CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Blood samples used in the generation of MoDCs

2.1.1.1 HIV-1 positive samples

Peripheral blood samples were collected into two of 7 ml EDTA tubes from each patient by the staff of Mae On and Phang hospital. Seven cases of HIV-1 positive patients with the level of $CD4^+$ T cells more than 200 cells/µl and 3 cases of HIV-1 positive patients with the level of $CD4^+$ T cells less than 200 cells/µl were enrolled. The patients must not receive highly active anti-retroviral therapy (HARRT) prior to study. Both groups of blood samples were used for generation of MoDCs. HIV-1 status of all blood samples were confirmed by using gelatin-particle agglutination (GPA) tests (SERODIA-HIV).

2.1.1.2 HIV-1 negative samples

Ten cases of HIV-1 negative volunteers were enrolled. Peripheral blood samples were collected into two of 7 ml EDTA tubes from each volunteer. All of the blood samples from HIV-1 negative volunteers were also tested for HIV-1 status by using gelatin-particle agglutination (GPA) tests.

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2.1.2 Blood samples used in preparation of T cells

Buffy coats from two blood donors were collected from Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital. Donor's buffy coats with only blood group O were selected and re-confirmed the ABO blood group by cell grouping with anti-A, anti-B and anti- A,B. Isolated PBMCs were frozen in 10% DMSO in heat inactivated fetal bovine serum (Biowest) at the concentration of $5x10^6$ cells per vial and kept in liquid nitrogen until needed.

2.1.3 Reagents used in generation of monocyte-derived dendritic cells

Recombinant human (rh) granulocyte macrophage colony stimulating factor (GM-CSF) (AbD Serotec) and recombinant human (rh) interleukin-4 (IL-4) (AbD Serotec) were used in generation of immature MoDCs. Lipopolysaccharide (LPS) (Sigma) was used to activate immature MoDCs into mature MoDCs. Mitomycin-C (Kyowa Hakko Kogyo Co,Ltd.) was used to inhibit the proliferation of the stimulators before performing proliferation assay.

2.1.4 Reagents used for purifying T cells for proliferation assay

Human Pan T cell Isolation Kit II (Miltenyi Biotec) was used for purifying T cells from PBMCs. This isolation kit is an indirect magnetic labeling system for the isolation of untouched T cells from human peripheral blood mononuclear cells (PBMCs). Non-T cells such as B cells, NK cells, dendritic cells, monocytes, granulocytes and erythroid cells are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A, as primary labeling reagent. Anti-Biotin MicroBeads was

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used as secondary labeling reagent. Isolation of highly purified T cells will be achieved by depletion of magnetically labeled cells. The magnetically labeled non-T cells are depleted by retaining them in a MACS® Column (MS column) in the magnetic field of a MACS® Separator, while the unlabeled T cells pass through the column.

2.2 Methods

2.2.1 Generation of monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll/Hypaque gradient centrifugation. PBMCs were maintained in complete culture medium which composed of RPMI-1640 (GibcoBRL) supplemented with 100 µg/ml streptomycin (M & H MANUFACTURING CO.,LTD.), 100 U/ml penicillin (M & H MANUFACTURING CO.,LTD.), 250 µg/l amphotericin B (Bristol-Myers), 10% heat inactivated fetal bovine serum and 5 mM HEPES buffer at the concentration of $2x10^6$ cells/ml. Monocytes were selected by allowing them to adhere to tissue culture flask for 1 hour at 37°C. Non-adherent cells were removed by rinsing the cultured flask with RPMI 1640 gently 3-5 times. Complete culture medium supplemented with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) was added to the adherent cells. Adherent cells were cultured at 37°C in humidified 5% CO_2 incubator for 6 days [9, 158, 159]. Morphology of cultured cells had been examined every day.

