In Vitro Determination of Canine Immunological Responses Due to Exposure to Recombinant *Wolbachia* Surface Protein

by

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Abstract

Dirofilaria immitis is the causative agent of heartworm disease in several hosts, including dogs and cats. Like other filarial parasites, *D. immitis* harbors a strain of *Wolbachia pipientis*, an obligate, intracellular, Gram-negative bacterium found in many arthropods and nematodes. During heartworm treatment, *Wolbachia* is released and may play a role in the host inflammatory response. I analyzed the immune response elicited from five heartworm positive dogs and three heartworm negative dogs when exposed to recombinant *Wolbachia* surface protein (rWSP). ELISA results indicated that heartworm positive dogs had developed antibodies specific to rWSP due to previous exposure to *Wolbachia*. When stimulated by rWSP, lymphocytes from heartworm positive dogs produced primarily IFN-γ, IL-4, and IL-12, with trends suggesting that increased concentrations of rWSP induce immunosuppression. Overall, in the present study, cytokine profiles did not indicate rWSP incited heartworm positive dogs to elicit an inflammatory response.

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Chapter I.

Literature Review: *Dirofilaria Immitis* and *Wolbachia*

Wolbachia

Wolbachia is a genus of obligate, intracellular, Gram-negative bacteria found within arthropods and nematodes. This genius belongs to the phylum Proteobacteria, class Alphaproteobacteria, order Rickettsiales, and family Rickettsiaceae (Masui, et al., 2000). *Wolbachia* is closely related to *Ehrlichia canis*, *Ehrlichia equi*, and *Anaplasma marginale*, parasites found within the blood of mammals (Werren, 1997).

Discovered in 1924, *Wolbachia pipientis* was found within the mosquito, *Culex pipiens* (Hertig and Wolbach, 1924). Although all members of the genus *Wolbachia* are classified as *Wolbachia pipientis*, there are eight different clades of *Wolbachia* (A-H) that were categorized based on *Wolbachia* surface protein (*wsp*) (Werren, et al., 2008). *Wolbachia* surface protein is a 23 kDa protein that is assumed to be on the surface of *Wolbachia* (Braig, et al., 1998). Clades C and D are found within nematodes, and the other clades are found in arthropods, whereby clades A and B are most common. Arguments have been made that not all bacteria under the genus *Wolbachia* should be given the scientific name *Wolbachia pipientis*; therefore, the convention is to refer to all strains as *Wolbachia* under specific clades and hosts (Werren, et al., 2008). Currently, ongoing studies are reanalyzing supergroup classification of different strains of *Wolbachia* using five conserved genes with multilocus sequence typing (MLST) because *wsp* has a high degree of recombination (Baldo and Werren, 2007). In addition, genome projects are in

progress for various strains of *Wolbachia*. Genome sequences are complete for a strain of *Wolbachia* within *Drosophilia melanogaster* (wMel) and a strain of *Wolbachia* found in *Brugia malayi* (wBm), belonging to supergroups A and D respectively (Foster, et al., 2005, Wu, et al., 2004). Therefore, one arthropod strain and one nematode strain have been sequenced. Genomes associated with other *Wolbachia* strains are in progress, including the strain associated with *Dirofilaria immitis* (wDim) and the strain associated with *Onchocerca volvulus* (wOv) (Werren, et al., 2008).

The exact distribution of *Wolbachia* is unknown; however, *Wolbachia* is found intracellularly in many arthropods and nematodes. Approximately 25-75% of insects are infected with *Wolbachia* (Kozek and Rao, 2007). In addition to insects, many other arthropods are infected, including some isopods, mites, and spiders (Werren, et al., 2008). As mentioned, nematodes infected with *Wolbachia* include filarial parasites, such as *D. immitis* and *O. volvulus* (Taylor, et al., 2005).

Wolbachia are pleomorphic bacteria, ranging in size from 0.2 μm to 4 μm (Kozek, 2005). *Wolbachia* may have multiple means of reproduction (Kozek, 2005). One method is binary fission, the most common form of reproduction in bacteria. Evidence suggests that *Wolbachia* may have a form of reproduction similar to the reproduction of *Chlamydia*. The process involves the condensation of the cytosol to produce dense inclusions. Smaller bacteria are formed from the dense inclusions within the parent bacterium. Although *Wolbachia* can be found in other locations, *Wolbachia* is often found within oocytes and other reproductive organs of hosts (Werren, et al., 2008). Therefore, *Wolbachia* is transmitted vertically from mother to offspring.

Much focus has been placed on the role of *Wolbachia* in arthropod reproduction. In the 1970s, *Wolbachia* was discovered to cause cytoplasmic incompatibility in *C. pipiens* (Yen and

Barr, 1973). Occurring as either uni-directional or bi-directional, cytoplasmic incompatibility is a reproductive barrier caused by the presence and/or absence of *Wolbachia* (Werren, et al., 2008). In regards to uni-directional cytoplasmic incompatibility, males harboring *Wolbachia* cannot produce offspring with females not infected with *Wolbachia*. However, females with *Wolbachia* can produce offspring with uninfected and strain-specific infected males. Bi-directional cytoplasmic incompatibility relates to the issue that a female arthropod with one strain of *Wolbachia* cannot produce offspring with a male arthropod of the same species if the male is infected with a different, incompatible strain of *Wolbachia*. The exact mechanism of cytoplasmic incompatibility is not understood. Incompatibility may result from male delayed activity of Cdk1, a kinase responsible for regulating the cell cycle, resulting in two pronuclei incapable of undergoing mitosis synchronously (Tram and Sullivan, 2002). In addition to cytoplasmic incompatibility, *Wolbachia* may cause male mortality, parthenogenesis, and feminization (Werren, et al., 2008).

Wolbachia can be microinjected into embryos of *Aedes aegypti*, a mosquito that does not naturally harbor *Wolbachia*, resulting in shorter lifespan (McMeniman, et al., 2009, Moreira, et al., 2009). Because of this, some strains of *Wolbachia* can interfere with the development and transmission of many agents of disease.

One of the underlying issues with *Wolbachia* is the confusion associated with how *Wolbachia* can pass from one species to another species, especially the capability to infect arthropods and nematodes. Through use of embryonic microinjection, *Wolbachia* strains have been transferred from one species to another species (McMeniman, et al., 2008, McMeniman, et al., 2009). Therefore, embryonic microinjection suggests the possibility of natural passage. However, strains of *Wolbachia* are not equally infective to all hosts (McMeniman, et al., 2008).

Wolbachia is capable of surviving outside of a host cell for a week at room temperature (Rasgon, et al., 2006). The ability of *Wolbachia* to survive suggests the possibility of natural transmission. Perhaps a better understanding of phylogenetic passage of *Wolbachia* will be achieved after analyzing new multilocus sequence typing (MLST) data (Baldo and Werren, 2007).

Dirofilaria immitis

Dirofilaria immitis is a filarial parasite that causes heartworm infection in many mammals, notably dogs and cats (Bowman and Atkins, 2009). As a filarial worm, *D. immitis* belongs to the phylum Nematoda, class Secernentea, order Spirurida, and family Onchocercidae (Bandi, et al., 1999). *Dirofilaria immitis* is found globally in tropical and temperate zones, with higher levels of infection in the United States, Japan, Australia, and Italy (Venco and Vezzoni, 2001).

