# CHAPTER V

# CONCLUSION AND DISCUSSION

## Characterization of proteins derived from *Wolbachia* of *Brugia malayi*

*Wolbachia* are of filarial nematode requirements for the normal larval development and reproduction. The significant biologic functions of *Wolbachia* have promoted applied researches to use *Wolbachia* as the target for a novel chemotherapy in filarial disease control (Hoerauf *et al.*, 1999; 2000; Bandi *et al.*, 1999; Taylor *et al.*, 2000b). However, the basic molecular biology of the mutualistic symbiosis between the filarial host and *Wolbachia* is yet to be defined. *Wolbachia* can also trigger the immunopathology of filariasis (Saint Andre *et al.*, 2002; Punkosdy *et al.*, 2003).

Potential drug targets as well as candidate immunomodulatory/ proinflammatory/ antigenic molecules of the bacteria can be selected by analyzing the filarial *Wolbachia* genome (Foster *et al.*, 2005). However, analysis of *Wolbachia* proteome is still needed to focus putative immunomodulatory/ proinflammatory/ antigenic molecule candidates by confirming their *in vivo* expression as well as antigenicity (Petricoin *et al.*, 2002; Nilsson, 2002; Ariel *et al.*, 2003). For discovery of drugs target to *Wolbachia*, the proteome analysis is necessary to identify drug targets for minimizing undesirable side effects in patients, and to prove that interaction with the cellular target is indeed the direct cause for the bacterial cell death (Bandow *et al.*, 2003a; 2003b).

Genomics is the study of an organism's entire genome. The genomics-based approaches have potential to expand understanding of the biology of bacteria, parasites, plants, animals, and human (Schlessinger, 1995; Bloom, 1995; Mushegian

and Koonin, 1996). The power and cost effectiveness of modern genome sequencing technology mean that, with the complete genome sequences of the major bacterial and parasitic pathogens, the sequence of every virulence determinant, every protein antigen, and every drug target are available (Bloom, 1995; Nowak, 1995; McCarthy, 1997; Hood, 1999). In term of proteomics, it is the large-scale study of protein, particularly their structures and functions (Anderson and Anderson, 1998). Proteomics is much more complicated than genomics (Anderson and Anderson, 1998; Haberkorn *et al.*, 2002; Yee *et al.*, 2003). Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell, and is constantly changing through its biochemical interactions with the genome and the environment. One organism has radically different protein expression in different parts of its body, in different stages of its life cycle, and in different environmental conditions. With completion of a rough draft of the human genome, many researchers are now looking at how genes and proteins interact to form other proteins. A surprising finding of the Human Genome Project is that there are far fewer protein-coding genes in the human genome than there are proteins in the human proteome (~20,000 to 25,000 genes vs. ~1,000,000 proteins) (Engel, 1993; Hardy; 2001; Little; 2005; Berg, 2006). The large increase in protein diversity is thought to be due to alternative splicing and post-translational modification of proteins. This discrepancy implies that protein diversity cannot be fully characterized by gene expression analysis alone, making proteomics a useful tool for characterizing cells and tissues of interest (Anderson and Anderson, 1998; Gerstein *et al.*, 2002; Ng and Tan, 2004).

A number of non-gel based proteomic approaches, such as multidimensional protein identification technology (MudPIT), isotope-coded affinity tag (ICAT$^{TM}$), surface-enhanced laser desorption–ionization time-of-flight (SELDI-TOF) MS, and protein arrays (antibody-based chips) are available. Their present advantages over gel-based techniques are the sensitivity, scope of analysis and dynamic range which lead to

increased the capability to analyze highly complex proteomic mixtures such as whole proteomes, organelles and protein complexes (Washburn *et al*, 2001; Wu *et al.*, 2003; Swanson and Washburn, 2005).

