CHAPTER III

MATERIALS AND METHODS

Materials

1. Filarial nematodes

The filarial nematodes, *Acanthocheilonema viteae, Brugia malayi* and *Dirofilaria immitis*, were female adults obtained from TRS Laboratories (Atlanta, GA). The adults of *B. malayi* were recovered from the peritoneal cavity of Mongolian jirds (*Meriones unguiculatus*) infected with the infective stage larvae 4–6 months earlier, while adults of *A. viteae* were recovered from the subcutaneous tissues of multimammate mouse (*Mastomys coucha*) infected 2–4 months prior. *D. immitis* adults were obtained from experimentally infected dogs. Infection was established by subcutaneous injection of the infective stage larvae at least 6 months before collection of adult *D. immitis*. Upon arrival, the female adults were then stored at -70 °C until used. Alive *B. malayi* adult worms were maintained in RPMI media for 1 week before collected for *Wolbachia* isolation.

2. Genotype of *Escherichia coli* strains

2.1 Genotype of one shot® TOP10 for cloning and plasmid propagation

Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

The *E. coli* strain, one shot® TOP10 (Invitrogen, Carlsbad, CA), was used for cloning of blunt-end PCR products into the pET TOPO® vectors. This strain was also

used for plasmid propagation. This *E. coli* strain carries the deletion of *hsd*R that is for efficient transformation of unmethylated DNA from PCR amplifications. The mutation on *mcr*A is for efficient transformation of methylated DNA from genomic preparations. The partially deletion of the *lac*Z gene (*lac*ZΔM15) of this *E. coli* allows α complementation of the β-galactosidase gene required for blue/white selection on X-gal agar plates. The mutated *end*A1 of this strain is for cleaner preparations of DNA, and better results in downstream applications due to the elimination of non-specific digestion by Endonuclease I. For reduced occurrence of non-specific recombination in cloned DNA, the *rec*A1 is mutated.

2.2 Genotype of BL21 star (DE3) for recombinant *Wolbachia* surface protein (rWSP) expression

Genotype: F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm rne131* (DE3)

The *E. coli* strain, BL21 star (DE3) one shot® (Invitrogen), was used as a bacterial host for an induced expression. The DE3 designation means that this strain contains the lambda DE3 lysogen, which carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase. The strain does not contain the *lon* protease. It also has a mutation in the outer membrane protease, OmpT. The lack of these two key proteases reduces degradation of heterologous proteins expressed in this strain. Moreover, the strain carries a mutated *rne* gene (*rne131*) which encodes a truncated RNaseE enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability.

3. Expression vector pET100/D-TOPO® for rWSP

Recombinant *Wolbachia* surface protein (rWSP) was induced to express in *E. coli* under the expression vector pET100/D-TOPO®. The map and diagrammatic features of pET100/D-TOPO® expression vector (Invitrogen) are shown in **Figure 7**. Features of the vector are including T7*lac* promoter for high-level, IPTG-inducible expression of the gene of interest in *E. coli* (Dubendorff and Studier, 1991; Studier *et al.*, 1990), directional TOPO® cloning site for rapid and efficient directional cloning of blunt-end PCR products, N-terminal fusion tags for detection and purification of recombinant fusion proteins, protease recognition site for cleavage of the fusion tag from the recombinant protein of interest, *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter in the pET TOPO® vector, and from the *lacUV5* promoter in the *E. coli* host chromosome, kanamycin and ampicillin antibiotic resistance marker for selection in *E. coli*, and pBR322 origin for low-copy replication and maintenance in *E. coli*.

