CHAPTER 2

Materials and methods

Materials and methods described in this chapter were commonly used in all three studies. The methods specific to a particular study are described in that chapter.

2.1 Human subject research ethics

All studies were approved by the Human Experimentation Committee of the Research Institute for Health Sciences (RIHES), Chiang Mai University (CMU), Thailand. The studies were conducted according to the international conference on harmonization of good clinical practice, the Belmont report and the World Medical Association Declaration of Helsinki. Potential participants were invited to take part in the studies when they came for regular visits at Maharaj Nakorn Chiang Mai hospital, or through advertising posters about the studies. All participants were informed about details of the studies and given time to ask any enquiries they had before enrolling into the studies with consent.

2.2 Blood collection

Venous blood was collected in vacutainer tubes containing acid citrate dextrose (ACD) solution A formulation (22.0 g/L trisodium citrate, 8.0 g/L citric acid, 24.5 g/L dextrose) as an anti-coagulant (BD Vacutainer® Blood Collection Tube, Becton Dickinson and Company, Franklin Lakes, NJ, USA). The volume of blood collected was dependent on each study as indicated in specific chapters.

2.3 Isolation of peripheral blood mononuclear cells (PBMCs) by gradient centrifugation

Whole blood was diluted with equal volume of plain RPMI 1640 medium (Life Technologies, Grand Island, N.Y., USA). Ficoll-Hypaque solution (Biochrome, Berlin, Germany) was underlayered under the diluted blood. After brake-off centrifugation,

PBMCs enriched interface separated from red blood cells (RBCs) and polymorpho-nuclear cells (PMNs) were collected to a new tube and washed with RPMI 1640. The remaining red blood cells (RBCs) were lysed by ammonium-chloride-potassium (ACK) lysing buffer. Then, PBMCs were washed twice with plain RPMI 1640 and cell numbers were assessed. PBMCs were adjusted up to 20 x 10^6 cells/mL in freezing medium (fetal bovine serum (FBS, Biochrome, Berlin, Germany) containing 10% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA)), depending on studies, and aliquoted into a cryotube for 1 mL per tube. After leaving overnight in a freezing box at −70 °C, PBMCs contained cryotubes were stored in liquid nitrogen until use.

2.4 Cell stimulations

Cryopreserved PBMCs from liquid nitrogen storage were thawed in 37°C water bath. Thawed PBMCs were transferred to a 15 mL conical tube containing pre-warmed RPMI 1640. After wash twice with RPMI 1640, cells were resuspended in complete RPMI (RPMI 1640 containing 10% FBS). Cell suspension was incubated in 37°C 5% CO_2 incubator overnight. Resting PBMCs were stimulated with various antigens as indicated in each study.

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

After overnight stimulation, 5 μg/mL of brefeldin A and 1 μM of monensin (Sigma-Aldrich, St. Louis, MO, USA) were added to the culture and incubated for 4 hours in 5% CO_2 37ºC incubator. The cells were washed with FACS buffer (phosphate buffer saline (PBS) containing 2% FBS and 0.05% NaN_3). Fc receptors were blocked by 30 min incubation with PBS containing 10% FBS and 0.05% NaN_3 (blocking buffer). Then, the cells surface markers were determined by staining with fluorochrome-conjugated monoclonal antibodies (MAbs), including anti-CD8-PE Alexa Fluor 610 (Life Technologies, USA), anti-CD4-APC/Cy7 and anti-CD45RO-Pacific Blue (Biolegend, San Diego, CA, USA), depending on the study. After 30 min incubation at 4 °C, cells were fixed and permeabilized with 4% paraformaldehyde in PBS and FACS

buffer containing 0.2% saponin (PBS-S), respectively. After Fc receptors blocking, the cells were incubated with anti-CD3-Krome Orange (Beckman Coulter, Brea, CA, USA), anti-TNF-α-FITC, anti-CD107a-PE, anti-IFN-γ-PerCP/Cy5.5, anti-IL-2-PE/Cy7 and anti-IL-10-APC (Biolegend, San Diego, CA, USA) at 4 °C for 30 min. After incubation, cells were washed with PBS-S and resuspended in 1% paraformaldehyde in PBS. Cytokine-secreting or granzyme-producing cells were determined by Cyan ADP 9-color flow cytometer (Beckman Coulter, Brea, CA, USA). Flow-cytometric analysis was performed by using Kaluza software (Beckman Coulter, Brea, CA, USA).

2.6 Flow-cytometric analysis

For all cytometric determinations, T cell populations were identified from CD3+ population by dot plot of CD3-versus-side scatter (SSC) (Fig. 2.1A). CD3+ T cells were then gated and single-positive of CD4 and CD8 populations were then identified from CD4-versus-CD8 dot plot (Fig. 2.1B). This gating strategy was used to ensure that only single positive CD4 or CD8 T cells were analysed, as it has been reported that CD4/CD8 double positive cells with memory phenotype circulate in peripheral blood during viral infections [232, 233]. To evaluate cytokine-production and functional markers for degranulation, IFN-γ, IL-2, IL-10 and CD107a were plotted against TNF-α (Fig. 2.1C).