Using MudPIT technology, all of the proteins in a sample are digested and loaded onto liquid chromatography (LC) columns. After the peptides are fractionated, they are fed into an MS/MS instrument for protein identification (Washburn *et al*, 2001). This method can identify thousands of protein, and can be used to detect membrane proteins. MudPIT is similar in concept to shotgun sequencing of DNA. Although recent study demonstrated that MudPIT can be used in conjunction with stable-isotope labeling to provide quantitative measurement, the proteomic altogether with high level of MS skill are still required. SELDI-TOF-MS is a proteomic tool that attempts to overcome the requirement for purification and separation of proteins before MS analysis. SELDI-TOF uses a variety of selective chips on which complex biomaterials (e.g., body fluids and cell extracts) can be spotted. This proteomic tool is hindered by the difficulty in identifying proteins of interest (Bischoff and Luider, 2004). Protein arrays have been developed to capture and separate known proteins selectively (Lopez and Pluskal, 2003; Wilson and Nock, 2003). The advantage of this kind of proteomic tool is the high throughput nature of the technology that makes it be applicable to routine testing. However, a high-quality antibody is needed for each protein of interest and each modification of that protein. The generation of antibodies remains a laborious task that is almost as much art as it is science. Finally, it should be remembered that sequence and structure knowledge are needed for individual protein to be analyzed (Biron *et al.*, 2005).

Although the gel-based approach, 2-D gel electrophoresis (2-DE) coupled with mass spectrometry, is a powerful tool, it has limitations in resolving proteins with extreme pI, proteins with hydrophobic characteristics, protein with very high or low

molecular weights, and less abundance (Rabilloud, 2003). In comparative analysis, 2-DE stands out by its remarkable quantitative capabilities (Rabilloud, 2002). Moreover, the separation power of 2-DE is highly suited for analysis of post-translational modifications. The proteomic tool is a well-established method and low cost (Biron *et al.*, 2005). In this study, we preferred to use the 2DE approach as a tool for proteome analysis of *Wolbachia* of *B. malayi*. A 2-DE reference map still represents the best starting point for future interpretation of functional proteomics experiments.

However, the obligatory nature of *Wolbachia* that places limitation on difficulty in obtaining the purified bacteria without host materials has hampered progress in proteomics in the detailed study of their biology. To achieve *Wolbachia* protein materials for analysis, the isolation protocol for filarial nematode *Wolbachia* was optimized. The use of NP-40 at 0.04% was useful to facilitate the proteomic studies. Since *Wolbachia* lack an efflux pump mechanism, the detergent would affect intact metabolism as well as DNA architecture (Foster *et al.*, 2005). Therefore, our purification procedures needed to be validated in other applications which require intact *Wolbachia* DNA materials, metabolism, and viable cells, etc. The modified protocol led to massive enrichment of *Wolbachia* proteins from the total parasite extracts. However, like purification of other strictly intracellular bacteria, there are co-purified host proteins, which are naturally found in cytoplasm of eukaryotic cells (Renesto *et al.*, 2005; Tucker *et al.*, 2005). We detected, for example, paramyosin, heat-shock protein, and beta-tubulin of *D. immitis* during procedures of the *Wolbachia* isolation (**Figure 18** and **Table 5**). The proteins would be closely associated with the bacteria by the fact that some of them function in maintaining the cytoskeleton architecture (Liu *et al.*, 2004; Buey *et al.*, 2006). Hence, they are difficult to trade-off during purification. Nevertheless, *Wolbachia* isolation from filarial nematodes based on an affinity column with antibodies raised against paramyosin, heat-shock protein,

and beta-tubulin of filarial nematodes would be helpful to obtain highly purified filarial nematode *Wolbachia*.

The *Wolbachia* prepared from this isolation method would be an optimal starting material for further extensive purification, such as an antibody-affinity column. Based on the immunorecognition profiles of *Wolbachia* purified from *D. immitis* and *B. malayi* as compared to *A. viteae*, the Western blot analysis by anti-*Wolbachia* antibodies could distinguish host proteins from the *Wolbachia* proteins. Purity of *Wolbachia* isolated from various pioneer protocols could be monitored by the immunoblot analysis. In addition, a purification column based on affinity of antibodies raised against *A. viteae* extracts would help to isolate *Wolbachia* proteins from filarial parasites with higher purity.

