheet)

2.3 Th17 and Treg determination

2.3.1 Th17 cells identification

To determine the percentage of Th17 cells, PBMCs from overnight cultured with RPMI were subjected to Th17 identification using the protocol modified from Guenova, *et al* [83]. Cells were plated into flat bottom 24 well cell culture plate (5×10^5/well, 4-6 wells/sample) and stimulated with 500 ng/mL of phorbol 12-myristate 13-acetate (PMA) (InvivoGen, San Diego, USA) and 500ng/mL ionomycin (Sigma Aldrich, St. Louis, USA) in the presence of 1µg/mL brefeldin A (Biolegend, San Diego, USA) for 4 hours at 37 °C in a 5% CO_2 incubator. Then the cells were harvested to flow cytometry tube (5×10^5- 1×10^6/tube) and blocked the Fc receptors on the cells with 20% AB serum for 30 minutes. Cells were washed twice and surface stained with 5 µL of antibody against CD3 (FITC anti-human CD3; BioLegend, San Diego, USA) and CD8 molecules (PE anti-human CD8; BioLegend, San Diego, USA) for 30 minutes in the dark at 4°C. After surface staining, cells were washed twice and then, fixed with 2% paraformaldehyde for 30 minutes followed by membrane permeabilization with 0.1% saponin (AMRESCO, Ohio, U.S.A) for 20 minutes. The fixed/permeabilized cells then were washed twice and stained with antibody against IL-17 (PerCP/Cy5.5 anti-human IL-17A; BioLegend, San Diego, USA), intracellularly. Cells were washed 2 times, re-suspended with 1% paraformaldehyde and analyzed by flow cytometry.

2.3.2 Treg cell identification

To determine the percentage of Treg cells, the expression of CD25 and lack of CD127 were used as markers. Treg was identified using the protocol modified from Liu, *et al* [62]. The remaining PBMCs from overnight cultured with RPMI were blocked with 20% AB serum for 30 minutes and washed twice before staining of the surface molecules with 5 µL of antibody against CD3 (FITC anti-human CD3; BioLegend, San Diego, California, USA), CD8 (PE anti-human CD8; BioLegend, San Diego, USA), CD25 (PE/Cy7 anti-human CD25; BioLegend, San Diego, USA) and CD127 (PerCP/Cy5.5 anti-human CD127; BioLegend, San Diego, USA) for 30 minutes in the dark at 4°C. Cells were washed 2 times, re-suspended with 1% paraformaldehyde and analyzed by flow cytometry.

2.4 Statistical analysis

SPSS for window version 22.0 was used for statistical analysis. ANA positive prevalence in each group was shown in percentage. A nominal scale factor such as sex and ANA result was compared between group by Chi-square test. Nonparametric statistic, Mann-Whitney U-test and Kruskal-Wallis ANOVA were performed for matched pairs. The statistical significant was considered at p-value of less than 0.05.