III. MATERIALS AND METHODS

A. Subjects

1. Normal persons

Monocytes were obtained from peripheral blood of healthy non-smoking volunteers using gelatin/plasma-coated flask method.

2. Tuberculosis Patients

Monocytes were obtained from peripheral blood of immunocompetent patient with multidrugs-resistant pulmonary tuberculosis volunteers using gelatin/plasma-coated flask method.

B. Isolation and purification of human peripheral blood monocytes

1. Preparation of gelatin-coated surface

Gelatin-coated surfaces were prepared by adding 10 ml of steriled gelatin solution (3% in water, Sigma) to each 75 cm^2 plastic tissue culture flask (Nunc). These flasks were incubated at 37°C for 2 hours. The gelatin solution was removed by aspiration, and the flasks were dried overnight at 50 °C. These flasks were stored at 37 °C in a dry incubator before use.

2. Preparation of human plasma

Plasma, which will be used as the source of fibronectin was prepared from human blood. Human blood was collected by venipuncture and mixed with heparin solution (5000 i.u./ml, Leo Pharmaceutical Products, Ballerup, Denmark), the final concentration of heparin was 10 i.u./ml. Platelet-rich plasma was prepared from this blood by centrifuge at 200 g for 20 min (Rotina 48R-V1 0.3). Platelet-poor plasma was obtained by centrifuge the platelet-rich plasma at 1200 g for 10 min to separate the platelet from plasma. Ten milliliters of autologous platelet-poor plasma were added to each gelatin-coated flask and incubated for 60 min at room temperature. The plasma was saved and the flask was washed twice with 10 ml steriled phosphate buffer saline (PBS) pH 7.2. Ten milliliters of steriled PBS was added to each

plasma/gelatin-coated flask and leave the flask at room temperature until the mononuclear cells were ready to adhere to the flask.

3. Preparation of Mononuclear cells

Mononuclear leukocytes were obtained from heparinized peripheral blood as described above. After removal of the plasma, the whole blood cells were resuspended with complete media (RPMI1640 supplemented with 10% heat inactivated fetal bovine serum) in an equal volume to the removal plasma. The cell suspension was carefully overlayered onto 10 ml of the Ficoll-Hypaque (Isoprep) solution, then centrifuged at 400 g for 30 min. Mononuclear cells were removed by steriled pasteur pipette and washed with 10 ml of RPMI1640 medium three times by centrifuge at 1500 rpm for 10 min. The mononuclear cells were resuspended in 30 ml of complete media. White blood cells count, viability count and nonspecific estersae staining were performed using 100 µl of the cell suspension and 15 ml of mononuclear cell suspension were added to each plasma/gelatin-coated flask then incubated for 60 min at 37°C, 5% CO_2. Nonadherent cells were removed and flasks were washed gently for four times with 10 ml of RPMI1640 medium prewarmed at 37°C. The nonadherent cells was successively washed out by adding a mixture of 100 µl, 0.5M EDTA, pH 8.0 in 10 ml of RPMI1640 medium to each flask for 1 min. The flasks were washed again for four times with 10 ml of RPMI1640 medium prewarmed at 37°C. The adherent cells were obtained by adding a mixture of 100 µl, 0.5M EDTA, pH 8.0 in 10 ml of complete medium prewarmed at 37 °C to each flask and incubated at 37 °C, 5% CO_2 for 10 min. The adherent cells were removed and washed with 10 ml of complete medium by centrifuged at 1500 rpm for 15 min. They were resuspended in 5.0 ml of complete medium and 100 µl of the cell suspension were used to perform white blood cells count, viability count and nonspecific esterase staining, respectively. These cells were adjusted to approximately $2.0x10^5$ cells/ml with complete medium. One milliliter of cell suspension was dispensed to each well of a 24-wells tissue culture plates (Nunc) which had been placed by a 12-mm-diameter sterile circular glass coverslip. Plates were incubated for 5 days at 37°C, 5% CO_2 to allowed the monocytes differentiated into the macrophage cells.

C. *Mycobacterium tuberculosis* strains

(1) *Mycobacterium tuberculosis* H37Ra was kindly obtained from Assistant Prof. Dr. Suchart Panchaisri, Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University. Thailand.

(2) *Mycobacterium tuberculosis* H37Rv KK11-20 strain (KK: Mycobacteria Collection, Research Institute of Tuberculosis, Tokyo.) was obtained from Mr. Sakarin Chanwong, Center of Tuberculosis Control Region 10, Chiang Mai, Thailand. Bacteria were grown in Middlebrook 7H9 broth base (Difco, Detroit, MI) supplemented with 10% albumin-dextrose-catalase (ADC) (Difco) and 0.05% (w/v) Tween 80 (Sigma) at 37°C for 1 month. Single cell suspension of the mycobacteria was prepared by the method of Wright et al (1996) with some modification. Briefly, the log phase growing bacteria was pelleted by centrifugation at 1000 g for 15 min. Supernatant fluid was discarded and the pellets were resuspended in sterile PBS. The bacterial clump was homogenated by vortexing with sterile glassbeads (4-mm-diameter). The bacterial suspension were allowed to settle at room temperature for 30 min. The upper portion of the bacterial suspension was collected in sterile vial (1ml/vial) and stored at –70 °C as the stock bacteria for all experiment.

