CHAPTER 4

Materials and methods

4.1 The experimental research plan

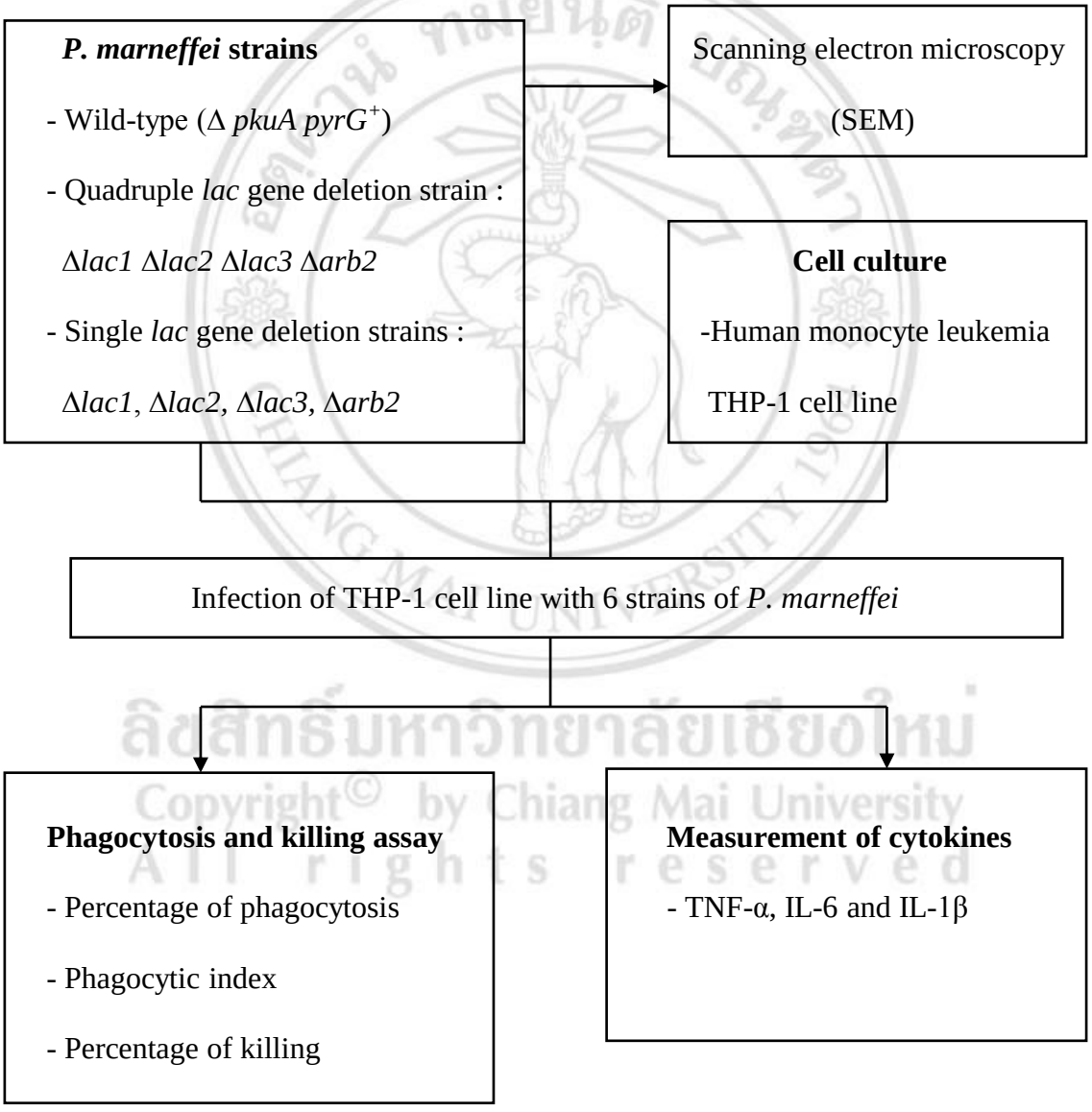

Figure 4.1 Diagram of the experimental research plan.