The antigenic detection based on 1-D immunoblot analysis indicated that the corresponding *Wolbachia* antigens from hosts, *D. immitis* and *B. malayi*, were observed at MW of 25.6/26 (WSP), 20, and 17 kDa (**Figure 22** and **23**). These proteins could represent genus specific antigens useful in developing a *Wolbachia* detection system. By comparing the protein profiles obtained from SDS-PAGE with the 2-DE profiles, the equivalent antigenic bands of *Wolbachia* derived from *B. malayi* observed were 83-kDa, 48-kDa, 26-kDa (WSP), 20-kDa and 17-kDa (**Figure 26** and **27**). However, the antigens having MW of 48, 26, and 20 kDa consisted of several protein species. In addition, antigenic spots comprising MW of 62, and 58 kDa were additionally revealed by separation of 2-DE. As other applications (e.g., detection of immunorelevant *Borrelia garinii* antigens, characterization of cell surface proteins of spiral-shaped and coccoid forms of *Helicobacter pylori*, and identification of human caspase-3 substrates), the advantage of 2-D immunoblotting is clearly improving the resolution, as compared to immunoblotting based on SDS-PAGE (Jungblut *et al.*, 1999; Nilsson *et al.*, 2000; Lee *et al.*, 2004).

The investigation on proteins expressed by *Wolbachia* of *B. malayi* revealed 27 reactive spots (appearing either as unique or as multiple isoforms) from which 17 spots were defined on the Coomassie blue-stained gels (**Figure 24** and **26**). The missing spots implied low level of expression, and the limited sensitivity of the staining technique. More sensitive staining procedures based on fluorescence could result in a higher number of spots (Sitek *et al.*, 2006; Hoorn *et al.*, 2006). In contrast, some stained spots did not react with the antibodies at all, leading to inability to yield the analysis. The explanations may be that their protein nature is non-antigenic, or that the anti-*Wolbachia* antibodies predominately react with WSP. Therefore, other antibodies generated from *Wolbachia* extracts that are depleted for WSP protein could facilitate more specific antibodies for detection of more *Wolbachia* proteins. Proteins of *Wolbachia* of *Drosophila* have been characterized by metabolic labeling on 2D-gels (Sasaki *et al.*, 1998). The advantages of our approach over such tool are not to use a radioactive material and no need of viable *Wolbachia* host. In contrast, our *Wolbachia* protein analysis was used to detect protein components of *Wolbachia*, and the protein analysis cannot be used to identify actively expressed proteins.

We mapped *Wolbachia* proteins based on the data that proteins of *A. viteae* are conserved in their MW and in recognition of anti-*Wolbachia* antibodies with proteins in *D. immitis* and *B. malayi* extracts. The *Wolbachia* protein defining approach would be reliable. The 6 WSP spots were clearly identified based on this approach. Although it was less likely, we could not exclude the possibility that a number of other spots were of *B. malayi*-specific proteins. Further identification of the defined *Wolbachia* spots is important to confirm the *Wolbachia* protein analysis, and *Wolbachia* protein function study. From the MALDI-TOF-MS data of the *Wolbachia* specific spots that weak mass spectra, and adequate mass spectra with no identification were obtained. To succeed the protein identification, the MALDI-TOF-MS analysis with an optimized in-gel digestion protocol, and in-house peptide mass fingerprint

searching for full protein sequences of *B. malayi Wolbachia*, as well as protein sequencing techniques, such as tandem MS, will be useful.

The *B. malayi Wolbachia* antigens with MW of 62, 58, 48, 26 (WSP), and 20 kDa comprised several protein species (**Figure 24**). In additional to the assignment to an individual protein, the protein species with the same MW derive from a gene, or from the occurrence of protein modifications. As confirmed by MALDI-TOF-MS, two WSP spots that were different in pI values were detected on the 2-DE gels. All efforts were made to minimize urea-induced modifications (carbamylation) by using freshly made urea solution, and to avoid using urea-containing solutions that was warm above room temperature (Zeindl-Eberhart *et al.*, 1994; Lebeau *et al.*, 2005). Apart from artefactual carbamylation, typical chain-like patterns are often attributed to small, charge-modifying modifications such as phosphorylation, and acylation including acetylation, formylation, pyroglutamylation, and myristoylation (Lebeau *et al.*, 2005; Bjellqvist *et al.*, 1994; Hecker *et al.*, 2003). However, complete elucidation of post-translational modifications of WSP was not pursed in this study, due to limitation of the filarial parasite material.