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2.2.2 Determination of purity and viability of generated monocyte-derived dendritic cells

After 6 days of culture, monocyte-derived dendritic cells will become non-adherent cells. Cells were harvested and re-suspended at the concentration of $2x10^5$ cells/ml. The purity of MoDCs were determined by staining cells with fluorescein isothiocyanate (FITC) conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and phycoerythrin-cyanin 5 (PC5) conjugated anti-HLA-DR. MoDCs were identified as the cells that positive for HLA-DR and negative for other linage markers. Finally, cells were stained with propidium iodide (PI) to assess viability of generated cells.

2.2.3 Preparation of MoDC-T cell co-culture

2.2.3.1 Preparation of T lymphocytes

The frozen PBMCs were thawed and transferred to RPMI 1640 medium supplemented with 5% heat inactivated fetal bovine serum. T cells were purified by using human Pan T cell Isolation Kit II (Miltenyi Biotec). After PBMCs were washed in RPMI 1640 medium supplement with 5% inactivated fetal bovine serum, cells were centrifuged at 300xg for 10 minutes. Cell pellet was re-suspended in 40 µl of separating buffer (Appendix) per 10^7 total cells. Then biotin-antibody cocktail was added at 10 µl per 10^7 total cells. Cells were incubated for 10 minutes at 4-8°C. Separating buffer was added at 30 µl per 10^7 total cells. Anti-biotin micro beads were then added at 20 µl per 10^7 total cells. Cells were incubated for an additional 15 minutes at 4−8°C. Cells were washed by adding 10−20x labeling volume of separating buffer and centrifuged at 300xg for 10 minutes. Finally, cells were re-

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suspended with 500 µl of separating buffer. Re-suspended cells were proceeded to magnetic separation.

2.2.3.2 Determination of purity and viability of purified T cells

The purified T cells were re-suspended at the concentration of $2x10^5$ cells/ml. The purity of T cells was determined by staining cells with fluorescein isothiocyanate (FITC) conjugated anti-CD14, anti-CD16, anti-CD19, anti-CD20 and phycoerythrin-cyanin 5 (PC5) conjugated anti-CD3. T cells were identified as the cells that positive for CD3 and negative for other linage markers. Finally, cells were stained with propidium iodide (PI) to assess viability.

2.2.3.3 Preparation of mature MoDCs

After 6 days of MoDC culture, cells were collected and stimulated with 1 µg/ml LPS for 48 hours to prepare mature dendritic cells. Mature dendritic cells were then treated with 10 µg/ml mitomycin C for additional 30 minutes.

2.2.4 MoDC-T cell co-culture

Purified T cells were prepared at the concentration of $2x10^7$cells/ml. Cells were diluted with 5 µM of working carboxy fluoroscein succinimidyl ester (CFSE) at the ratio of 1:1 to make the final concentration of T cells and CFSE at $1x10^7$ cells/ml and 2.5 µM respectively. Cells were incubated at room temperature and agitated gently for 10 minutes. Stained cells were washed with RPMI 1640 medium supplement with 5% heat inactivated fetal bovine serum and re-suspended at the concentration of $1x10^6$ cell/ml. Cells were then co-cultured with $1x10^5$ cells/ml of mitomycin C treated

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mature dendritic cells in 24 –well tissue culture plate for 7 days in humidified 5% CO_2 incubator. Proliferation of T cells was assessed by flow cytometer.

2.2.5 Intra-cellular cytokine staining

Six hours before harvesting, 1 µg/ml of brefeldin A (Sigma) was added to the culture to inhibit cytokine secretion by T cells. Cells were then harvested and stained with PC5 conjugated anti-CD3 and energy couple dye (ECD) conjugated anti-CD4, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% saponin. Finally, cells were stained with either phycoerythrin (PE) conjugated anti-IFN-γ or anti-IL-4 for determining Th1/Th2 cytokine responses of T cells.

2.2.6 Statistical analysis

The statistic analysis was presented by using Mann-Whitney U Test to compare T cells responses which were activated by MoDCs from different groups of patients.

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