The life cycle of *D. immitis* involves both intermediate and definitive hosts. Many species of mosquitoes can serve as intermediate hosts of *D. immitis*. Definitive hosts of *D. immitis* range from sea lions to ferrets; however, most research is focused on dogs and cats (Geraci and St Aubin, 1987, McCall, 1998). The life cycle of *D. immitis* varies between dogs and cats (Bowman and Atkins, 2009). Regardless of definitive host, the development of *D. immitis* within a female mosquito requires approximately 10 to 14 days after acquisition of a blood meal containing microfilaria. After larvae have developed to the third stage (L3) within the mosquito, the mosquito can infect a new definitive host by transferring L3 during a subsequent blood meal. Within the definitive host, L3 molt twice and mature to adult worms. Once microfilariae are produced in the host, mosquitoes can feed on the blood of the infected definitive host and continue to spread the parasite.

Adult female heartworms range in size from 10 to 14 inches; male heartworms are smaller at 5 to 7 inches (Haddock, 1987). Adult heartworms tend to be found in the pulmonary arteries, sometimes moving into the right ventricle or right atrium of the heart (Venco and Vezzoni, 2001). In caval syndrome, heartworms are found in the caudal vena cava, leading to valvular insufficiency (Strickland, 1998). Ectopic locations for adult worms include the eye, brain, and peripheral vasculature in dogs and cats; however, ectopic locations are more common in cats than dogs (Venco and Vezzoni, 2001).

Clinical signs associated with canine heartworm infection depend on worm burden. Although dogs can experience no signs, signs generally include exercise intolerance, enlarged pulmonary arteries, and decreased blood flow to the lungs (Bowman and Atkins, 2009). In regards to caval syndrome, canine symptoms include heart murmur, sudden onset of lethargy, and hemoglobinuria (Bowman and Atkins, 2009, Strickland, 1998). Feline heartworm burden tends to be limited, with cats experiencing signs when worms appear within the vasculature or at the time of worm death (Bowman and Atkins, 2009, Dillon, 1998). Signs of feline heartworms include chronic cough, emesis, and rarely death. Most infected cats remain asymptomatic. Some infected cats may experience heartworm associated respiratory disease (HARD), a condition that is often misdiagnosed as asthma or bronchitis (Bowman and Atkins, 2009).

Several macrocyclic compounds are available that can be administered to prevent canine and feline heartworm infections. However, preventives are generally only effective through two months post infection, with effectiveness decreasing thereafter (Bowman and Atkins, 2009). Currently, the only FDA approved drug treatment for canine heartworm infection is melarsomine dihydrochloride (Immiticide®, Merial) (Bowman and Atkins, 2009, McCall, et al., 2008). Treatment consists of either two intramuscular injections (2.5 mg/kg given 24 hours apart) or

three intramuscular injections (2.5 mg/kg given 1 month apart with a third injection 24 hours after the second injection). The American Heartworm Society (AHS) recommends use of three injections (McCall, et al., 2008). There is no FDA approved drug treatment for cats (Bowman and Atkins, 2009). Melarsomine dihydrochloride can be toxic to dogs and cats (Bowman and Atkins, 2009). In regards to heartworms in dog, treatment using melarsomine dihydrochloride often leads to pulmonary thrombosis (Kramer, et al., 2008). Although not FDA approved, a slow kill method can be used to treat canine heartworms. The slow kill method involves treating the dog with ivermectin, a macrocyclic lactone, over a period of 36 months (McCall, et al., 2001).

Dirofilaria immitis* and *Wolbachia

Like many filarial nematode species, *D. immitis* is host to *Wolbachia* as well. Current evidence suggests that *Wolbachia* of *D. immitis* (wDim) belongs to the C clade (Bandi, et al., 1998). *Wolbachia* is located within clusters in the lateral hypodermal cords of male and female heartworms (Kramer, et al., 2003). In addition, *Wolbachia* is located in the female oocytes and embryos of *D. immitis* (Kramer, et al., 2003). The location of *Wolbachia* within *D. immitis* follows a similar pattern to *Wolbachia* localization within other filarial worms (Kramer, et al., 2003).

Dirofilaria immitis and *Wolbachia* seem to exhibit an endosymbiotic relationship (Taylor, et al., 2005). All developing stages of *D. immitis* appear to harbor *Wolbachia* (Kozek, 2005). *Wolbachia* seems necessary for *D. immitis* to continue development within a definitive host. Although *Wolbachia* was still detected after use of the antibiotic, tetracycline, the molt of *D. immitis* from L3 to L4 was prevented (Smith and Rajan, 2000). Other filarial worms tend to show an increase in *Wolbachia* from the L3 to L4 molt, with *Wolbachia* loads stabilizing in males but increasing in females through the development of oocytes and embryos (McGarry, et

al., 2004). Adult heartworm exposure to tetracycline correlated with fewer pretzel shaped and stretched microfilarial stages within the uterus (Bandi, et al., 1999). Thus, *Wolbachia* seems to be necessary for the molt from L3 to L4 larval stages and embryogenesis. *Wolbachia* may produce glutathione, a compound that could protect worms from stress and immunological harm, as well as haeme, a substance that hormones may need in regulation of molting (Kramer, 2006).

Because of the potential toxicity of melarsomine dihydrochloride, other treatment options are being investigated. Although ivermectin could be used as a slow kill agent, the time necessary to kill heartworms and owner compliance may be an issue in resolving the infection. Use of doxycycline with or without ivermectin has been investigated for treatment of heartworm infections. Results of one study indicated that 78.3% of intravenously transplanted adult worms were killed after treatment with ivermectin and doxycycline over a course of 36 weeks (McCall, et al., 2008). In the same study, doxycycline alone had an efficacy of 8.7% and ivermectin alone had an efficacy of 20.3% (McCall, et al., 2008). Therefore, doxycycline and ivermectin may prove beneficial as potential adulticides. In another study, experimentally infected dogs were treated with different drugs over a course of 36 weeks to analyze pathologic effects (Kramer, et al., 2008). The group treated with doxycycline, ivermectin, and melarsomine dihydrochloride had fewer pulmonary pathological effects compared to the group treated with melarsomine dihydrochloride alone (Kramer, et al., 2008). Bacterial components of *Wolbachia* are known to initiate host innate immune responses when expelled from a dead filarial worm (Saint André, et al., 2002). Thus, elimination of *Wolbachia* may reduce pathologic changes due to death of *D. immitis*.

In addition to the pathological problems associated with *Wolbachia* during the treatment and death of *D. immitis*, *Wolbachia* may have a role in the immunological response of a host

infected with *D. immitis*. Both antigens from *D. immitis* and antigens from *Wolbachia* have been shown to stimulate an immune response in dogs, cats, and humans (Simón, et al., 2007). Because *Wolbachia* may not contain lipopolysaccharide (LPS), *Wolbachia* must stimulate inflammatory reactions in ways other than via LPS (Wu, et al., 2004, Taylor, et al., 2001). To determine the effects of *Wolbachia*, recombinant *Wolbachia* surface protein (rWSP) has been developed due to the homologous relationship WSP shares with primary antigenic proteins, such as a 30 kDa outer membrane protein of *Ehrlichia canis* (Bazzocchi, et al., 2000a, Ohashi, et al., 1998). In cats, IgG has been detected against rWSP and antigens derived from *D. immitis* separately (Bazzocchi, et al., 2000a, Morchón, et al., 2004). In one study, death of feline heartworm larval stages led to an increase in IgG specific to rWSP, whereas IgG specific to antigens of *D. immitis* decreased (Morchón, et al., 2004). Such a strong IgG response may indicate that *Wolbachia* plays a role in the inflammatory reaction during feline heartworm disease (Morchón, et al., 2004).