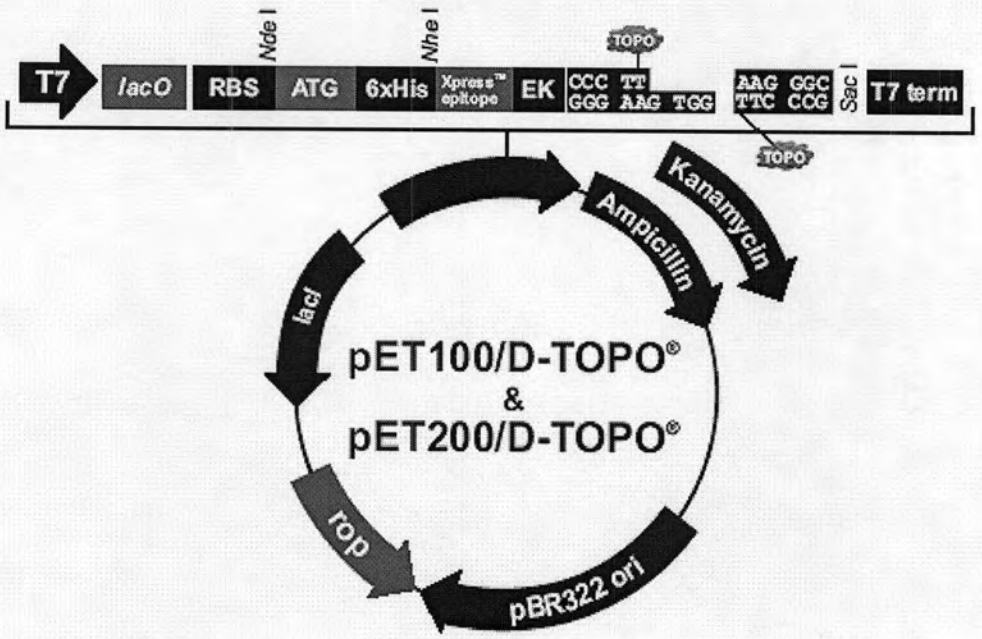

Figure 7 Map of the expression vector pET100/D-TOPO® for cloning and expression of recombinant *Wolbachia* surface protein (rWSP) derived from *Brugia malayi Wolbachia*.

4. Murine macrophage RAW 264.7 cell line

The RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) was obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Organism: *Mus musculus* (mouse)

Strain: BALB/c

5. Synthetic oligonucleotides (or primers)

The oligonucleotides for PCR amplification of *wsp* gene, and of cDNA of murine interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and β-actin (Overbergh *et al.*, 1999) were synthesized, and purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). The details of oligonucleotide sequences and lengths of the PCR products are shown in **Table 4**. The amplification of *wsp* gene was cloned into the pET100/D-TOPO® expression vector. For relative quantification of mRNA of the proinflammatory cytokines by real-time RT-PCR, the mRNA of murine IL-1β, IL-6, TNF-α, and β-actin was transcribed into the cDNA, which was subsequently amplified.

Table 4 PCR primer sequences for *wsp* gene, and murine IL-1β, IL-6, TNF-α, and β-actin genes. The amplification of *wsp* gene was cloned into the pET100/D-TOPO® expression vector. Real-time RT-PCR of murine IL-1β, IL-6, TNF-α, and β-actin genes was for quantification of the proinflammatory cytokine mRNA.

Gene	Primer	Sequence (5'⟶3')	Amplicon length (bp)	PCR amplification on gDNA
wsp	Forward	CAC CAT GGA TCC TGT TGG TCC AAT AGC	658	+
	Reverse	TTA GAA ATT AAA CGC TAT TCC AGC		
IL-1β	Forward	CAA CCA ACA AGT GAT ATT CTC CAT G	152	-
	Reverse	GAT CCA CAC TCT CCA GCT GCA		
IL-6	Forward	GAG GAT ACC ACT CCC AAC AGA CC	141	-
	Reverse	AAG TGC ATC ATC GTT GTT CAT ACA		
TNF-α	Forward	CAT CTT CTC AAA ATT CGA GTG ACA A	175	-
	Reverse	TGG GAG TAG ACA AGG TAC AAC CC		
β-actin	Forward	AGA GGG AAA TCG TGC GTG AC	148	+
	Reverse	CAA TAG TGA TGA CCT GGC CGT		

Methods

1. Developing a *Wolbachia* isolation method from filarial nematodes

The purification procedures for filarial nematode *Wolbachia* were modified from the protocol previously described (Sun *et al.*, 2001). According to the protocol, *Wolbachia* was extracted by using 0.85% NaCl supplemented with 0.001% Nonidet P-40 (NP-40) non-ionic detergent as a homogenization buffer. It has been suggested that higher percentage of NP-40 would increase yield of the bacterial extraction as well as decrease host parasite contamination. Therefore, we adjusted a proper condition for the *Wolbachia* isolation by varying concentrations of NP-40 (Sigma, St. Louis, MO) ranging from 0.005% to 0.04% in the homogenization buffer. Adult females of *D. immitis* were used as a starting material. Protein profiles of the *Wolbachia* extraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting with chicken anti-recombinant *Wolbachia* surface protein (rWSP) IgG antibodies (New England Biolabs, Beverly, MA) and with rabbit anti-*Onchocerca volvulus* antibodies. Intact *Wolbachia* cells were observed under microscopy, and the immunofluorescence with anti-rWSP IgG antibodies (New England Biolabs).