Although, post-translational modifications, such as phosphorylation, glycosylation, acylation, and methylation are often extremely important for the functions of proteins, most of these modifications cannot yet be predicted from genomic or mRNA sequences (Washburn *et al.*, 2003; Gygi *et al.*, 2000). It is now evident that post-translational modifications, such as protein glycosylation, phosphorylation, and methylation are abundant in prokaryotes (Schaffer *et al.*, 2001; Benz and Schmidt 2002; Hecker *et al.*, 2003). Utilizing the proteomic approach, multiple isoforms of the 28-kDa outer membrane proteins (p28-Omp) of *Ehrlichia chaffeensis* observed on the 2-DE proteome blots are identified as the result of glycosylation, and phosphorylation which may involve in the pathogen's adaptation

(Singu *et al.*, 2005). In characterization of the vaccine candidate, the outer membrane protein B (OmpB) of *Rickettsia prowazekii*, based on LC-MS/MS, lysine residues are heavily methylated in OmpB from virulent strains, but hypomethylated in those from attenuated strains (Chao *et al.*, 2004). The methylation on the OmpB of *R. prowazekii* probably defines their virulence.

In addition to the WSP, protein species of 58 kDa appeared as the major antigen (**Figure 24**). The WSP and 58-kDa proteins would play an important role in the pathogenesis of lymphatic filariasis associated with the antibody responses. There are already evidences for supporting the role of WSP in the antibody responses associated with lymphatic filariasis in patients (Punkosdy *et al.*, 2001; 2003). Proteome analysis of *R. conorii* by 2-DE coupled with MALDI-TOF-MS reveals intense silver-stained spots identified as GroEL with MW and pI corresponding with the detected 58-kDa spot (Renesto *et al.*, 2005). The GroEL is also identified as the major antigen recognized by rabbit and the patient sera. The four 58-kDa spots may be associated with GroEL of *Wolbachia*. The MW of 58 kDa, however, is not close to those of the heat-shock protein belonging to filarial nematodes. GroEL is one of the heat-shock proteins that is constitutively expressed, and ubiquitously distributed. It is highly conserved among bacteria (Gupta, 1995). The key role of GroEL is to mediate protein folding within the cell to guarantee normal protein function (Craig *et al.*, 1993).

In conclusion, the modified isolation protocol was applicable to the analysis of proteins expressed by *Wolbachia* of *D. immitis* and *B. malayi*. The immunoblot analysis with anti-*Wolbachia* antibodies could map proteins unique to *Wolbachia* of *B. malayi* on the 2-DE gels, and those spots should be ready for structural and functional assays. The immunoblot analysis should be able to apply on studies of *Wolbachia* antigens among filarial hosts.

**Characterization of proinflammatory activity of WSP to murine macrophage**

It has been thought that *Wolbachia* play an important role in the pathogenesis of lymphatic filariasis, and onchocerciasis with respect to their potential to activate intense innate inflammatory responses (Taylor *et al.*, 2000; Brattig *et al.*, 2001; 2004; Saint Andre *et al.*, 2002), and adaptive immune responses (Punkosdy *et al.*, 2003). Evidences of *Wolbachia*-derived products mediating severe inflammatory responses following DEC treatment in lymphatic filariasis, and onchocerciasis have been postulated (Haarbrink *et al.*, 2000; Cross *et al.*, 2001; Keiser *et al.*, 2002). Highly purified *Wolbachia* are absolutely needed to be used to characterize an underlying molecular mechanism of the immune responses. In addition, *Wolbachia* molecules with proinflammatory activity are needed to be identified, and characterized. In the absence of purified *Wolbachia*, and ideal controls (*B. malayi* strain without *Wolbachia* and *B. malayi* depleted all *Wolbachia*), the rWSP derived from *B. malayi Wolbachia* was used to test proinflammatory activity against cells of the innate immune system, the murine macrophage RAW 264.7 cells.

Several factors are thought to contribute to the pathogenesis of lymphatic filariasis, including the parasite, the immune response, and opportunistic infection (Ottesen, 1987; 1992; Freedman, 1998; Olszewski *et al.*, 1997). Host immunity and inflammatory reactions are thought to be major factors contributing to the progression of lymphatic filariasis (Ottesen, 1992; Freedman, 1998), leading to immunopathology either present as either acute or chronic inflammation. However, the pathogenesis of lymphatic filariasis is largely complex. The filarial nematodes have complex life cycles that involve several discrete developmental stages within the human host. The immune response to these parasites involves multiple effector pathways directed against different parasite stages, each of which occurs simultaneously within the same host (Nutman and Kumaraswami, 2001). The early lymphatic filariasis infection is

