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THE ROLE OF *WOLBACHIA* IN THE INFLAMMATORY AND IMMUNE RESPONSE IN *D. IMMITIS* INFECTED ANIMALS

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INTRODUCTION

From the moment Sironi et al (1995) discovered that *Dirofilaria immitis* harbours *Wolbachia*, the scientific community realized that a major discovery had been made, one that would likely change the way they looked at filarial disease. Indeed, as a gram-negative bacteria, *Wolbachia* have the potential to play an important role in the pathogenesis and immune response to filarial infection. The immunopathology of filarial disease is extremely complex and the clinical manifestations of infection are strongly dependent on the type of immune response elicitted by the parasite. Furthermore, the fact that adult parasites can survive for years in otherwise immunecompetent hosts is likely due to the parasite's ability to avoid/modulate the immune system of the host. It is therefore extremely important to identify which components of the parasite interact with the host's immune system, including *Wolbachia*. This manuscript will outline what is currently known about the interaction between *Wolbachia* and the filarial worm-infected host, including dogs and cats infected with *D. immitis*.

WOLBACHIA-DERIVED MOLECULES AS PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPS)

The role of *Wolbachia* in the host response to filarial infection may include interaction between bacterial molecules and the innate and adaptive immune system. The innate immune system represents a defense mechanism against molecular structures that are conserved among a wide range of organisms. It consists of the recognition of specific "markers" (pathogen-associated molecular patterns, PAMPs) that signal the presence of "generic" pathogens. The consequent recognition of these PAMPs by Toll-like receptors (TLR) on the surface of antigen-presenting cells leads to the production of reactive oxygen species, pro-inflammatory cytokines and to the up-regulation of co-stimulatory molecules that assist in development of an adaptive immune response. Potential PAMP candidates for *Wolbachia* include the *Wolbachia* Surface Protein (WSP) and GroEL.

HOW DOES THE FILARIAL WORM-INFECTED HOST INTERACT WITH WOLBACHIA?

The first reports of a possible role for *Wolbachia* in the immunopathogenesis of filarial infection come from studies on *Onchocerca volvulus* and *Brugia malayi/Wuchereria bancrofti* in humans. *O. volvulus* is a skin-dwelling filarial nematode that causes subcutaneous nodules due to the presence of the adult worms and the release/migration of microfilariae into the surrounding tissue. If adult worms are present on the head, microfilariae may also migrate to the cornea, causing so-called “river blindness”. *B. malayi* and *W. bancrofti* are agents of lymphatic filariasis, where adults reside in lymphatic vessels and microfilariae are released into the bloodstream. In-vivo and in-vitro studies of these parasitic infections have demonstrated that: 1) adverse reactions to filariacidal therapy (ivermectin, DEC) are associated with the presence of *Wolbachia* and/or its DNA in the bloodstream and peak levels of *Wolbachia* correlate with levels of pro-inflammatory cytokines like TNFa; 2) *O. volvulus*-induced skin nodules feature neutrophil infiltration around adults and microfilariae; this inflammation subsequently subsides following antibiotic-mediated removal of *Wolbachia.* Interstingly, a major surface protein of *Wolbachia* from *D. immitis* has been shown to provoke chemiokinesis and IL-8 production in canine neutrophils in vitro; 3) filarial worm extracts stimulate cells in vitro to produce pro-inflammatory cytokines in a TLR-dependent manner and this effect is abolished with antibiotic-mediated removal of *Wolbachia*. Furthermore, this effect is not present with extracts of filarial worms that do not harbour *Wolbachia*; 4) chronic pathology in lymphatic filariasis (elephantiasis, hydrocele) is correlated with a strong specific humoral response to the *Wolbachia* Surface Protein (WSP) (for review see Hise et al, 2004). Most evidence indicates that the filarial-infected host comes into contact with *Wolbachia* following the death of worms (macro-microfilariae through natural attrition, microfilarial turnover and/or pharmacological intervention). However, Kozek et al (2005) have recently hypothesized that living worms may release *Wolbachia* and/or their products, possibly from uterine debris, which promote inflammatory responses adjacent to the worms.

We recently tested the hypothesis that *D. immitis*-infected dogs also come into contact with *Wolbachia* either through microfilarial turnover or natural death of adult worms (Kramer et al, 2005). In our study, intense staining for the *Wolbachia* Surface Protein was observed in various tissues from dogs who had died from natural heartworm disease. Bacteria were observed in the lungs and particularly in organs like the kidney and liver, where microfilariae normally circulate (Figure. 1). Interestingly, immunecomplex glomerulonefritis is a frequent complication of heartworm disease and the localization of WSP in glomeruli is suggestive of a role for *Wolbachia* in renal pathology. It has been reported that infection in dogs with *Ehrlichia canis*, a bacteria closely related to *Wolbachia*, features immune-complex formation that may be responsable for renal lesions. Furthermore, when we looked at specific antibody responses to *Wolbachia*, we observed a stronger humoral response in dogs with circulating microfilariae compared to dogs with occult infection, supporting the hypothesis that microfilarial turnover is an important source of *Wolbachia* in dogs with heartwrom disease. Interestingly, preliminary results of cytokine studies in naturally infected dogs indicate that the presence of both pro-inflammatory mediators (interleukin-2, inducible