Heartworm positive dogs produce IgG2 against rWSP in occult and microfilaria positive infections; however, only microfilaria positive dogs produced IgG1 against rWSP (Kramer, et al., 2005). Therefore, microfilaria status is important in the development of immune response. For dogs, a predominant IgG2 response relates to a higher T-helper 1 (Th1) response, whereas a predominant IgG1 response correlates with a higher T-helper 2 (Th2) response (Simón, et al., 2007). Th1 responses correlate with inflammatory reactions, whereas Th2 responses relate to neutralizing or anti-inflammatory reactions. According to one study using murine models, immunostimulation using antigens of *D. immitis* resulted in a Th2 response, whereby antigens of *Wolbachia* induced a Th1 response, implying that *Wolbachia* may be a more important stimulator of inflammatory reactions than *D. immitis* (Marcos-Atxutegi, et al., 2003). In

addition, rWSP stimulates the production of IL-8 in canine neutrophils (Bazzocchi, et al., 2003). Other research findings indicate that TNF-α, iNOs, IL-2, IL-4, and IL-5 are present in microfilaria positive and microfilaria negative infections (Simón, et al., 2007). Production of IL-10 is expressed more in mircrofilaremic infections than in occult infections (Simón, et al., 2007). However, the studies did not address the specific role of *Wolbachia* in causing the stimulation of the cytokines. More research is necessary to properly characterize the Th1/Th2 responses associated with *Wolbachia* involved with hosts infected with *D. immitis* (Kramer, 2006).

Research Objectives

Currently, there are few published in vitro immunological studies that address the immune response and pathologic changes caused by *D. immitis* (Simón, et al., 2007). In addition, the Th1/Th2 responses to *D. immitis* and *Wolbachia* have not been well characterized (Kramer, et al, 2005). Often, determination of an immunological response as either Th1 or Th2 has been based upon production of IgG1 and IgG2 to specific antigens. However, Th1 and Th2 responses are classified by the production of particular cytokines.

Because W*olbachia* strains from filarial worms have not been cultured, research has usually focused on rWSP as the sole antigen from *Wolbachia* (Werren, et al., 2008, Simón, et al., 2007). Unfortunately, rWSP may not reflect the complete stimulation *Wolbachia* has on the immune system of a host infected with *D. immitis*; however, WSP is probably the main antigenic protein of Wolbachia as WSP shares similarities to the main antigenic protein of *Ehrlichia canis* (Bazzocchi, et al., 2000a, Ohashi, et al., 1998). When heartworms die, the heartworm infected animal is likely exposed to *Wolbachia* antigens (Saint André, et al., 2002, Taylor, et al., 2001). Because inflammatory reactions occur following the death of *D. immitis* and the release of

Wolbachia, *Wolbachia* may be responsible for a significant portion of the host pathologic response to heartworm death (Taylor, et al., 2001).

Therefore, the objectives of this study are to determine heartworm infection status of dogs. In addition, prior canine exposure to *Wolbachia* will be determined using PCR detecting *wsp* gene. Secondly, an ELISA will be done using canine plasma and rWSP to determine antibody response. Lastly, reverse transcription polymerase chain reaction (RT-PCR) will determine if canine lymphocytes when exposed to rWSP produce various pro-inflammatory and anti-inflammatory cytokines. I hypothesize that heartworm positive dogs will produce a detectable immunological response to rWSP, producing pro-inflammatory cytokines.

Chapter II.

Characterization of Canine Heartworm Infection Status

Introduction

Wolbachia is a Gram-negative bacterial endosymbiont of several arthropod and nematode species, including *Dirofilaria immitis* (Kozek, 2005, Masui, et al., 2000). *Wolbachia* is found in all developmental stages of *D. immitis* (Kozek, 2005, Taylor, et al., 2005). Currently, the exact nature of the endosymbiotic relationship is unknown. *Dirofilaria immitis* may rely on *Wolbachia* for the production of glutathione, a compound that could protect the parasite (Kramer, 2006). *Wolbachia* may also produce haeme, a substance that could help in the regulation of molting (Kramer, 2006).

The life cycle of *D. immitis* is complicated, involving the use of an intermediate host and a definitive host (Bowman and Atkins, 2009). Intermediate hosts include several species of mosquitoes, whereas definitive hosts include dogs, cats, and other small mammals. Once a dog has been infected with L3 larvae through the bite of a female mosquito, approximately 5-7 months pass before the L3 larva develops into an adult. As an adult, the heartworm typically occupies the pulmonary arteries, and sometimes migrating into the right atrium and right ventricle (Venco and Vezzoni, 2001). Adult worms will reproduce sexually and produce microfilariae that pass into the blood stream. A mosquito picks up the microfilaria during a blood meal, whereby the microfilaria develops into the L3 larva stage.

In vitro research with *Wolbachia* is difficult to conduct because of the necessity of isolating and maintaining stock cultures. *Wolbachia* has been cultured from a mosquito cell line derived from the embryos of *Aedes albopictus*, a naturally infected host of *Wolbachia* (O'Neill, et al., 1997). However, to date, no cultures of *Wolbachia* from nematodes have been established.

Because there are no cultures of nematode-derived *Wolbachia*, recombinant *Wolbachia* surface protein was developed (Bazzocchi, et al., 2000a). Some work has been done characterizing the immune response canines produce when exposed to recombinant *Wolbachia* surface protein (rWSP). Much of the work has centered on use of ELISAs to establish production of immunoglobulin G (IgG) (Bazzocchi, et al., 2000a, Kramer, et al., 2005, Morchón, et al., 2007a, Morchón, et al., 2004). Heartworm positive dogs produce more IgG than heartworm negative dogs (Kramer, et al., 2005). Microfilaremic dogs can produce IgG1 and IgG2 when stimulated with rWSP; however, amicrofilaremic dogs only produce IgG2 (Kramer, et al., 2005). Therefore, the presence of heartworms and microfilaria affect immune response to *Wolbachia*. The objective component of my research is to characterize heartworm infection status in dogs and determine if dogs have been exposed to *Wolbachia*.

Materials and Methods

Determination of Canine Heartworm Infection Status

Three groups of dogs were analyzed. All dogs were female beagles, gang-housed in an indoor facility at Auburn University College of Veterinary Medicine. The first group consisted of four experimentally infected heartworm positive beagles that were microfilaremic. Group two consisted of one beagle that was experimentally infected with heartworms. The beagle was heartworm antigen positive but amicrofilaremic, due to the prepatency of the infection. Group three consisted of three beagles that were heartworm antigen negative.