1.1 *Wolbachia* isolation from *Dirofilaria immitis*

Adult females of dog heartworm, *D. immitis*, were cut into small pieces using a sterile razor blade, and then homogenized in 0.85% NaCl supplemented with 0.04% NP-40. Inclusion of this NP-40 concentration into the homogenization buffer was found to provide the best *Wolbachia* yields per milligram of starting parasite materials. After incubation for 15 min on ice, the homogenate was passed through two layers of cheesecloth, and the suspension was centrifuged 350 *g* at 4°C for 40 min to pellet the

nematode nuclei. The supernatant was collected, and the next centrifugation (4,100 *g*) was performed at 4°C for 5 min. After washing with the homogenization buffer in an equal volume of the collected supernatant, the final pellet enriched for *Wolbachia* cells was collected by another centrifugation.

1.2 *Wolbachia* isolation from *Brugia malayi*

Wolbachia cells were isolated from adult females of the lymphatic filarial parasite *B. malayi* following the protocol described above, with modification of the centrifugation force. The homogenization was performed by using the buffer 0.85% NaCl + 0.04% NP-40. After incubation on ice, the suspension was centrifuged 350 *g* at 4°C for 40 min to pellet the nematode nuclei. The nuclei-depleted supernatant was collected, and the next centrifugation (16,000 *g*) was performed at 4°C for 20 min. The *Wolbachia* pellet was re-suspended with the homogenization buffer in an equal volume of the collected supernatant, and the *Wolbachia* cells were collected by the next centrifugation.

1.3 Indirect immunofluorescence of *Wolbachia* by anti-rWSP IgG antibodies

Five microliters of *Wolbachia* cell suspensions were dropped onto clean glass slides, and allowed to air dry. The slide was then fixed with 100% methanol at –20 °C for 5 min. The fixed slide was rinse twice with 1X-phosphate buffered saline (1X-PBS; 138 mM NaCl, 10 mM PO_4, and 2.7 mM KCl, pH 7.4). Fifty microliters of anti-chicken rWSP IgG antibodies (New England Biolabs), a dilution of 1:100, were applied onto the slide. After incubation at 4 °C overnight, the slide was washed in three changes of 1X-PBS/1% Triton X-100 for 5 min each. Subsequently, the slide was incubated with 50 microliters of fluorescein isothiocyanate (FITC)-rabbit anti-

chicken IgG conjugate (Zymed, San Francisco, CA), a dilution of 1:20, at room temperature for 40 min. The slide was washed in three changes of 1X-PBS/1% Triton X-100. After mounting with 100% glycerol, detection of *Wolbachia* cells was performed under a fluorescence microscope.

1.4 Western blot analysis of *Wolbachia* by anti-rWSP IgG antibodies

Proteins of *Wolbachia* extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) were transferred electrophoretically to nitrocellulose or polyvinylidine difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by using transfer buffer (0.05% SDS, 20% methanol), and applying current of 45 mA at 4 °C for 1 h. Each membrane was blocked with 5% non-fat dry milk (Carnation, Wilkes-Barre, PA) in 1X-PBS/0.1% Tween-20 at room temperature for 2 h. After the blocking step, the blot was incubated with anti-rWSP antibodies (New England Biolabs) diluted in the blocking buffer (1:2,000) at 4°C overnight. The blot was then washed in four changes of 1X-PBS/0.1% Tween-20 for 5 min. The secondary antibody, peroxidase-rabbit anti-chicken/turkey IgG conjugate (Zymed) diluted in the blocking buffer (1:2,000) was applied, and followed by incubation at room temperature for 1 h. After washing in four changes of 1X-PBS/0.1% Tween-20 for 10 min each, the blot was incubated with chemiluminescent substrate (Pierce, Rockford, IL) for 5 min, and then exposed to an X-ray film.