associated with establishment of parasite specific delayed-type hypersensitivity responses (Nutman, 1991), parasite-specific lymphocyte proliferatation, cytokine production with a mixed IL-2, interferon (IFN)-γ, IL-4 and IL-5 phenotype (Mahanty *et al.*, 1992; Mahanty *et al.*, 1993; Dimock *et al.*, 1996), marked eosinophilia and elevated levels of parasite-specific immunoglobulin (Ig)E (McCarthy *et al.*, 1994) in the absence of antifilarial IgG4. With the onset of patency (development of adult worms and/or appearance of microfilariae in the blood), certain immune responses are profoundly altered. Most notably, there is a diminution of parasite-specific lymphocyte proliferation, IL-2 and IFN-γ production, an increase in antifilarial IgG4 and the production of the regulatory cytokine IL-10 (King et al., 1993; Maizels *et al.*, 1995; Dimock *et al.*, 1996; Mahanty *et al.*, 1996; Sartono *et al.*, 1997). The development of this downregulated immune response is determined not only by the duration of infection, but also by parasite burden (number of adult worms and microfilariae), both of which can be a function of the intensity of transmission. Notably, most of these patients are asymptomatic microfilaraemia.

A spectrum of clinical manifestations of lymphatic filariasis is associated with a diverse range of inflammatory immune responses. Inflammatory responses also occur following anti-filarial drug treatment particularly in patients with high parasite burdens. Severe adverse reactions are associated with the increase in systemic proinflammatory cytokines and inflammatory mediators (Haarbrink *et al.*, 2000). Raised levels of IL-6 and IL-8 emerged as markers of acute as well as chronic disease, while increased TNF-α was a feature found only in acute filariasis (Satapathy *et al.*, 2006). Although, the inflammatory pathogenesis of lymphatic filariasis is thought to be resulted from both adaptive and innate immune responses, in an animal model, innate immune response plays an important role (Ottesen, 1992; Freedman, 1998). Infection of immunodeficient mice with *Brugia* species results in the development of lymphedema in the absence of T cells and opportunistic infection (Vincent *et al.*,

1984), and is associated with the local production of proinflammatory cytokines including IL-1, IL-6, and TNFα in parasitized lymphatics. The induction and regulation of inflammatory responses has been shown to be under the control of key proinflammatory cytokines including IL-1β and TNF-α (Dinarello *et al.*, 1997). These cytokines are produced predominantly by macrophages and result in a cascade of inflammatory mediators and physiological responses that serve to amplify, and regulate innate immunity (Ulevitch and Tobias, 1999). Together with the development of inflammation after death of filarial parasites, and the possible role of *Wolbachia* derived molecules as major stimuli for innate immune response, the role of the candiate rWSP of *B. malayi Wolbachia* in the induction of proinflammatory responses was investigated.

The dose-response data characterized by mark induction of IL-1β mRNA, followed by IL-6 and TNF-α mRNAs in 3 h suggest that IL-1β mRNA generation were fast, and required lower inoculation (1 μg/ml) of *Wolbachia* than IL-1β and IL-6 transcription (3 μg/ml) (**Figure 39** and **40**). The activity of the rWSP that could strongly induce transcription of IL-1β was in consistent with the characteristics of inflammatory induction by human granulocytic ehrlichiosis (HGE) agent, the closely related bacteria of *Wolbachia* (Kim and Rikihisa, 2000). When compared to LPS stimulation, *D. immitis* rWSP induced a release of similar amounts of IL-6 in human PBMC (Brattig *et al.*, 2004), while the *B. malayi* rWSP induced a generation of comparable levels of TNF-α and IL-1β mRNAs in the murine macrophages (**Figure 40**). The different feature of the immune responses against rWSP could be from the host cells or rWSP of different filarial nematode hosts. However, the potent inflammatory activity appears to require a similar dose (9-10 μg/ml) of rWSP.