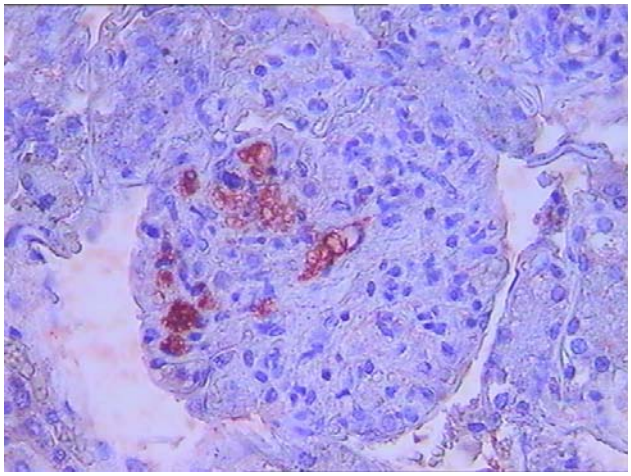

Figure 1. Immunohistochemistry (ABC-HRP method) for the localization of *Wolbachia* Surface Protein in dogs with natural *D. immitis* infection:glomerular positivity for anti-WSP (40X) (from Kramer et al, 2005).

nitric oxide synthetase (iNOS)) and the immunomodulatory cytokine IL-10 are characteristic of patent heartworm disease in dogs. Is it possible that the fine balance of inflammatory pathology/long-term survival of adult worms may in some way be dependent on continuous exposure to *Wolbachia*? Brattig et al (2004) have reported that blood cells from patients with *Onchocerca volvulus*, when incubated in vitro with the *Wolbachia* Surface Protein, produced high levels of IL-10 and the authors suggest that *Wolbachia* may contribute to the down-regulation of pro-inflammatory mediators, thus establishing the necessary hoemostasis for chronic infection. O'Connor et al. (2003) reviewed the role of NO in filarial disease, reporting evidence that several filarial antigens (microfilarial extracts, filarial cystatins) are capable of inducing NO production in vivo and in vitro. The authors suggest that this, in turn, may induce peripheral tollerance through NO-mediated apoptosis of antigen-specific T lymphocytes. They also cite the potential immunoregulatory influence of *Wolbachia* in NO production during filarial infection.

Interaction between *Wolbachia* and the humoral immune system has also been reported by several authors in different hosts infected with different species of filariae (Bazzocchi et al., 2000; Pundosky et al., 2003; Simón et al., 2003; Brattig et al, 2004; Morchon et al, 2004; Kramer et al, 2005). Specific antibody recognition of *Wolbachia* is also a feature of infection with Dirofilaria immitis. Naturally infected cats produce antibodies to that recognize WSP in Western Blot analysis. In a more recent study, the antibody response against specific molecules of *D. immitis* and *Wolbachia* endosymbionts in both naturally and experimentally infected cats with and without larvicidal (ivermectin) treatment, was evaluated. Increased antibody production against filarial antigens and WSP was observed in experimentally infected cats without treatment. However, in experimentally infected cats treated with a larvicidal drug, there was a transient increase in anti-*D. immitis* IgG that decreased dramatically in association with the death of the larvae, while the anti-WSP IgG response increased constantly until the end of the experiment (6 months). The immune response to *Wolbachia* antigens was detected as early as 2 months after infection, before detection of specific antibodies against *D. immitis* antigens. These findings suggest that Wolbachia also plays an important role in the immune response to heartworm infection in cats that may also have diagnostic value. Specific immune responses against WSP have also been studied in dogs with natural heartworm infection. As mentioned above, higher anti-WSP total IgG titres were observed in dogs with circulating microfilariae (mf+) compared to dogs with occult infection (mf-) (Figure 2). There was also a predominance of IgG2 antibodies, indicating a bias towards cell-mediated immunity against *Wolbachia*. Perhaps one of the most interesting results seen so far with infection by *D. immitis* concerns human dirofilariaisis. Simòn et al (2003) have reported spcific humoral recognition of WSP in patients with pomonary nodules due to migration of *D. immitis* and have suggested the use of this antibody response in the differential diagnosis of the disease.

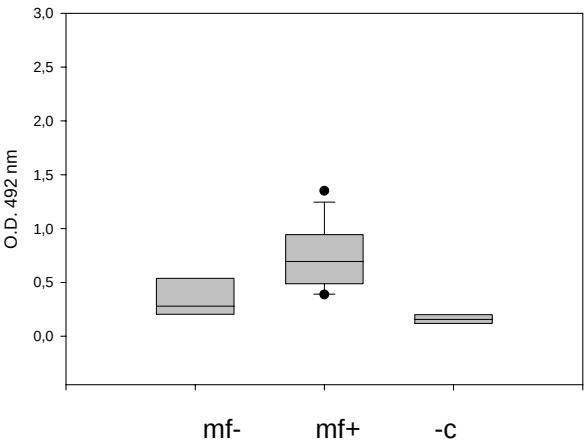

Figure 2. Anti-WSP total IgG antibodies in dogs with natural heartworm infection. mf- = amicrofilariemic; mf+ = microfilariemic; c= healthy controls (from Kramer et al, 2005).

Little data is currently available for the potential proinflammatory/immunomodulatory effect of GroEL. The protein from the *Wolbachia* of *D. immitis* has been produced in recombinant form by C. Bazzocchi at the Univerity of Milan and has been used in preliminary inoculation trials in mice. When inoculated alone, *Wolbachia* GroEl does not appear to stimulate pro-inflammatory responses: however, when inoculated in combination with WSP, there is a stronger innate inflammatory response compared to WSP alone (F. Simòn, pers. com.).

In conclusion, there is increasing evidence that *Wolbachia* participates in the inflammatory and immune response to *D. immitis* infection. Areas of future research should include the possibile diagnostic use of specific immune responses to *Wolabchia*, it's potential immunomodulatory activity (prevention) and the effects of antibiotic treatment in infected animals.

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