1.5 Western blot analysis of *Wolbachia* by anti-*Onchocerca volvulus* antibodies

Each blotted membrane was blocked with 5% non-fat dry milk (Carnation) in 1X-PBS/0.1% Tween-20 at 4 °C overnight. The blot was then incubated with rabbit anti-*O. volvulus* antibodies diluted in the blocking buffer (1:3,000) at room

temperature for 2.5 h. After incubation of the primary antibodies, the blot was washed in four changes of 1X-PBS/0.1% Tween-20 for 5 min. The secondary antibody, peroxidase-goat anti-rabbit IgG conjugate (Pierce) diluted in the blocking buffer (1:20,000) was applied, and followed by incubation at room temperature for 40 min. After washing in four changes of 1X-PBS/0.1% Tween-20 for 10 min each, the blot was incubated with chemiluminescent substrate (Pierce) for 5 min, and then exposed to an X-ray film.

2. Characterization of proteins of *Brugia malayi Wolbachia*

For protein analysis, *Wolbachia* cells were isolated from filarial nematodes using the protocol with optimization, and extracted their proteins. However, our preliminary data showed that there was co-purification of parasite proteins. To determine proteins specific for *Wolbachia* of *B. malayi*, and complexity of the *Wolbachia* extraction, proteins of the *Wolbachia* extracts were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and probed by specific reactivity with anti-*Wolbachia* antibodies (kindly provided by Dr. O'Neill, University of Queensland, QLD, Australia). The antibodies were raised against *Wolbachia* isolated from *Drosophila* spp. We expected that there should be less cross-reactivity of the anti-*Wolbachia* antibodies with any co-purified parasite proteins. However, protein extracts of *Acanthocheilonema viteae* (a related nematode that does not contain *Wolbachia*) were included in all analysis to check cross-reactivity of the anti-*Wolbachia* antibodies to any co-purified parasite proteins in *Wolbachia* preparations. Protein spots specific for *Wolbachia* of *B. malayi* were then identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS).

2.1 Two-dimensional gel electrophoresis

Sample preparation

Proteins were extracted in the lysis buffers; either the extraction buffer II (8M urea, 4% w/v CHAPS, 40 mM Tris, and 0.2% w/v Bio-Lyte 3/10 ampholyte) or III (5M urea, 2M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris, and 0.2% w/v Bio-Lyte 3/10 ampholyte) (Bio-Rad) containing 1% protease inhibitor cocktail (Sigma). The lysate was cleared by intermittent sonic oscillation, and centrifugation. The protein extracts were then estimated for protein concentration by Bradford reagent (Bio-Rad).

The first dimension (Isoelectric focusing)

A prepared protein sample was added tributyl phosphine (TBP) (Bio-Rad) to the final concentration of 1% immediately before isoelectric focusing. The protein sample was then loaded onto an immobilized pH gradient (IPG) strip (Bio-Rad) by applying voltages of 50 V at 20 ^{o}C overnight. Proteins were then focused initially at 250 V for 1 h, and increased to 500 V for 1 h. The voltage was increased to 8,000 V. The final focusing was conducted for 20,000 V·h (for a 7-cm IPG strip), or 60,000 V·h (for an 11-cm IPG strip) at a maximum of 8,000 V using PROTEAN® IEF Cell (Bio-Rad).

The second dimension (SDS-PAGE)

Reduction and alkylation of proteins were performed before the second dimension by first equilibrating the IPG strips in dithiothreitol (DTT) equilibration buffer (6M urea, 2% SDS, 0.05M Tris pH 8.8, 20% glycerol, and 2% DTT) for 10 min, and then in iodoacetamide equilibration buffer (6M urea, 2% SDS, 0.05M Tris pH 8.8, 20% glycerol, and 2.5% iodoacetamide) for 10 min. After the equilibration, the IPG

strips were embedded onto a 10-20% gradient polyacrylamide gel (Bio-Rad), and SDS-PAGE was performed at 150 V for 1.5 h.

2.2 Coomassie blue G-250 staining

The Coomassie blue staining was achieved as described (Neuhoff *et al.*, 1988). A protein gel was fixed with 50% methanol and 10% acetic acid for 10 min, and then washed with distilled water for 5 min. For staining, the gel was covered overnight with the staining solution, 8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie blue G-250 (Bio-Rad), and 20% methanol, and de-stained with Milli-Q water.