In order to determine heartworm status, dogs were screened using several tests. A SNAP semi quantitative canine heartworm antigen test (Idexx, Westbrook, ME) was performed on each dog according to manufacturer's instructions. Secondly, a modified Knott's test (MKT) assessed presence and numbers of microfilaria in heartworm antigen positive dogs. Briefly, a MKT consists of mixing 1 ml canine whole blood with 2% formalin in a test tube. Upon spinning the contents at 1600 rpm in a table top centrifuge, contents of the tube were removed, and the test tube was inverted for 15 min. Approximately 1 drop of methylene blue was added. The entire content of the tube were analyzed under low power field for microfilaria. In addition, a complete blood count (CBC) was conducted to confirm to the extent possible the health of the animals. All CBCs were performed by the Clinical Pathology Laboratory at Auburn University College of Veterinary Medicine.

Detection of *Wolbachia* within Microfilaria and Canine Blood

To determine if *Wolbachia* was present within microfilaria and canine blood, polymerase chain reaction (PCR) was performed using primers (Forward: 5'-TAGCTACTACATTCGCTTGCA-3': Reverse: 5'- CCAACAGTGCCATAAAGAAC-3') to detect a 619 bp amplicon specific to *Wolbachia* surface protein (*wsp*) gene (Bazzocchi, et al., 2000b, Lee, et al., 2008). Approximately 4 ml of peripheral blood was collected into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) via cephalic venipuncture from four heartworm positive, microfilaremic dogs and one heartworm negative dog. Methods for purification of microfilaria were derived from Grieve, et al. (1984). Blood was placed in 45 ml 0.2% saponin in 0.85% NaCl (1:11 dilution of blood to saponin), inverted, and incubated at 37°C for 15 min to lyse red blood cells. Incubation was followed by centrifugation at 850 x g for 5 min. Supernatant was decanted into a container containing 6% sodium hypochlorite. To clean

the pellet, 15 ml of .01 M phosphate buffered saline (PBS) warmed to approximately 37°C was added. After mixing the pellet with PBS, the sample was centrifuged at 850 x g for 5 min. Following centrifugation, the supernatant was decanted. The pellet was washed in 1 ml minimum essential medium (MEM) plus (5% fetal calf serum and penicillin-streptomycin).

DNA was extracted from the pellet using proteinase k and ethanol precipitation. PCR reaction tubes contained approximately 0.1-0.5 µg of DNA, 0.5 mM of each primer, 500 mM dNTPs, 1 x PCR buffer, 1.5 mM $MgCl_2$, and .5 µl Taq polymerase (Applied Biosystems, Foster City, CA) (Lee, et al., 2008). PCR involved 35 cycles of 94°C for 30 s, 58°c for 1 min, and 72°C for one min. Samples were loaded into a 1% agarose gel (1 g agarose/100 ml TBE) at 75 V for approximately 1 hr 5 min. Relevant bands were detected after staining with ethidium bromide.

Results

Eight female beagles were used during the study. Beagles were cared for by Lab Animal Health at Auburn University College of Veterinary Medicine. Seven of the beagles were cared for through funding provided to Dr. Byron L. Blagburn. One beagle was cared for through funding provided to Dr. Bruce Smith. All dogs were gang-housed in indoor facilities. According to complete blood counts (CBCs) performed by Clinical Pathology at the Auburn University College of Veterinary Medicine, all beagles were healthy. However, heartworm positive beagles had elevated numbers of eosinophils. Elevated eosinophils are typical of a heartworm infection (Bowman and Atkins, 2009)

All heartworm positive dogs were previously infected with L3 larvae. Of the eight beagles, five beagles were experimentally infected with *Dirofilaria immitis*. Four of the five heartworm positive beagles were high antigen positive and microfilaremic according to SNAP semi-quantitative heartworm antigen test (Idexx, Westbrook, ME) and Modified Knott's Test

(MKT) results (Table 2.1). Microfilaria numbers ranged from approximately 6000 to 33,000 microfilaria/ml. The fifth heartworm positive beagle was low antigen positive and amicrofilaremic at the time of the blood collection used for the study (Table 2.1). Three beagles were antigen negative (Table 2.1). A MKT was not performed on heartworm negative beagles at time of blood collection as they were assumed amicrofilaremic.

Results from PCR indicated presence of *Wolbachia* through detection of a 619 bp amplicon of *Wolbachia* surface protein (*wsp*) gene within microfilaria and blood cells prepared from four heartworm positive, microfilaremic dogs (Figure 2.1). Failure to amplify *wsp* when using varying amounts of DNA from the heartworm negative dog indicated that *Wolbachia* was not detected in blood cells collected following similar procedure used to isolate microfilaria.

Table 2.1. Overview of heartworm and microfilaremic status of research dogs.

Dog	SNAP Semi-Quantitative HW Antigen Test	Presence of Microfilaria
1	High Ag Positive	Yes
2	High Ag Positive	Yes
3	High Ag Positive	Yes
4	High Ag Positive	Yes
5	Low Ag Positive	No
6	Negative	No
7	Negative	No*
8	Negative	No*

*MKT was not performed on dog7 and 8 because they were heartworm antigen negative and were assumed not to harbor microfilaria.

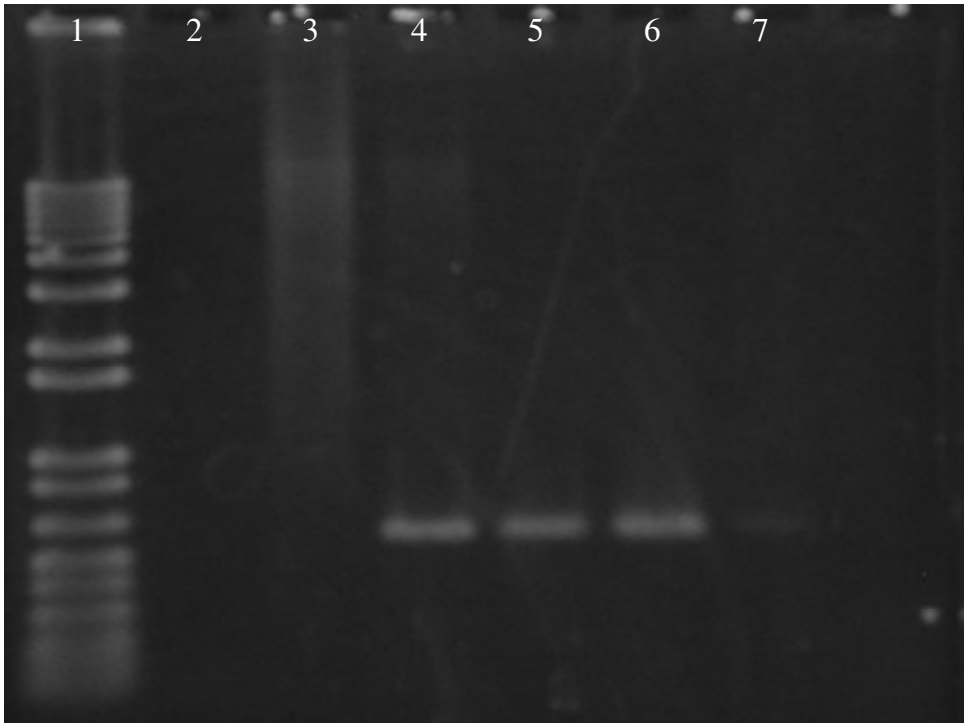

Figure 2.1. PCR amplicon products detecting *wsp* from microfilaria and blood cells pelleted together. The amplicon of *wsp* was 619 bp. PCR products were separated on a 1% agarose gel that was stained with ethidium bromide. Lane 1, 1-kb molecular weight marker; lane 2, dog 6 (HW-) (0.1 µg DNA); lane 3, dog 6 (HW-) (0.5 µg DNA); lane 4, dog 1 (HW+, mf+) (0.5 µg DNA); lane 5, dog 2 (HW+, mf+) (0.5 µg DNA); lane 6, dog 3 (HW+, mf+) (0.5 µg DNA); lane 7, dog 4 (HW+, mf+) (0.1 µg DNA); lane 8, negative control. Abbreviations: HW+, heartworm positive; HW-, heartworm negative; mf+, microfilaremic.