4.2 Fungal strains and media

Penicillium marneffei wild-type (Δ*pkuA pyrG*$^{+}$), quadruple *lac* gene deletion strain (Δ*lac1* Δ*lac2* Δ*lac3* Δ*lac4*), single *lac* gene deletion strains (Δ*lac1*, Δ*lac2,* Δ*lac3* and Δ*arb2*) were obtained from Dr. Ariya Sapmak (Sapmak *et al*., 2012). The cultures were maintained at 25^{o}C on *Aspergillus nidulans* synthetic medium (ANM). After incubation for 10 to 14 days, the conidia were harvested by suspension in normal saline solution (NSS) containing 0.01% tween 80. The resulting conidia suspension was then filtered through sterile glass wool (Corning, MA, USA), where fungal hyphae were trapped on the surface and conidia were small enough to pass through the glass wool. The eluate containing mycelium-free conidia was centrifuged for 10 minutes at 4,500 rpm and then the pellet was washed twice in phosphate buffered saline (PBS), pH 7.4. The conidial suspension was counted with a hemocytometer; and at concentration 10^{8} conidia/ml was used immediately to infect the macrophage cell line, THP-1 as described in 4.3.

4.3 Macrophage culture and infection with *P. marneffei*

The human monocyte leukemia THP-1 cell line (ATCC TIB-202) was cultured in RPMI 1640 medium, Catalog NO. 30-2001 (GIBCO, Invitrogen Corporation, CA, USA) supplemented with 0.05 mM 2-mercaptoethanol and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO). Cell line was incubated at 37^{o}C in 5% CO_2 at a humid atmosphere. For the induction of cell differentiation, cells ($2x10^{6}$ per ml) were seeded into 24-well culture plates (Costar, Corning Inc., Corning, NY) in 1 ml of RPMI 1640 medium with 10% (v/v) FBS and 100 ng/ml phorbol myristate acetate (PMA) (Sigma, St. Louis, Mo) and placed in 5% CO_2 at 37^{o}C for 72 hours. After incubation, adherence of THP-1 cells were observed under microscopy (usually 80% of cells), and

nonadherent cells were removed by gentle washing with prewarmed (37°C) PBS. The conidia suspended in RPMI 1640 medium with 10% (v/v) FCS, were added to the wells containing a monolayer (about $2x10^6$ cells) of THP-1 to obtain a ratio of 2 conidia to 1 macrophage.

4.4 Phagocytosis assay

The phagocytosis assay was initiated by adding $4x10^6$ conidia to $2x10^6$ macrophage cells in each well (MOI=2). The samples were then incubated in 5% CO_2 at 37°C. THP-1 cells were allowed to ingest conidia at different time points, 15, 30, 60 and 120 minutes. Supernatants were discarded, and the wells were washed gently 3 times with PBS pH 7.2 to remove an excess of conidia. THP-1 were lifted from the wells with 0.25% trypsin-EDTA (GIBCO) for 5 minutes at 37°C and washed twice more to remove trypsin-EDTA, and then fixed by adding 0.5% paraformaldehyde in PBS (PFD; Sigma-Aldrich Gmbh, Steinheim, Germany). The phagocytosis was assessed by light microscopy, the percentage of phagocytosis is the total number of macrophage cells from 100 cell count that can internalize fungal conidia. The phagocytic index was determined by counting the intracellular conidia and calculating for the average number of conidia per macrophage cell as follow:

$$\text{Phagocytic index} = \frac{\text{Total number of intracellular conidia}}{\text{Number of phagocyte cells}}$$

4.5 Killing assay

To measure macrophage killing of conidia by serial dilution, THP-1 cells were allowed to ingest conidia for 2 hours. Medium containing nonadherent, nonphagocytosed conidia was removed, the monolayers were washed with 50 μg/ml of nystatin (Sigma-Aldrich) to kill the extracellular conidia. The monolayers were then washed with warm PBS to remove the nystatin. Macrophages were supplemented with fresh media and incubated for 2, 4 and 8 hours. After 2, 4 and 8 hours post infection, macrophages were lysed with 1% Triton X-100 (USB Corporation, Cleveland, USA) for colony forming unit (CFU) count. Cell lysates were diluted and plated on PDA and incubated at 25-28ºC. The CFU from cell lysates after 2 hours of phagocytosis were considered as the initial inocula and using as the baseline values for intracellular killing analysis. CFU at 2, 4 and 8 hours were used to calculate the killing of fungal cells in macrophages.