The characteristic of the early marked induction of IL-1β mRNA, and peak of induced TNF-α transcripts in 3 h in response to the rWSP was consistent with the fact

that the induction, and regulation of inflammatory responses have been shown to be under the control of key proinflammatory cytokines including IL-1β and TNF-α (**Figure 40**) (Dinarello *et al.*, 1997). The induced transcription of IL-6 appeared to result not only from the rWSP stimulation, but also from IL-1β, and TNF-α cytokines that result from the responses (Dinarello *et al.*, 2006). This expectation was also supported by the time-point analysis, which showed that proinflammatory responses of the macrophage RAW 264.7 cell line to the rWSP activation were characterized by the later peak of transcription of IL-6 in 6 h. This will be important data for selection of drugs in alleviating WSP-mediated severe immune responses. Drugs target on the production and activity of IL-1β and TNF-α, e.g., pyridinyl-imidazole compounds, IL-1 receptor antagonist (IL-1Ra), and neutralizing antibodies to TNF (also called cachectin) are also vulnerable (Dinarello *et al.*, 2006).

IL-1Ra is the naturally occurring inhibitor of IL-1, and is on phase III clinical trail (Fisher *et al.*, 1994a). The initial (phase II) with IL-1Ra administration to patients with septic shock is found to be a dose dependent improvement in 28-day mortality (Fisher *et al.*, 1994b). In addition, there is a dose-related fall in the circulating levels of IL-6 at 24 h after the initiation of IL-Ra infusion. Pyridinyl-imidazole compounds reduce translation of IL-1 and TNF due to their abilities to bind to, and to inactivate two related mitogen activating protein (MAP) kinases (Lee *et al.*, 1994). The cytokine synthesis-inhibiting drugs bind, and inactivate these MAP kinases in cells stimulated with LPS or hyperosmolarity (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994). Anti-TNF antibodies have a dramatic effect in reducing the circulating levels of IL-1 and IL-6 (Fong *et al.*, 1989). However, a large, randomized, placebo-controlled, double-blind study of a murine antihuman TNF-α monoclonal antibody is performed in patients with the "sepsis syndrome" (Abraham *et al.*, 1995). There is no overall benefit in 28-day all-cause mortality in patients receiving the antibody. A clinical trial

with antihuman TNF-α monoclonal antibody will require a far greater amount of time to complete a large pivotal study (Dinarello *et al.*, 2006).

The IL-1β, IL-6, and TNF-α transcription responses to the rWSP were corresponding to the LPS stimulation throughout the analysis period of 24 h. Stimulation by the rWSP probably shares signaling pathways with the LPS activity. Upregulation of IL-1β, IL-6, and TNF-α mRNAs is directly induced by the rWSP, but not by LPS (**Figure 41**). The supported reasons were (i) pretreatment of proteinase-K drastically abrogated the transcription of the proinflammatory cytokines induced by the rWSP, while the same treatment had no effect on the activity of LPS, (ii) the proinflammatory activity of the rWSP was slightly decreased by the polymyxin B treatment (a LPS-neutralizing agent), and the inhibitory effect was independent of the rWSP concentration, (iii) endotoxin concentration, as determined by the *Limulus* assay, was less than 0.1 EU/ml of rWSP preparation and this endotoxin content does not correlate with pro-inflammatory activity on the murine macrophage RAW 264.7 cell-line (Hume, 2001). However, one of the following questions, that arises are the issue of how WSP interacts with receptors on the murine macrophages. An alignment of a number of WSP sequences clearly showed areas that are more conserved than others. Heat inactivation and enzyme digestion data suggested that the recognition site is not conformational. Further characterization of innate recognition based on the conserved sequence, and surface orientation will be novel knowledge of molecular immunology.

Inflammatory activity derived from *Wolbachia* has also been demonstrated in the lymphatic filarial parasites. Soluble extracts of *B. malayi* adults, or microfilariae induce a potent innate inflammatory response *in vitro* and *in vivo* (Taylor *et al.*, 2000a, Saint-Andre *et al.*, 2002, Gillette-Ferguson *et al.*, 2004). The activation of innate inflammation requires CD14, and Toll-like receptor 4 (TLR-4) pattern recognition

receptors. The activity is lost following antibiotic depletion of bacteria and it is absent from soluble extracts derived from aposymbiotic species (*A. viteae* and *L. loa*, Taylor *et al.*, 2000a; 2005). The severe adverse reactions are associated with the increase in systemic proinflammatory cytokines and inflammatory mediators (Haarbrink *et al.*, 2000). PCR and immunoelectron microscopy analysis of plasma samples following the treatment of *B. malayi* with DEC showed the persistent presence of *Wolbachia* in patients with severe systemic inflammation (Cross *et al.*, 2001). In animal models, the production of TNF-α following the chemotherapy of *B. malayi* microfilariae only occurred in mice with an intact TLR-4 receptor, suggesting that the release of *Wolbachia* is responsible for this inflammation (Taylor *et al.*, 2000).