To assess cytokine-production and CD107a-expression by memory T cell populations. Gating on CD3+ cells from CD3-versus-SSC dot plot (Fig. 2.2A), CD4 or CD8 single-positive populations were identified from CD4-versus-CD8 dot plot (Fig. 2.2B). Memory T cells were identified as CD45RO+ population. Expression of intracellular cytokines or surface CD107 of memory T cells were then evaluated from intracellular cytokines or surface CD107-versus-CD45RO dot plot (Fig. 2.2C).

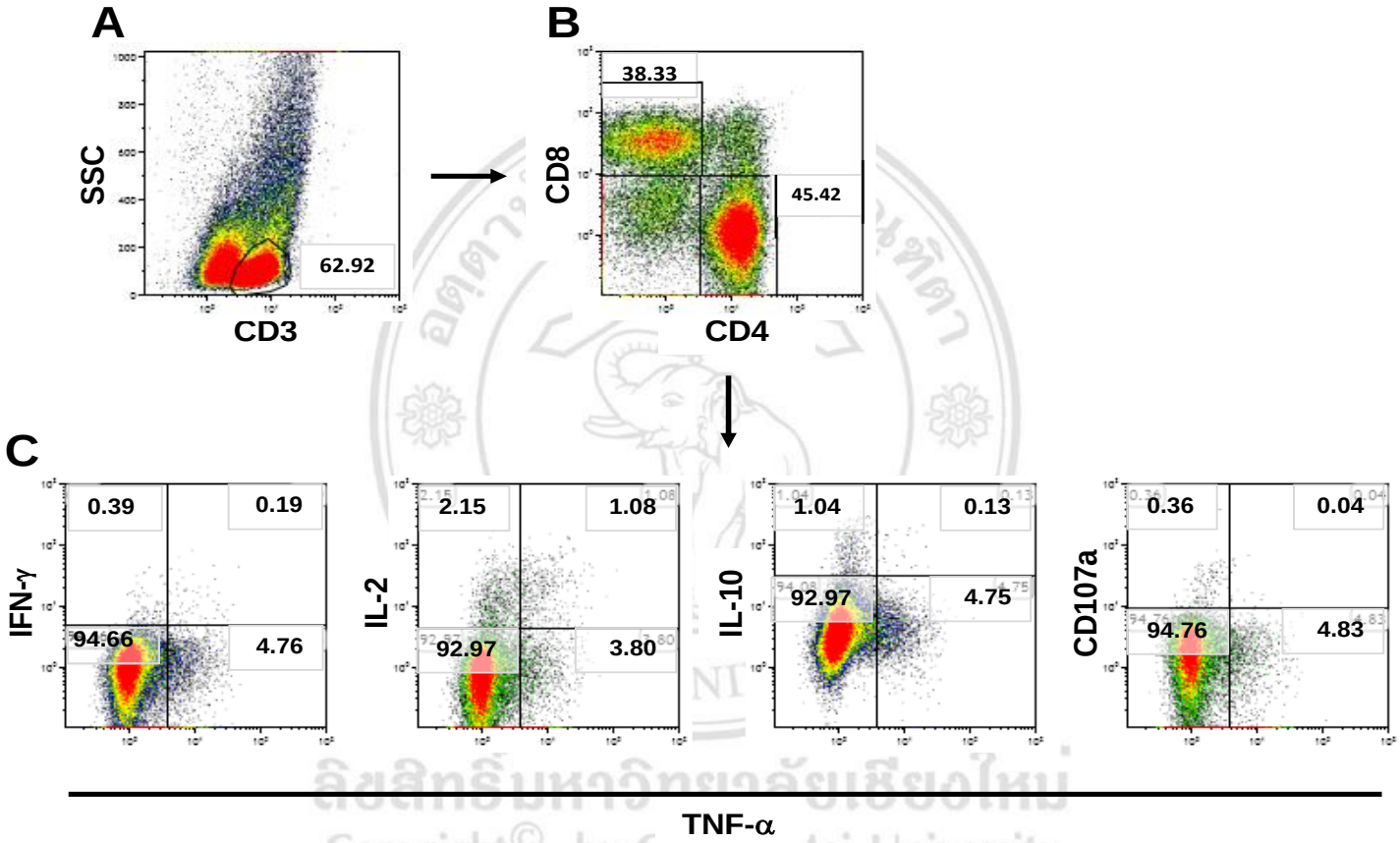

Figure 2.1. Analysis of cytokine-producing and CD107a-expressing T cells. The population of T cells was identified as CD3+ population from CD3-versus-side scatter (SSC) dot plot (A). Single positive of CD4 or CD8 population were gated from CD4-versus-CD8 dot plot (B). Plots of IFN-γ, IL-2, and IL-10 cytokine production, and surface CD107a expression, versus TNF-α, were analysed (C).