The percentage of killing in CFU was calculated as follow:

$$\text{Percentage of killing} = 100 \times \left(1 - \frac{\text{CFU test}}{\text{CFU control}}\right)$$

4.6 Measurement of cytokines

The macrophage cells were infected with conidia at a MOI of 50, and cytokine levels were measured in cell-free supernatants following 2, 8 and 24 hours postinfection. The macrophage cells were centrifuged at 2,000 rpm for 5 minutes to remove any debris, and then the supernatants were harvested. Samples were kept at −70°C until the assay was applied. The human TNF-α, IL-6 and IL-1β levels were

measured by enzyme-linked immunosorbent assay (ELISA), purchased from BioLegend, Inc. (San Diego, CA). Determination of the cytokine levels in a culture supernatants were carried out in triplicate and followed the company's instruction. Briefly, the ELISA were optimized by using 96-well plates (MaxiSorp; Nalge Nunc International Corp., Naperville, IL) coated with 100 µl of capture antibody solution. The plates were then incubated at 4°C for overnight and washed with PBS-0.05% tween 20 for 3 times. Non-specific binding sites were blocked by incubation of the wells for 1 hour at room temperature with 200 µl of 1X Assay diluents A per well. After blocking, plates were washed 3 times with PBS-0.05% tween 20. Then, 100 µl of samples and cytokine standards diluted in 1X Assay diluents A were added into the well. Then, the plates were incubated for 2 hours at room temperature, and plates were washed 3 times with PBS-0.05% tween 20. One hundred µl of biotinylated detection antibody that diluted in 1X Assay diluents A was added to each well. After that, the plates were incubated for 1 hour at room temperature before washing as described earlier. Avidin-HRP diluted in 1X Assay diluents A were added at 100 µl per well. After each of the above experimental steps, plates were washed 5 times with PBS-0.05% tween 20. One hundred µl of tetramethlybenzidine (TMB) substrate solution was added to each well and the reaction was developed by incubating the plate for 15 minutes in the dark. The reaction was stopped by the addition of 100 µl of 2 N H_2SO_4 to each well and the optical density (OD) at 450 nm was determined by reading the plate on ELISA plate reader (BioTek, U.S.A.). Concentrations of cytokines in the experimental samples were calculated according to the optical densities obtained from wells containing standards of cytokine. The measurable concentrations of TNF-α and IL-6 ranged from 7.8 to 500 pg/ml, and IL-1β ranged from 2.0 to 125 pg/ml.

4.7 Scanning electron microscopy

Briefly, for scanning electron microscopy the conidia were fixed overnight in a 2.5% (w/v) glutaraldehyde solution in PBS, then resuspended conidia of *P. marneffei* with 1% Osmium Tetroxide (OsO_4). Fixed conidia were dehydrated through a 20% increment of ethanol concentration from 30% to 90%, followed by subsequent dehydration steps, in absolute ethanol. The specimens were dried in a critical point drying apparatus and sputter-coated with gold and viewed using a LV-scanning electron microscope, JSM 5910 LV.

4.8 Statistical analysis

All data were expressed as mean ± SD of the number of determinations carried out in duplicate for the percentage of phagocytosis, phagocytic index and percentage of killed CFU. Variables were tested for normality and then the different groups were compared using the One-Way ANOVA, where $P < 0.05$ was considered as statistically significant between the groups.

Differences in the cytokine production between wild-type and laccase mutants were analyzed by One-Way ANOVA. The level of significance was set at $P < 0.05$.