Recently, clinical trials have provided further evidence to support the role of *Wolbachia* in the presentation of adverse reactions. In patients infected with *W. bancrofti*, prior treatment with a 3-week course of doxycycline to deplete *Wolbachia* prevented moderate adverse reactions to albendazole and ivermectin. In individuals in the placebo group levels of *Wolbachia* released into plasma were related to the incidence of adverse reactions, levels of plasma proinflammatory cytokines and pre-treatment microfilarial load (Turner *et al.*, 2006). Therefore, the inflammatory stimulatory activity released by dead worms is derived from endosymbiotic *Wolbachia* bacteria, rather than the nematode.

This finding led to a proposed mechanism by which repeated exposure to *Wolbachia*-mediated inflammation may lead to the damage of the infected lymphatics, and tolerization of innate immunity that lead to susceptibility to the opportunistic infections commonly associated with lymphoedema and elephantiasis (Taylor *et al.*, 2001). Indeed, experimental infections of monkeys with *B. malayi* have been reported, in which antibody responses to WSP develop prior to and throughout episodes of lymphoedema (Punkosdy *et al.*, 2001). Moreover, human antibody responses to WSP

are elevated in individuals with hydrocoele and lymphoedema. This suggested that acquired immune responses to *Wolbachia* may be associated with the development of chronic pathology (Punkosdy *et al.*, 2003). The pilot studies mentioned above in human lymphoedema patients benefiting from doxycycline treatment further underscore these findings, and suggest that chemotherapy to *Wolbachia*, in addition to its anti-parasitic activity, may also have a beneficial effect on pathology.

Genomic sequencing has shown that *Wolbachia* lack the genes required for LPS biosynthesis (Wu *et al.*, 2004; Foster *et al.*, 2005). The search for the molecular nature of the stimulatory activity has therefore focused on other candidate molecules found on the surface membrane of the bacteria. A series of experiments using purified rWSP from *D. immitis Wolbachia* have shown that it can activate innate inflammatory activity. The rWSP of *D. immitis Wolbachia* activates IL-8 transcription, and stimulates chemokinesis in canine neutrophils (Bazzocchi *et al.*, 2003). Further studies showed that the rWSP induces proinflammatory cytokine production from murine macrophages, and dendritic cells and human whole blood cell cultures (Brattig *et al.*, 2004). The stimulation of innate responses depends upon both TLR-2, and TLR-4 in transfected human fibroblastoid cells, and in murine gene knockout and mutant cells. The rWSP of *D. immitis Wolbachia* also stimulated anti-inflammatory IL-10, prostaglandin E2 responses, and IFN-$\gamma$ production from peripheral blood cells together with IgG1 antibody responses from onchocerciasis infected individuals (Brattig *et al.*, 2004). In this study, the rWSP of *B. malayi Wolbachia* is capable of directly inducing proinflammatory responses in the murine macrophage RAW 264.7 cell line, supporting the previous experiments of the *D. immitis* rWSP. Taken together, WSP is a major inducer of the inflammatory activity of *Wolbachia*.

Recent accumulating evidence has shown that innate immunity recognition can be attributed mainly to the Toll-like receptor (TLR) family. TLR signaling can induce

the production of proinflammatory cytokines and upregulated expression of costimulatory molecules, thereby activating not only innate but ultimately also adaptive immunity. To date, ten human and nine murine transmembrane proteins have been shown to belong to the mammalian TLR family (Rock *et al.*, 1998; Takeuchi *et al.*, 1999; Du *et al.*, 2000; Hemmi *et al.*, 2000; Chuang and Ulevitch, 2001). Toll and TLR family proteins are characterized by the presence of extracellular domains with a leucine-rich repeat and intracytoplasmic region called the Toll/Interleukin-1 receptor (IL-1R) homology (TIR) domain, so designated by their similarity to IL-1R and IL-18R.