Discussion

Three groups were established based on heartworm infection and presence of microfilaria (Table 2.1). These groups of dogs may experience different immunological responses to *Wolbachia*. The first group of dogs included four heartworm positive, microfilaremic dogs. The second group included dog 5, a heartworm positive, amicrofilaremic dog. Three heartworm negative dogs made up the third group.

All stages of *Dirofilaria immitis* (from microfilaria to adult) contain *Wolbachia* (Kozek, 2005). Therefore, heartworm infected dogs are likely to have been exposed to *Wolbachia*. The turnover rate of microfilaria is a possible mechanism behind canine exposure to *Wolbachia*, as *Wolbachia* is released from dying worms (Keiser, et al., 2002). However, microfilaria can survive for up to 2.5 years (Bowman and Atkins, 2009).

Heartworm negative animals may not have been exposed to *Wolbachia*. If a heartworm negative animal is infected with L3 larvae of *D. immitis*, the L3 are a source of *Wolbachia* (Kozek, 2005). However, the three heartworm negative dogs were kept in indoor facilities away from mosquitoes.

Dog 5's infection period was a contributing factor to its amicrofilaremic status. Dog 5 had been experimentally infected less than six months previous to this study. Therefore, worms from dog 5 were not sexually mature. Presence of microfilaria typically occurs between six and eight months post-infection (Bowman and Atkins, 2009). Other causes for amicrofilaremia include single sex infection and host immunological attack of microfilaria (Kramer, et al., 2005). This dog's data was separated from the other heartworm positive dogs because previous research has shown that lack of microfilaria produces a different immunological response than infected dogs with microfilaria (Kramer, et al., 2005). Kramer, et al. (2005) showed that naturally

infected, amicrofilaremic dogs do not produce IgG1 at as high levels as naturally microfilaremic dogs. Other studies have indicated that microfilaria may induce immunosuppression (Brattig, et al., 2002, Morchón, et al., 2007b, Simón, et al., 2007).

PCR results indicated that heartworm positive, microfilaremic dogs contained *Wolbachia* (Figure 2.1). Failure to detect DNA from *Wolbachia* surface protein (*wsp*) within blood cells of the heartworm negative dog gives some claim that the dog has not been exposed to *Wolbachia*. As all stages of *D. immitis* contain *Wolbachia*, evidence of *Wolbachia* within microfilaria and blood cells from heartworm positive, microfilaremic dogs is not unexpected (Kozek, 2005, Taylor, et al., 2005). Perhaps a better indicator of whether dogs have been exposed to *Wolbachia* would be analyzing tissue with anti-WSP polyclonal antibody (Kramer, et al., 2003, Kramer, et al., 2005). Previoius work showed WSP within lungs, livers, and kidneys of heartworm positive dogs, but WSP was not detected in the tissues of heartworm negative dogs (Kramer, et al., 2005). However, analysis of tissue would require sacrifice of animals.

Chapter III.

Determination of Canine Humoral Response to Recombinant *Wolbachia* Surface Protein

Introduction

Wolbachia is a Gram-negative bacterial endosymbiont of *Dirofilaria immitis*, the causative agent of heartworm disease in several animal species (Kozek, 2005, Masui, et al., 2000, Taylor, et al., 2005). Heartworm disease is of significant importance in canines, especially in endemic areas like the Southeastern United States.

Currently, the only approved FDA drug for treatment of canine heartworms is melarsomine dihydrochloride (Immiticide®, Merial), an arsenic-based compound (Bowman and Atkins, 2009, McCall, et al., 2008). Treatment involves two or three intramuscular injections using 2.5 mg/kg (Bowman and Atkins, 2009, McCall, et al., 2008). The two injection option involves injections 24 hours apart. However, the three injection option involves one treatment followed by a second treatment a month later, with the third shot given 24 hours later. Treatment using melarsomine dihydrochloride is dangerous, often leading to pulmonary thrombosis in dogs (Kramer, et al., 2008).

Evidence suggests that dead adult heartworms and larval stages release *Wolbachia* (Keiser, et al., 2002). Some heartworm infected dogs have been shown to have *Wolbachia* specific proteins within the glomerulus of the kidney and the kidney tubules (Kramer, et al., 2005). In addition, a demonstrable inflammatory response may occur in the lungs following the death of adult heartworms (Saint André, et al., 2002). Research has been done to determine

benefits of removing *Wolbachia* during drug treatment of filarial parasites. For example, use of doxycycline, an antibiotic against *Wolbachia* and other bacteria, has been shown to reduce the severity of pathologic changes in canine lungs when used with ivermectin and melarsomine dihydrochloride (Kramer, et al., 2008).

Studies have been conducted using recombinant *Wolbachia* surface protein (rWSP) since there is no established nematode-derived culture of *Wolbachia* (Bazzocchi, et al., 2000a, Kramer, et al., 2005, Morchón, et al., 2007a, Morchón, et al., 2004). Many publications have used ELISAs, with canine serum exposed to rWSP to determine immunoglobulin G (IgG) production (Kramer, et al., 2005). According to Kramer, et al. (2005), heartworm infection and presence of microfilaria affected production of IgG. Microfilaremic infected dogs produced more total IgG than amicrofilaremic dogs (Kramer, et al., 2005). Both microfilaremic and amicrofilaremic dogs produced IgG1 specific to rWSP; however, only microfilaremic dogs produced rWSP specific IgG2 (Kramer, et al., 2005). For this study, the objectives were to determine canine antibody response to rWSP based on heartworm infection status. I hypothesized that heartworm positive dogs would have IgG specific to rWSP.

Materials and Methods

ELISA

An ELISA was performed using canine plasma and recombinant *Wolbachia* surface protein (rWSP) following methods previously published (Kramer, et al., 2005, Morchón, et al., 2004). Recombinant WSP was gifted by Dr. Claudio Genchi, from the University of Milano, Italy. An overview of the technique involved coating each well of a 96-well plate with 50 µl of 1.0 µg/ml rWSP overnight at 4°C. The following day, the contents of the wells were emptied and washed with superblock blocking buffer (ThermoScientific, Rockford, IL) three times.