2.3 Silver staining

After gel electrophoresis, a protein gel with silver staining was carried out according to the manufacturer's recommendations (Bio-Rad). A protein gel was incubated in the fixative enhancer solution (10% fixative enhancer concentrate, 50% methanol, and 10% acetic acid) for 20 min with gentle agitation. After decanting of the fixative enhancer solution, a protein gel was rinsed in deionized distilled water for 10 min, and the rinse step was repeated. For staining step, a protein gel was incubated in the staining solution (5% silver complex solution containing NH_4NO_3 and $AgNO_3$, 5% reduction moderator solution containing tungstosilicic acid, 5% image development solution containing formaldehyde, and 50% development accelerator solution containing Na_2CO_3).

2.4 Western blot analysis of *Wolbachia* by anti-*Wolbachia* antibodies

A PVDF membrane blotted with proteins was blocked with 5% non-fat dry milk (Carnation) in 1X-tris-buffered saline (1X-TBS; 10 mM Tris, pH 7.4, 137 mM NaCl)/0.1% Tween-20 at 4°C overnight. The blot was then incubated with anti-*Wolbachia* antibodies diluted in the blocking buffer (1:150) at room temperature for 2.5 h. After washing in four changes of 1X-TBS/0.1% Tween-20 for 5 min each, the secondary antibody, peroxidase-goat anti-rabbit IgG conjugate (Pierce) diluted in the blocking buffer (1:20,000), was applied, and followed by incubation at room temperature for 1 h. The blot was then washed in four changes of 1X-TBS/0.1% Tween-20 for 10 min each, and incubated with chemiluminescent substrate (Pierce) at room temperature for 5 min. The exposing to an X-ray film was performed.

2.5 Image analysis

Data were captured using a Fuji LAS-1000 Imaging System CCD camera (Fuji, Tokyo, Japan), and images were annotated and assembled in PDQuest 2-D analysis software (Bio-Rad). The observed pI values were deduced from the pI as received from the supplier, while observed molecular masses were deduced from the molecular weight standards.

2.6 Protein identification by MALDI-TOF-MS and peptide-mass fingerprint searching

In gel digestion for MALDI-TOF-MS (Voyager DE-STR, Applied Biosystems, Foster City, CA, USA) was performed as described (Shevchenko *et al.*, 1996; Gharahdaghi *et al.*, 1999). Protein spots were excised, and rinsed twice with 50% acetonitrile (CH_3CN) for 10 min. The gel pieces were incubated with 50 mM

ammonium bicarbonate (NH_4HCO_3) for 10 min, and then dehydrated with 100% CH_3CN incubation for 10 min. The steps were repeated once. After drying, the gel pieces were incubated in 10 ng/μl trypsin (Promega, Madison, WI), and 50 mM (NH_4HCO_3) for 60 min on ice. The trypsin solution was discarded. The gel pieces were then incubated with 50 mM NH_4HCO_3 at 37°C overnight. The supernatant was saved after brief centrifugation. More tryptic peptides were extracted once with 25 mM NH_4HCO_3, and triple with 50% CH_3CN, and 5% trifluoroacetic acid (TFA). All the saved supernatants were combined, and dried. For mass analysis by MALDI-TOF-MS, the dried peptides were re-dissolved in 50% CH_3CN, and 0.3% TFA, and the peptide solution was subsequently mixed with an equal volume of α-cyano-4-hydroxy-cinnamic acid (CHCA) saturated in 50% CH_3CN, and 0.3% TFA.

PMF searching was performed against NCBI database using the MS-FIT program (http://prospector.ucsf.edu/ucsfhtml/msfit.htm), or against the *Wolbachia* local database using the Mascot program. The internal program contained 1310 translated protein sequences of *Wolbachia* of *B. malayi* (kindly provided by Dr. Slatko, New England Biolabs, Beverly, MA) which is incomplete for the genome sequence. Mass spectra were calibrated using an external calibration. Partial enzymatic cleavages leaving one cleavage site, oxidation of methionine, modification of cysteine by acrylamide, and a mass cut-off of 50 parts per million (ppm) were considered in this search.

Interpretation for protein identification includes concordance between observed MW/pI of an analyzed protein, and theoretical MW/pI of a matched protein within 20%, and concordance between analyzed species, and matched species in the protein databases. In addition, we considered positive protein identification based on criteria, including $\geq$ 4 matched peptides, and 20% sequence coverage. For searching against

Mascot program, evidences for interpretation are concordance between observed MW/pI of an analyzed protein, and theoretical MW/pI of matched protein in the databases with a significant score (Mascot score $\geq$ 40).