Pathogens possess several components that are not found in the host which have been referred to as pathogen-specific molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). Upon infection, macrophages recognize these patterns as nonself through the TLRs. TLR signaling can stimulate macrophage activation by inducing production of proinflammatory cytokines and anti-microbial small molecules such as nitric oxide. Then, activated macrophages function to eliminate microorganisms during the early phase of infection. In contrast to macrophages, which mainly function to eradicate pathogens, dendritic cells (DCs, an immune cell expressing TLRs) are important for sensing the invading pathogen and for instructing the adaptive immune system which is thereby recruited to fight against the infection. TLRs activate signal transduction cascades leading to expression of immune response genes following recognition of their respective ligands.

TLR signaling pathways arise from intracytoplasmic TIR domains, which are conserved among all TLRs. Recent accumulating evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-$\beta$ (TRIF), modulate TLR signaling pathways (Takeda and Akira, 2006). MyD88 is essential for the induction of

inflammatory cytokines triggered by all TLRs. TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathway. Thus, TIR domain-containing adaptors provide specificity of TLR signaling.

In conclusion, we have demonstrated that the rWSP of *B. malayi Wolbachia* is capable of directly inducing proinflammatory responses in the murine macrophages RAW 264.7. The WSP may play a role in the initiation of inflammatory responses in human patients.

**Prospective**

The 2-DE reference map specific for *B. malayi Wolbachia* proteins could be useful to evaluate differences in the expressed protein form, and levels within different physiological conditions or parasite stages, thereby serving to elucidate *Wolbachia* protein functions important to filarial nematodes (Porksakorn *et al.*, 2003). The preliminary data of the first proteome of *B. malayi Wolbachia* was reported here, however, to uncover all proteins expressed by *B. malayi Wolbachia*, it is necessary to integrate protein expression data by using many proteomic tools. Protein data, based on the non-gel based proteomic approaches, are important to study the entire proteome of *B. malayi Wolbachia.*

Additional to the elucidation of the *Wolbachia*-filarial nematode relationship, the *B. malayi Wolbachia* antigens would be candidates for further investigation of the role of *Wolbachia* in antibody responses in human filariasis. The human antibody responses to WSP of *B. malayi Wolbachia* are elevated in individuals with hydrocoele and lymphoedema, suggesting that acquired immune responses to *Wolbachia* may be associated with the development of chronic pathology (Punkosdy *et al.*, 2003). The

aspartate aminotransferase (AspAT) of *Wolbachia* from the human pathogenic filarial parasite *Onchocerca volvulus* (Ov-WolAspAT) is an additional *Wolbachia* antigen detected in the patient sera (Fischer *et al.*, 2003). In antibody responses associated with the lymphatic filariasis pathogenesis, limited number of *B. malayi Wolbachia* antigens has been studied. It is interesting to clone and express the defined *Wolbachia* antigens as recombinant proteins, and to analyze specific antibody responses to those candidate *Wolbachia* proteins in patients with lymphatic filariasis. Beside serology testing, the *Wolbachia* antigens should be applied to develop a *Wolbachia* detection system, e.g., a sandwich ELISA or protein array. The *Wolbachia* detection systems will be useful to study the role of *Wolbachia* proteins in antibody and inflammatory responses associated with the pathogenesis of lymphatic filariasis in systematic manner.

This study supported the role of WSP as a major inducer of the proinflammatory activity of *Wolbachia*. The remaining questions are whether WSP is the exclusive inflammatory molecule from *Wolbachia*, or from other filarial species. Whether other *Wolbachia* molecules are with proinflammatory activity is the subject to be studied. The clarification of WSP as the most inflammatory molecule of *Wolbachia* will be a value to alleviate or protect the severe inflammatory responses associated with the pathogenesis of lymphatic filariasis that also include the drug-induced adverse reactions, by targeting the WSP. However, further characterization of the innate immune response to rWSP in details of signaling pathways, and receptor usages is also required to provide a basic molecular mechanism of the immune response. It can be helpful for selection of inhibitors specific for WSP receptors or activated signaling pathways. Several approaches could be appropriate to block the WSP activation, such as protein modeling for analogous WSP with lack of proinflammatory activity, production of anti-peptide antibodies against WSP, and RNA interference (RNAi) induction using small interfering RNA (siRNA)-mediated knockdown of *wsp* gene.