D. Infection of monocyte- derived macrophages with *M. tuberculosis*

Monocyte-derived macrophages, $2.0x10^5$ cells/ml, were infected with *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv. The multiplicity of infection (MOI) was 10 mycobacteria per macrophage. After incubation at 37°C 5% CO_2 for 4 hours, the extracellular mycobacteria were removed by washing three times with RPMI1640 medium. The acid fast stain was performed to determine the percentage of phagocytosis. Macrophages were counted at least 500 cells. Percent phagocytosis was calculated as the following formula.

% Phagocytosis = (Number of ingested macrophages/ Total of counted macrophages) x100

E. Apoptosis Assay

Monocyte-derived macrophages, $2.0x10^5$ cells/ml, were infected with *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv. The multiplicity of infection (MOI)

was 10 mycobacteria per macrophage. After incubated at 37°C 5% CO_2 for 4 hours, the extracellular mycobacteria were removed by washing three times with RPMI1640 medium. The cells were cultured for 48 hours and the apoptosis of *M. tuberculosis* - infected macrophages was determined. Actinomycin D, an inhibitor of RNA polymerase enzyme, was used as the positve control. Uninfected macrophages were used as the negative control of the experiment.

Apoptosis of *M. tuberculosis*-infected macrophages were determined by using Annexin-V-FLUOS Staining Kit (Roche, Germany). This method based on the fact that in live, non-apoptotic cells phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Thus, while phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, phosphatidylserine is almost exclusively located on the inner surface. Early during apoptosis this asymmetry is broken and phosphatidylserine becomes exposed on the outside of the plasma membrane. Because the anticoagulant protein annexin V binds with high affinity to negatively charged phospholipids like phosphatidylserine, the fluoresceinated annexin V has found an application as a marker of apoptotic cells. In the course of apoptosis the cells become reactive with annexin V after the onset of chromatin condensation but prior to the loss of plasma membrane ability to exclude cationic dyes such as propidium iodide (PI). Therefore, by staining cells with a combination of fluorescein-conjugated annexin V and PI, it is possible to detect unaffected, non-apoptotic cells (annexin V negative /PI negative), early apoptotic cells (annexin V positive/PI negative), and late apoptotic or necrotic cells (PI positive) by fluorescence microscope or flow cytometry (Studzinski, 1999). Hence Annexin V conjugated to fluorescein can be used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and is therefore suited for detecting apoptotic cells. The procedure was followed according to the manufacture leaflet. Briefly the cells were incubated with the mixture of 2 µl of Annexin-V-Fluorescein and 2 µl of propidium iodide in 100 µl of Hepes buffer for 15 min in the dark place. Analysis the apoptotic cells was performed under a fluorescence microscope.

F. Non specific esterase staining of cells

Esterases present in white blood cells that are capable to hydrolyzing various aliphatic and aromatic short chain esters are known as nonspecific esterases. These esterases are cell specific provided selective substrate and pH condition are used. For instance, either α-naphthyl acetate or α-naphthyl butyrate is split by esterases (under acidic condition) found only in cell of the monocyte series.

1. Fix smears by submerging slides in ice-cold buffered formalin-acetone fixative (pH6.6) for 30 sec. Rinse slides in distilled water.
2. Let the fixed smears air dry at room temperature for 30 min.
3. Incubate fixed smears in 40.2 ml phosphate buffer/hexazotized pararosaniline/α-naphthyl acetate 45 min at room temperature in the dark place.
4. Rinse slides in distilled water and air dry.
5. Mount smear with a suitable mounting medium and examine stained smear under a light microscope.
6. The cells were counted at least 500 cells to determine the percentage of the nonspecific esterase positive cells and calculated as the following formula.

% Nonspecific esterase positive cells = (The nonspecific esterase positive cells/ Total of counted cells) x 100

G. The viability of monocytes/macrophages

The viable cells in cell suspension were determined based on the principle of dye exclusion. The viable cells which have intact membrane can exclude trypan blue whereas dead cells do not. In this experiment, the cell suspension were mixed with trypan blue solution in the ratio 1:1 and allow to stain for 10 min at room temperature. Transfer a small amount of the suspension to a hemacytometer chamber and count the cell at least 500 cells. Nonviable cells will stain blue.

H. Acid -fast stain

The acid-fast stain was performed by using Kinyoun method. The monolayer of *M. tuberculosis*-infected macrophages adhering on coverglass were fixed with 2% paraformaldehyde for 10 min, air dry and stain with AFB stain (see appendices). After staining and air dry, mount the cells with suitable mounting medium. The number of *M. tuberculosis*-ingested macrophages were counted under a light microscope and percent phagocytosis was calculated.

% Phagocytosis = (The number of ingested macrophages/ Total number of macrophages) x100

I. Statistical analysis

Phagocytosis and apoptosis data were compared using Student's *t* test using SPSS version 2.0 (SPSS) or Microsoft EXCEL. All data were expressed as mean±SE and p value < 0.05 were considered statistically significant.