2.7 Western blot by anti-phosphorylated protein antibodies

A PVDF membrane blotted with proteins was blocked with 5% BSA, and 0.1% Tween 20 in Tris buffered saline, pH 7.4 (TBS) at 4 °C overnight, and rinsed twice with 0.1% Tween 20 in TBS. Phosphoserine, phosphothreonine, and phosphotyrosine proteins were then detected by incubating the blot with 1 μg/ml rabbit anti-phosphorylated protein antibodies (Zymed, San Francisco, CA) in TBS, 5% BSA and 0.1% Tween-20 at room temperature for 2 h. Then, washing procedure was performed in four changes of TBS, and 0.1% Tween 20 for 5 min. After the washing, the blot was incubated in peroxidase-anti-IgG conjugate diluted 1: 20,000 in TBS, 5% non-fat dry milk, and 0.1% Tween 20 at room temperature for 1 h. The blot was washed in four changes of TBS, and 0.1% Tween 20 for 10 min. Exposing to an X-ray film was performed after incubating the blot with chemiluminescent substrate (SuperSignal, Pierce) for 5 min.

2.8 Dephosphorylation of proteins by calf intestinal alkaline phosphatase

Proteins in crude *B.malayi* extracts were dephosphorylated by incubating with 1 unit/μg protein calf intestinal alkaline phosphatase (CIP) (Roche Applied Science, Mannheim, Germany) in 50 mM Tris-HCl, 0.1 mM EDTA, and a mixture of protease inhibitors at pH 8.5 at 37 °C for 30 min. The reaction was stopped by the addition of a mixture of phosphatase inhibitors to a final concentration of 10 mM NaF, 5 mM sodium vanadate, 10 mM sodium pyrophosphate, and 5 mM sodium phosphate. In control extracts (incubation with no CIP) the inhibitors were added to the extracts before the 37 °C incubation. Both samples were then precipitated for proteins by

trichloroacetic acid (TCA) treatment. Briefly, the samples on ice were added with TCA to a final concentration of 1%, and incubated for 30 min. Centrifugation was performed to collect pellet, which was then sonicated with ice-cold acetone. After centrifugation of the suspension, pellet was air dried on ice. The pellet was solubilized in the sample solubilization solution, and used as a sample for 2D gel electrophoresis. A 2-DE blot was immunostained by anti-WSP antibodies to detect shifting of WSP protein.

3. Characterization of proinflammatory cytokine mRNA responses to rWSP of *Brugia malayi Wolbachia* in murine macrophages RAW 264.7 cell line.

We set up an *in vitro* study to test whether rWSP of *Wolbachia* of *B. malayi* could induce proinflammatory cytokine mRNA expression in the murine macrophage RAW 264.7 cells. The macrophage cell line was derived from a mouse, an animal model that has been widely used for study on immune responses related to the pathogenesis of filariasis (Lawrence, 1996). The role of rWSP in the innate immunity could be studied by examination of proinflammatory responses in the murine macrophage RAW 264.7 cell line, since the macrophage is an important effecter cell of an innate immune system, and can recognize microorganisms resulting in the activation of many immune-response genes, including inflammatory cytokines (Medzhitov and Janeway, 2000). The WSP was selected as a candidate *Wolbachia* protein that could be an inflammatory molecule for the murine macrophage RAW 264.7 cells. WSP of *Wolbachia* from *B. malayi* shares conserved regions with that of *Wolbachia* from other filarial parasites, and with outer membrane proteins of the closely related bacteria. Furthermore, the WSP appeared to be a major protein in our protein analysis. The *wsp* gene was cloned, and induced to express as the rWSP protein.

3.1 Sequence of *wsp* gene in *Wolbachia* of *Brugia malayi*

The DNA sequence and the deduced amino acid sequence of *wsp* was retrieved from all databases available (GenBank, EMBL, SWISS-PROT, PDB, and dbEST) distributed by the National Center for Biotechnology Information (NCBI).

