CHAPTER III

MATERIALS and METHODS

3.1 *Medium and Reagents*

3.1.1 Culture media

Culture media for all experiments consists of RPMI 1640 supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, 80μg/ml of gentamycin (Gibco Laboratories, Grand Island, NY) and 10% heat-inactivated autologous human serum. Ficoll-Hypaque (Histopaque 1.077) was obtained from Sigma (St Louis, MO). WF10 (Immunokine®) was obtained from OXO Chemie (Bangkok, Thailand).

3.1.2 *Monoclonal antibodies*

Table 2 lists the monoclonal antibodies (mAbs) used in the experiments. All mAbs were purchased from Becton Dickinson (San Jose, CA).

Table 2: Monoclonal antibodies used for flow cytometric analysis

mAbs	Populations
Anti- CD8	T cell subset
Anti- CD4	T cell subset
Anti- γ/δTCR	T cell subset
Anti- CD20	Pan B cells
Anti- CD56	NK cells
Anti- CD69	Activation antigen
Mouse IgG isotype control	

3.2 *Subject selection*

Peripheral blood (50-100ml) were obtained from 10 healthy adult volunteers. These subjects had not taken antibiotics or anti-inflammatory drugs during a period of three months prior to the beginning of the study. None of them had symptoms of infection. Each subject had healthy periodontium or gingivitis with probing pocket depth less than 4 mm.

3.3 *Bacterial preparation*

Porphyromonas gingivalis FDC-381 were used. Briefly, the bacteria were grown at 37°C in the anaerobic chamber (Forma Scientific, USA) in trypticase soy broth. The bacterial purity was checked by Gram's stain and colony morphology on trypticase soy agar plates.The bacteria were harvested by centrifugation (Beckman Instruments, Irvine, CA) at 2060 × g for 15 minutes, washed twice in sterilized phosphate bufferred saline (PBS,0.15M,pH7.2) and subjected to sonication with high intensity ultrasonication (High Intensity Ultrasonic Processor, microprocessor controlled 600-Watt Model, Sonic and Material Inc., Danbury, CT) at 4°C for 20 minute-elapsed time, with pulse on 2.5 seconds and pulse off 2 seconds. Complete breakage of cells as the results of sonication was examined under microscope. Then, the protien concentration of *P. gingivalis* was determined by using the Bio-Rad protein assay. Sonicates extract of *P. gingivalis* were aliquoted and kept at –20°C until use.

3.4 *Peripheral blood mononuclear cell (PBMC) preparation*

PBMC were prepared as previously described by Boyum (1968). Briefly, heparinized peripheral blood was collected from the median

cephalic vein, layered on Ficoll-Hypaque and centrifuged for 30 minutes at 700 × g at 25°C. PBMC were washed twice with PBS and then resuspended in RPMI 1640 medium with 10% heat inactivated autologous serum. Cells were counted in haemocytometer. Viability of isolated cells was examined using Trypan blue (Sigma). The viable cells appeared clear under microscope compared to the blue nonviable cells.

3.5 *Treatment of PBMC with WF 10*

PBMC (3×10^6 cells/ml) were treated with various concentrations of WF 10 (1:900, 1:300 and 1:100 final dilution) at 37°C in a humidified atmosphere of 5% CO_2. To obtain final dilutions of 1:900, 1:300 and 1:100 in PBMC culture, 10 µl of 1:9, 1:3 dilution of WF10 in PBS, and undiluted WF10 were added to 990 µl of PBMC suspension. After 24 h, cells as well as the supernatants were collected for analysis of activation marker (CD69 expression) and cytokine production (IFN-γ, TNF-α, and IL-12), respectively.

3.6 *Treatment with P. gingivalis and WF 10*

PBMC (3×10^6 cells/ml) were co-cultured with different concentrations of *P. gingivalis* sonicated extracts (0,0.001,0.01,0.1,1.0,10 µg/ml) in the presence or absence of WF10 in 24 well tray at 37°C in a humidified atmosphere of 5% CO_2. After 24 h. of culture period, culture supernatants were collected and analyzed for cytokine production (TNF-α and IL-1β).

3.7 *Phenotypic analysis of activated cells by flow cytometry*

PBMC incubated with varying concentrations of WF10 were aliquoted (1×10^5 cells in 100μl) and then stained at 4 °C for 30 minutes with 1 of 5 mAb cocktail combinations:

1). Anti-CD4(PE) + anti-CD69 (FITC)

2). Anti-CD8 (PE) + anti-CD69 (FITC)

3). Anti- γ/δTCR(PE) + anti-CD69 (FITC)

4). Anti- CD20(PE) + anti-CD69 (FITC)

5). Anti- CD56 (PE) + anti-CD69 (FITC)

Mouse isotype control antibodies conjugated with PE or FITC were used in each arm. Stained cells were washed with PBS containing 0.1% albumin and 0.01% sodium azide and then reconstituted in 1% paraformaldehyde. Fifty thousand stained cells were then analysed for expression of CD69 by 2-color flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

3.8 *Detection of cytokines*

Commercial enzyme-linked immunosorbent assays (ELISA) kits (Genzyme Co., Cambridge, MA) were used to measure the production of IL-1β, IL-12, TNF-α, and IFN-γ. The assay was performed according to commercial instructions. The concentration of each cytokine was calculated with a standard curve of corresponding cytokine.

3.9 *Statistic analysis*

The data were analyzed using the computer program SigmaStat for DOS (Jandel Scientific, San Rafael, CA). Results were expressed as means ± standard errors (S.E.). Student's t test was used and p values of 0.05 or less were regarded as significant.