In order to obtain plasma, canine whole blood was collected via cephalic vein into ethylenediaminetetraacetic acid (EDTA) tubes and spun at 1600 rpm for five min. Once separated, canine plasma was diluted to 1:20 in PBS-Tween-20 as per Kramer, et al. (2005). At that dilution, background absorbance values were high. Therefore, plasma was diluted to 1:50 in PBS-Tween 20. Wells received 50 µl of diluted plasma. Following addition of plasma, the plate was sealed and incubated at 37°C for 1 h.

After incubation, the plate was emptied and washed three times with PBS-Tween-20. Anti-dog IgG horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) was diluted 1:5000 in PBS-Tween 20. Each well received 50 µl of diluted anti-dog HRP, and the plate was sealed and incubated at 37°C for 1 h. Following incubation, the plate was washed three times in PBS-Tween-20 and 50µl of Fast o-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO) (1 tablet OPD + 20 ml dd H_2O) substrate was added to each well. The plate was allowed to sit for 15 min at room temperature until the reaction was stopped by addition of 25 µl 3M NaOH to each well. Absorbance was read at 450 nm by BioRad Model 680 Microplate Reader (BioRad, Hercules, CA).

Results

Canine plasma incubation with 1 µg/ml recombinant *Wolbachia* surface protein (rWSP) indicated IgG production in heartworm positive canines. The results from the ELISA are a representation of five experimentally infected heartworm positive dogs and three heartworm negative dogs. All dogs were female beagles housed in indoor facilities. Four of the five heartworm positive dogs were producing microfilaria. Based on optical density (O.D.) values obtained using spectrophotometric analysis, the mean IgG production by heartworm positive dogs was 0.208 ± .011, and the mean antibody production by heartworm negative dogs was

0.070 ± .023. Therefore, heartworm positive dogs were producing more IgG specific to rWSP than heartworm negative dogs (Figure 3.1). The results obtained from the one amicrofilaremic, heartworm positive dog, though slightly more elevated, appeared similar to microfilaremic, heartworm positive dogs. All results are located in appendix A.

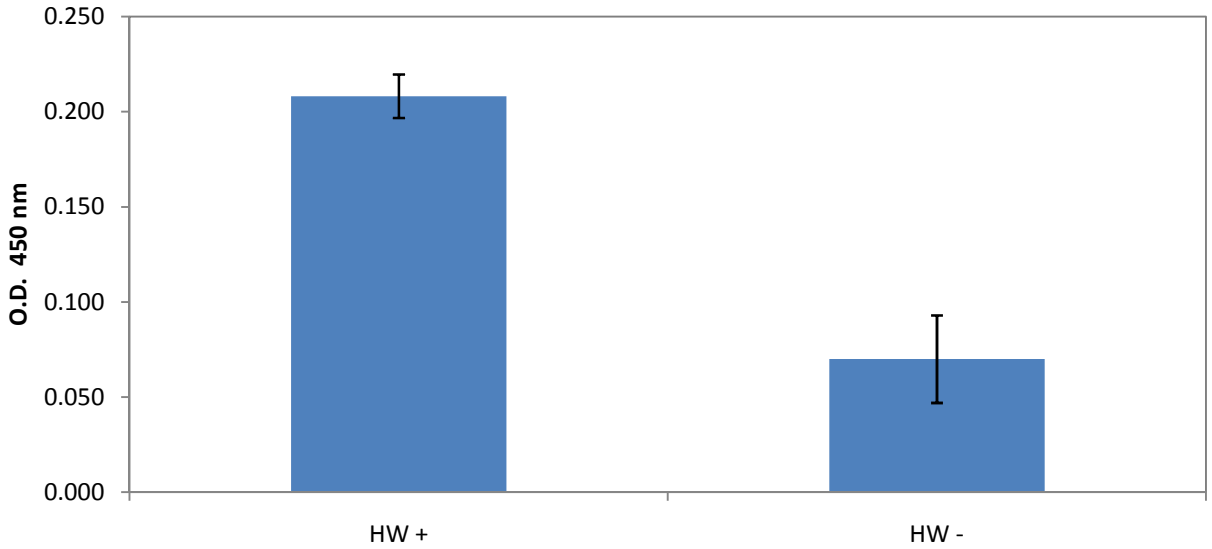

Figure 3.1. Average IgG production by heartworm positive (HW+) and heartworm negative (HW-) dogs when binding to 1 µg/ml rWSP. Canine plasma was diluted to 1:50 in PBS-Tweeen 20. Absorbance was read at 450 nm. Results are indicative of five heartworm positive dogs and three heartworm negative dogs.

Discussion

Recombinant *Wolbachia* surface protein (rWSP) was developed by Bazzocchi, et al. (2000a). Since the development of rWSP, several studies have been conducted using rWSP in ELISAs (Kramer, et al., 2005, Marcos-Atxutegi, et al., 2003, Morchón, et al., 2004). High levels of IgG specific to rWSP have shown the immunostimulatory role of WSP (Kramer, et al., 2005, Morchón, et al., 2004). *Wolbachia* surface protein may be the immunodominant antigen of *Wolbachia* (Ohashi, et al., 1998).

Results indicated that heartworm positive dogs developed a strong IgG response to rWSP (Figure 3.1). These results are similar to previously published data that heartworm positive dogs produced rWSP specific IgG, with heartworm positive dogs having a significantly greater optical density (O.D.) reading than heartworm negative dogs (Kramer, et al., 2005). High antibody production by heartworm positive dogs indicates that their infections have led to previous exposure to *Wolbachia*. The lower O.D. values from heartworm negative dogs represented nonspecific binding of antibody.

One heartworm positive dog used was amicrofilaremic due to infection period. The dog was experimentally infected less than six months prior to this study. The other four experimentally infected heartworm positive dogs were microfilaremic. Results from the study indicated that the antibody specific to rWSP was similar from all heartworm positive dogs, although the amicrofilaremic canine antibody was slightly elevated. The small variation could indicate immune suppression due to microfilaremic or more established heartworm infection.

Future work could include analyzing more experimentally infected heartworm positive dogs that are amicrofilaremic due to sexually immature worms or single sex infections. Although there was some indication of an elevated response by heartworm positive,

amicrofilaremic dogs, the results from this study are not conclusive data due to there being only one dog in this group. Kramer, et al. (2005) indicated that amicrofilaremic dogs produced less IgG than microfilaremic dogs. However, evidence indicates that dogs with patent infections of *Dirofilaria immitis* have higher levels of circulating anti-inflammatory cytokines than dogs with occult infections (Morchón, et al., 2007b). Finally, more work could be done to determine if treatment of *D. immitis* infected dogs leads to an increase in antibody specific to rWSP. Previous work has demonstrated that treatment of cats infected with L3 larvae leads to an increase in IgG specific to rWSP (Morchón, et al., 2004). Therefore, dogs treated for heartworms may elicit a greater humoral response to rWSP when worms die and release *Wolbachia*.

Chapter IV.

Evaluation of Canine Cytokines Produced in Response to Recombinant *Wolbachia* Surface Protein

Introduction

Dirofilaria immitis, the causative agent of heartworm disease in numerous hosts, contains a Gram-negative bacterial endosymbiont, *Wolbachia*, in all its developmental stages (Kozek, 2005, Masui, et al., 2000, Taylor, et al., 2005). *Wolbachia* is found within the lateral hypodermal chords of both sexes and oocytes within female worms (Kramer, et al., 2003). Evidence suggests that *D. immitis* requires *Wolbachia* for survival, perhaps due to the ability of *Wolbachia* to produce glutathione and haeme (Kramer, 2006).