3.2 Analysis of deduced amino acid sequence of *wsp* gene

The deduced amino acid sequence of *wsp* gene was obtained, and characterized its predicted antigenic epitopes using the computer algorithms for the hydrophilicity (Kyte and Doolittle, 1982), surface probability (Emini *et al.*, 1985), and antigenicity (Jameson and Wolf, 1988). In addition, the deduced amino acid sequences of *wsp* gene from various filarial species were aligned using the DNASTAR program (DNASTAR, Inc., Madison, WI).

3.3 Cloning and expression of *wsp* gene

The entire coding sequence of the *wsp* gene excluding the predicted N-terminal signal sequence was directionally cloned from crude *B. malayi* genomic DNA by PCR into pET100/D-TOPO expression vector (Invitrogen). *B. malayi* genomic DNA was isolated from a pool of adult worms through standard phenol-chloroform procedures (Sambrook *et al.*, 1989). The forward primer, 5'-CACC ATG GAT CCT GTT GGT CCA ATA GC-3', had 4 additional bases (underlined) at the 5' end to enable directional cloning into the pET100/D-TOPO vector. The sequence of reverse primer was 5'- TTA GAA ATT AAA CGC TAT TCC AGC-3'. The PCR reaction was performed in 50-μl volumes under the following final conditions: 2X Pfx amplification buffer, 1 mM $MgSO_4$, 0.3 mM of each dNTP, 0.3 μM each of forward and reverse primers, and 1.25 unit of Platinum Pfx DNA polymerase (Invitrogen). The

PCR amplification was performed by 35 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 60 s. The PCR products were purified, and cloned in the pET100/D-TOPO expression vector, and transformed into *E. coli* one-shot TOP10 cells. The plasmids containing inserts were selected by growth on Luria-Bertani (LB) plates containing amplicilin, and sequenced to confirm that the *wsp* gene represented correct orientation.

For expression, the plasmids containing the *wsp* gene were extracted from TOP10 cells, and transformed into *E. coli* BL21 (DE3) one shot cells (Invitrogen). A rWSP fusion protein was induced to express by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Invitrogen) into the cultivated clone at O.D.600 about 0.5-0.8. The cultivated clone was harvested at 1 h after the induction by centrifugation (10,000 *g*) at 4°C for 10 min. The cell pellet was then lysed by sonication. Soluble and insoluble parts were separated by centrifugation (16,000 *g*) at 4°C for 5 min. Expression profiles were analyzed by SDS-PAGE, and the Western blot analysis with anti-rWSP antibodies, and INDIA HisProbe-HRP (Pierce).

3.4 Purification of rWSP fusion protein

After induction with IPTG, the *E. coli* BL21 (DE3) cells containing the expression vector were harvested at 1-h incubation by centrifugation (10,000 *g*) at 4°C for 10 min. The cells were lysed by B-PER bacteria protein extraction reagent (Pierce) supplemented with 1% protease inhibitor cocktails (Sigma). The inclusion bodies were then isolated from the crude cell lysate by centrifugation (10,000 *g*) at 4°C for 15 min. The rWSP fusion protein was firstly purified by treatment of lysozyme and washing with B-PER bacteria protein extraction reagent as manufacturer's instructions.

Affinity-purified rWSP fusion protein was performed by chromatography with Ni-NTA resin (QIAGEN, Valencia, CA) under denaturing condition. The insoluble

protein was solubilized in denaturing binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 1 mM β-mercaptoethanol, 6M Urea, pH 7.9), and incubated on ice for 1 h. Prior to loading onto the column containing Ni-NTA resin, the solution was clarified by filtration through a 0.45-μm nylon membrane (Millipore, Billerica, MA), and the purification column was prepared by washing with 5-column volume of the binding buffer. Then the sample was loaded onto the column, and wash with 10-column volume of the binding buffer. The column was washed with another 10-colume volume of denaturing washing buffer (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 1 mM β-mercaptoethanol, 6M Urea, pH 7.9). The rWSP fusion protein was eluted with denaturing elution buffer (1M imidazole, 500 mM NaCl, 20 mM Tris-HCl, 2 mM β-mercaptoethanol, 6M Urea, pH 7.9). The purified rWSP fusion protein was dialyzed against 20 mM Tris-HCl (pH 8.5) containing 0.1 mM DTT and finally 20 mM Tris-HCl (pH 8.5). Protein concentration of the purified and refolded rWSP fusion protein was measured by a bicinchoninic acid (BCA) protein assay (Pierce).