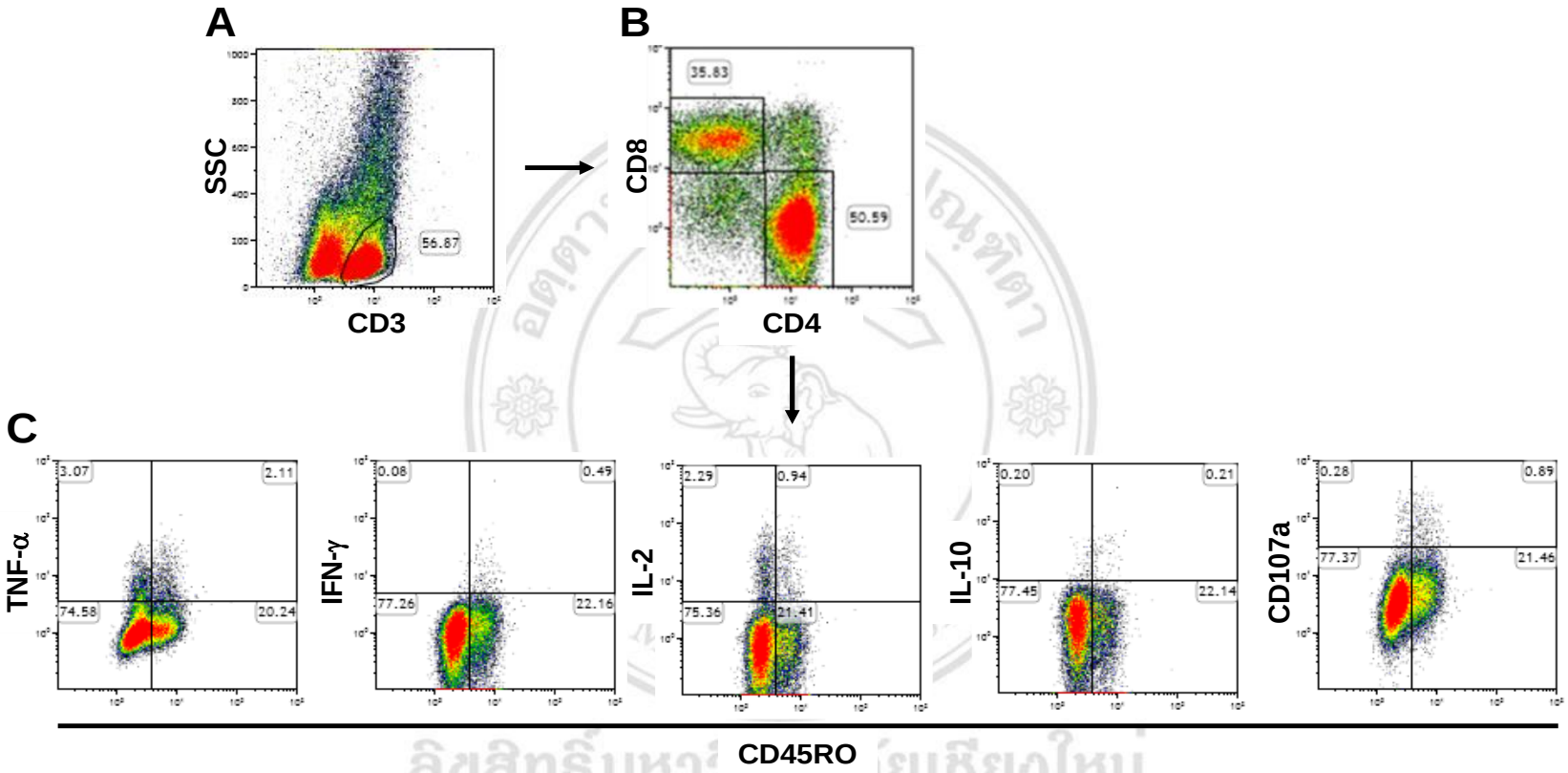

Figure 2.2. Analysis of cytokine-producing and CD107a-expressing memory T cells. The population of T cells was identified as CD3+ population from CD3 versus side scatter (SSC) dot plot (A). Single positive CD4 or CD8 populations were gated from CD4-versus-CD8 dot plot (B). Cytokine production and expression of surface CD107a of memory (CD45RO+) CD4+ or CD8+ T cell subpopulation were then evaluated from dot plot of TNF-α, IFN-γ, IL-2, IL-10 and CD107a -versus-CD45RO (C).

2.7 Statistical analysis

Statistical difference between 2 groups was analysed by Student t test or Mann-Whitney U test depending on data distribution. Comparison of more than 2 groups was analysed by using One-Way ANOVA or Kruskal-Wallis test depending on data distribution. Statistical analysis was performed using GraphPad Prism Version 5 software (GraphPad Software, La Jolla, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.