Melarsomine dihydrochloride (Immiticide®, Merial) is the only FDA approved drug for the treatment of canine heartworm infection (Bowman and Atkins, 2009, McCall, et al., 2008). Often canine treatment with melarsomine dihydrochloride results in pulmonary thrombosis (Kramer, et al., 2008). Host immunological response also results upon death of heartworms (Saint André, et al., 2002). When stages of *D. immitis* die within the host, *Wolbachia* is released (Keiser, et al., 2002). Consequently, an immunological response could be due to both death of *D. immitis* adults and *Wolbachia*. Studies have shown that use of doxycycline, an antibiotic used to treat *Wolbachia*, during elimination of adult heartworms results in less lung damage, suggestive that *Wolbachia* may play a large role in canine inflammatory reactions during heartworm treatment (Kramer, et al., 2008). Evidence indicates that *Wolbachia* can remain alive

outside of a host cell for as long as a week (Rasgon, et al., 2006). Thus, early removal of *Wolbachia* before treatment with melarsomine dihydrochloride could be beneficial to dogs.

Previous research conducted to clarify the immunological role of *Wolbachia* used recombinant *Wolbachia* surface protein (rWSP) (Bazzocchi, et al., 2000a, Kramer, et al., 2005, Morchón, et al., 2007a, Morchón, et al., 2004). Although *Wolbachia* may contain other antigenic peptides, WSP may be the immunodominant antigen of *Wolbachia* as WSP is homologous to the main antigenic peptide of *Ehrlichia canis* (Bazzocchi, et al., 2000a, Ohashi, et al., 1998). Studies have indicated that rWSP has elicits production of primarily IgG2, an antibody indicative of a T-helper 1 (Th1) type immune response in dogs infected with heartworms (Kramer, et al., 2005, Simón, et al., 2007). However, IgG1 has been detected only in microfilaremic positive infections, indicative of a T-helper 2 (Th2) type response (Kramer, et al., 2005, Simón, et al., 2007). A better understanding of immune responses to *Wolbachia* is needed for heartworm infected dogs (Kramer, 2006). Culturing canine lymphocytes in the presence of rWSP would allow a more thorough assessment of canine immune response to *Wolbachia*. Cytokine production would provide information to discern if *Wolbachia* stimulates a pro-inflammatory or anti-inflammatory response.

The goal of this study was to determine cytokine responses resulting from exposure of canine lymphocytes to rWSP. A cytokine profile is a good indicator of the host's immune response in producing pro-inflammatory and anti-inflammatory responses. An understanding of the role of cytokines in the immunopathology of heartworm disease will be helpful in management and treatment of infected animals. Because *Wolbachia* was anticipated to incite inflammation, I hypothesized that canine lymphocytes would be stimulated by rWSP to produce pro-inflammatory cytokines.

Materials and Methods

Lymphoblastogenesis

All procedures were carried out under aseptic conditions. Approximately 5-8 ml of peripheral blood was drawn into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) by cephalic venipuncture from heartworm positive and heartworm negative dogs. After collection, blood was centrifuged for 10 min at 2200 rpm. The buffy coat was removed using a pasteur pipette and placed in a sterile 15 ml tube. Hanks' balanced salt solution (HBSS) without magnesium and calcium (Mediatch, Inc, Manassas, VA), containing penicillin-streptomycin (p/s) (0.1 ml/100 ml medium) was added to the buffy coat of each dog at a ratio of 2:1. The final volume of each sample was approximately 12 ml. The mixture of buffy coat and HBSS was layered onto 4 ml Histopaque-1077 (Sigma, St. Louis, MO) (Spencer, 1993). Samples were centrifuged at 2200 rpm for 25 min with brakes and acceleration lowered to prevent the lymphocyte layer from being disrupted. Lymphocytes were removed from Histopaque 1077 using a pasteur pipette, placed into a sterile 15 ml tube, and spun at 2200 rpm for 12 min. After centrifugation, the supernatant was decanted, and 3 ml HBSS without magnesium and calcium was added to the lymphocyte pellet. As a final washing step, samples were centrifuged at 2200 rpm for 12 min, and the supernatant was decanted. Following the wash, 1 ml RPMI media containing L-glutamine (HyClone Laboratories, Inc, Logan, UT), 10% heat inactivated fetal bovine serum (FBS), and p/s was added to each sample. To determine lymphocyte concentration, lymphocytes in RPMI were mixed with Camco Quik Stain II Buffered Differential Wright-Giemsa Stain at a dilution of 1:9 and counted in a hemocytometer. Canine lymphocyte concentration was adjusted to 2×10^6 cells/ml in RPMI medium.

The lymphocytes of eight dogs were exposed to recombinant *Wolbachia* surface protein (rWSP). Dogs were heartworm positive and microfilaremic, heartworm positive and amicrofilaremic, or heartworm negative. Lymphocytes concentrated at 2 x 10^6 cells/ml were added to the wells of a 96 well sterile round bottom microplate (90 µl per well). Each well received either 5 µg/ml rWSP, 10 µg/ml rWSP, or 10 µg/ml concanavalin A (ConA) (Sigma Aldrich, St. Louis, MO). Negative wells contained lymphocytes in RPMI with L-glutamine. Recombinant WSP was gifted by Dr. Claudio Genchi, University of Milano, Italy. The supply of rWSP was limited, however. Plates were incubated at 37°C in 5% CO_2 for 48 h or 72 h. Following incubation, cells were harvested and centrifuged at 13,000 rpm for 10 min to remove the supernatant. The lymphocyte pellets were stored at -20°C until processed by reverse transcription polymerase chain reaction (RT-PCR).

Isolation of mRNA

Messenger RNA (mRNA) was isolated from previously pelleted lymphocytes exposed to rWSP or ConA using RNA Stat-60 total RNA/mRNA isolation reagent (Tel-Test, Friendswood, TX) according to manufacturer's instructions. Initially, each pellet of lymphocytes was resuspended in 500 µl RNA Stat-60. Once cells had sat for 5 min at room temperature, 100 µl chloroform was added to each sample and tubes were shaken vigorously. Afterwards, samples were kept at room temperature for 3 min and centrifuged for 15 min at 4°C. Following centrifugation, the upper aqueous phase was transferred to a sterile tube with 250 µl isopropanol and mixed. Samples were left at room temperature for 10 min and centrifuged for 10 min at 4°C. Once the supernatant was decanted, the pellet was washed in 500 µl 75% ethanol and centrifuged for 10 min at 4°C. The pellet was air dryed after the removal of ethanol. Finally, the pellet was resuspended in 20 µl diethylpyrocarbonate (DEPC) treated water (Invitrogen, Carlsbad, CA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR was performed using an AccessQuick RT-PCR System kit (Promega, Madison, WI) according to manufacturer's instructions. Approximately 0.5 – 1.0 μg of mRNA was used per RT-PCR reaction. The following cytokines were assayed: IFN-γ, TNF-α, IL-4. IL-5, IL-6. IL-10, and IL-12p40, with GAPDH used as a positive control. These cytokines were chosen because they are produced during pro-inflammatory reactions, or Th1-type responses, or because they are produced during anti-inflammatory reactions, representative of a Th2-type response. Primers specific to cytokine genes were previously designed (Table 4.1) (Chamizo, et al., 2001, Olivry, et al., 1999). Each RT-PCR reaction contained 1 μl of 10 μM concentration of a forward and reverse primer. All RT-PCR used cycles consisting of 45°C for 45 min, 95°C for 2 min, 40 cycles of amplification using 95°C for 30 s and 1 min at annealing temperature, final extension at 72°C for 7 min, and hold at 4°C. Annealing temperatures were 62°C for GAPDH, 64°C IFN-γ, 60°C for TNF-α, IL-4, IL-6, IL-10, and 55°C for IL-5 and IL-12p40 (Chamizo, et al., 2001, Olivry, et al., 1999). Products from RT-PCR were loaded into a 1.8% agarose gel (1.8 g agarose per 100 ml TBE), with gel electrophoresis at 70 V for approximately 1 hr 30 min to separate products. Afterwards, the gel was stained with ethidium bromide. Results were based on presence or absence of a band.