The rWSP preparation was also determined for any Gram-negative bacterial endotoxin contamination by the *Limulus* amebocyte lysate (LAL) assay according to the manufacturer's instructions (BioWhittaker, Inc., Walkersville, MD). Two-fold dilutions of the purified rWSP stock were prepared. The rWSP test samples were added into the LAL lysate vials. The lysate vials were tilted, and gently swirled until the contents were in solution. The positive control, *E. coli* LPS B026:B6 (Sigma), and negative control, LAL reagent water, were included, and prepared as a test sample. The prepared vials were incubated immediately after reconstitution at 37 °C for 1 h. At the end of the incubation period, each vial was carefully removed, and inverted 180°. A positive test is characterized by the formation of a solid gel which remains intact after inversion. This should be observed in the positive control vial, and in the positive sample control vial. A negative test is characterized by the absence of solid clot after inversion. This should be observed in the negative control vial. The lysate

may show an increase in turbidity or viscosity. This is considered a negative result. To determine the endotoxin concentration of rWSP, the serial two-fold dilutions were tested until an endpoint is reached. The geometric mean dilution as before was calculated, and multiplied by the labeled lysate sensitivity.

3.5 Murine macrophage RAW 264.7 cell culture

The murine macrophage RAW 264.7 cell line (ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air.

3.6 Treatments of murine macrophage RAW 264.7 cells with rWSP

The 10^5 RAW 264.7 cells were placed into a well of 6-well plates, and incubated at 37 $^{\circ}$C overnight. Duplicated wells were used for each treatment. After the incubation, when cells are about 70-80% confluent, cells were used for a treatment. The purified rWSP was added into medium to the final concentrations of 0.1-9.0 μg/ml for the dose-response analysis. Cells were incubated with *E. coli* LPS B026:B6 (0.1 μg/ml) (Sigma). For proteinase-K treatment, 100 μg of the rWSP was treated with 1 mg of proteinase K (Sigma) at 55 $^{\circ}$C overnight, and the proteinase K was inactivated at 95 $^{\circ}$C for 10 min. Immunoblotting with anti-rWSP antibodies was confirmed the completed digestion of rWSP. Boiled rWSP was prepared by heating at 95 $^{\circ}$C for 10 min. Cells were incubated with each treatment at 37 $^{\circ}$C for 3 h, except for the time-course experiment.

3.7 Relative quantification of proinflammatory cytokine mRNA by real-time RT-PCR

The RAW 264.7 cells were extracted for total RNA by using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA (1-5 μg) was used for first-stand cDNA synthesis in a 20-ul-reaction volume under the following condition; 0.5 μg of oligo$(dT)_{12-18}$ (Invitrogen), 0.5 mM dNTP mix, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 40 units RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and 200 units of Superscript II RNase H^- RT (Invitrogen).

PCR primers for IL-1β, TNF-α, IL-6, and β-actin were shown in **Table 4** (Overbergh *et al.*, 1998). A 50-μl PCR reaction containing 1X-SYBR Green PCR master mix (Applied Biosystems), 50 nM of each forward and reverse primer, and 2 μl of a cDNA template. The PCR-reaction mixtures were incubated in the ABI-prism 7700 sequence detector (Applied Biosystems) at 94 °C for 10 min, followed by a total of 40 two-temperature cycles (at 94 °C for 15 s and at 60 °C for 1 min). Dissociation protocol was included in the final step. Each sample was performed in duplicate.

For relative quantification using the standard curve method for each gene, PCR products were purified by the PCR product purification kit (QIAGEN), the concentration of the purified PCR product DNA was measured by OD_{260}, and the number of copies/ml was calculated using the following formula:

$$\text{Copies/ml} = \frac{6.023 \times 10^{23} \times C \times OD_{260}}{MWt}$$

C = 5 x 10^{-5} g/mL for DNA, and MWt = molecular weight of cytokine PCR products (base pairs x 6.58 x 10^{2} g)

Stock solution of the standard was made and diluted 10-fold for making a standard curve.

3.8 Data analysis

For relative quantification by real-time PCR, data were analyzed with the Sequence Detector 7000 software (Applied Biosystems), and exported to Microsoft Excel 6.0 program. Copy numbers of each gene were determined from a standard curve, and normalized by dividing with copy numbers of β-actin gene in each sample. Data were represented as folds changed relative to a negative control.