Table 4.1. Primers used in RT-PCR for the detection of canine cytokine genes.

Target Sequence	Primer Set (5'-3')	Product Size
IFN-γ*	CATTCAAAGGAFGCATGGATACC GACTCCTTTTCCGCTTCCTTAG	202 bp
TNF-α*	GCCCAGACAGTCAAATCATC CTTGATGGCAGAGAGTAGGTTG	302 bp
IL-4*	CCTCCCAACTGATTCCAACTC GATTTCATTCATAGAACAGGTC	316 bp
IL-5**	GGTGAAAGAGACCTTGGCAC CACTCGGTGTTCATTACACC	305 bp
IL-6*	GAACTCCCTCTCCACAAGC TTCTTGTCAAGCAGGTCTCC	325 bp
IL-10*	GAGCTCCGAGCTGCCTTC GATGAAGATGTCAAACTCACTC	374 bp
IL-12p40**	GAGATGCTGGCCAGTATACC GTCTCTGATGAAGAAGCTGC	451 bp
GAPDH*	GTGATGCTGGTGCTGAGTATG GTGATGGCATGGAC($^{G}_{T}$)GTGG	282 bp

*Primer designed previously (Chamizo, et al., 2001)
**Primer designed previously (Olivry, et al., 1999)

Results

The lymphocytes of eight female beagles were stimulated with either 5 μg/ml recombinant *Wolbachia* surface protein (rWSP), 10 μg/ml rWSP, 10 μg/ml concanavalin A (ConA), or RPMI media only. Dogs were divided into three groups initially: heartworm positive and microfilaremic, heartworm positive and amicrofilaremic, and heartworm negative. Four dogs represented the heartworm positive, microfilaremic group. Only one dog was included in the heartworm positive, amicrofilaremic group. Three dogs were heartworm negative. However, all heartworm positive dogs were combined for discussion purposes due to similar results. Results were determined by production of appropriate sized bands after gel electrophoresis using reverse transcriptase polymerase chain reaction (RT-PCR) products. The production of seven cytokines (IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10, and IL-12p40) and the house keeping gene, GAPDH, were analyzed (Figure 4.1). All results can be viewed in appendix B.

The lymphocytes of all dogs produced the house keeping gene GAPDH regardless of stimulation and incubation time.

Lymphocytes from heartworm positive dogs produced IFN-γ when incubated for 48 h with 5 μg/ml rWSP; however, one of the five heartworm positive dogs did not produce detectable IFN-γ when incubated for 72 h with 5 μg/ml rWSP (Figure 4.2). The four heartworm positive, microfilaremic dogs produced IFN-γ when stimulated for 48 h and 72 h with 10 μg/ml rWSP. The heartworm positive, amicrofilaremic dog produced IFN-γ when incubated at 48 h and 72 h with 5 μg/ml rWSP and 48 h with 10 μg/ml rWSP but did not produce IFN-γ when incubated with 10 μg/ml rWSP for 72 h. All heartworm negative dogs produced IFN-γ when incubated 48 h and 72 h with 5 μg/ml rWSP (Figure 4.2). When using 10 μg/ml rWSP, no heartworm negative dog produced IFN-γ at 48 h, although two of three heartworm negative dogs

produced IFN-γ at 72 h (Figure 4.2). By 72 h, all dogs produced IFN-γ when incubated with ConA. Most dogs did not produce IFN-γ when incubated with RPMI media only.

Lymphocytes from all dogs produced TNF-α when stimulated with ConA by 72 h (Figure 4.3). All heartworm negative dogs produced TNF-α when stimulated with 5 μg/ml rWSP at 48 h, but no production was detected at 72 h (Figure 4.3). No heartworm negative dogs produced TNF-α when stimulated with 10 μg/ml rWSP. However, one heartworm negative dog produced TNF-α when unstimulated. Only one heartworm positive, microfilaremic dog produced TNF-α when stimulated with 5 μg/ml rWSP and 10 μg/ml rWSP at 48 h only, although the dog did not produce detectable TNF-α when stimulated with rWSP at 72 h.

The lymphocytes from heartworm negative dogs did not produce detectable IL-4 when stimulated with rWSP, ConA, or RPMI media only (Figure 4.4). Four of five heartworm positive dogs produced IL-4 when stimulated with 5 μg/ml rWSP at 48 h (Figure 4.4). However, the fifth heartworm positive dog produced IL-4 when stimulated with 10 μg/ml rWSP at 48 h along with one other heartworm positive dog. No heartworm positive dog produced IL-4 when lymphocytes were stimulated with rWSP at 72 h (Figure 4.4). Two of the five heartworm positive dogs produced IL-4 when stimulated with ConA at 48 h, although no heartworm positive dogs were stimulated by ConA to make IL-4 at 72 h. No detection of IL-4 was seen when lymphocytes were stimulated with only RPMI media (Figure 4.4).

No production of IL-5 was detected. Canine lymphocytes did not produce IL-5 when stimulated with rWSP, ConA, or RPMI only.

Lymphocytes from all dogs were stimulated to produce IL-6 when incubated at 72 h with rWSP, ConA, or RPMI media only (Figure 4.5). At 48 h incubation, lymphocytes from all dogs were stimulated to produce IL-6 when exposed to 5 μg/ml rWSP or 10 μg/ml rWSP (Figure 4.5).

When incubated with ConA for 48 h, four of five heartworm positive dogs and two of three heartworm negative dogs produced IL-6. Canine lymphocyte incubation with only RPMI media for 48 h led to detectable stimulation of IL-6 by one heartworm positive, microfilaremic dog and two heartworm negative dogs.

No production of IL-10 was detected. Canine lymphocytes did not produce IL-10 when stimulated with rWSP, ConA, or RPMI only.

All dogs were stimulated to produce IL-12p40 when incubated at 48 h with 5 μg/ml rWSP (Figure 4.6). Only one heartworm positive dog produced IL-12p40 when stimulated with 10 μg/ml rWSP at 48 h. In addition, this dog produced IL-12p40 when exposed to 10μg/ml rWSP for 72 h. Canine lymphocytes did not produce IL-12p40 when incubated with ConA or RPMI media only (Figure 4.6).

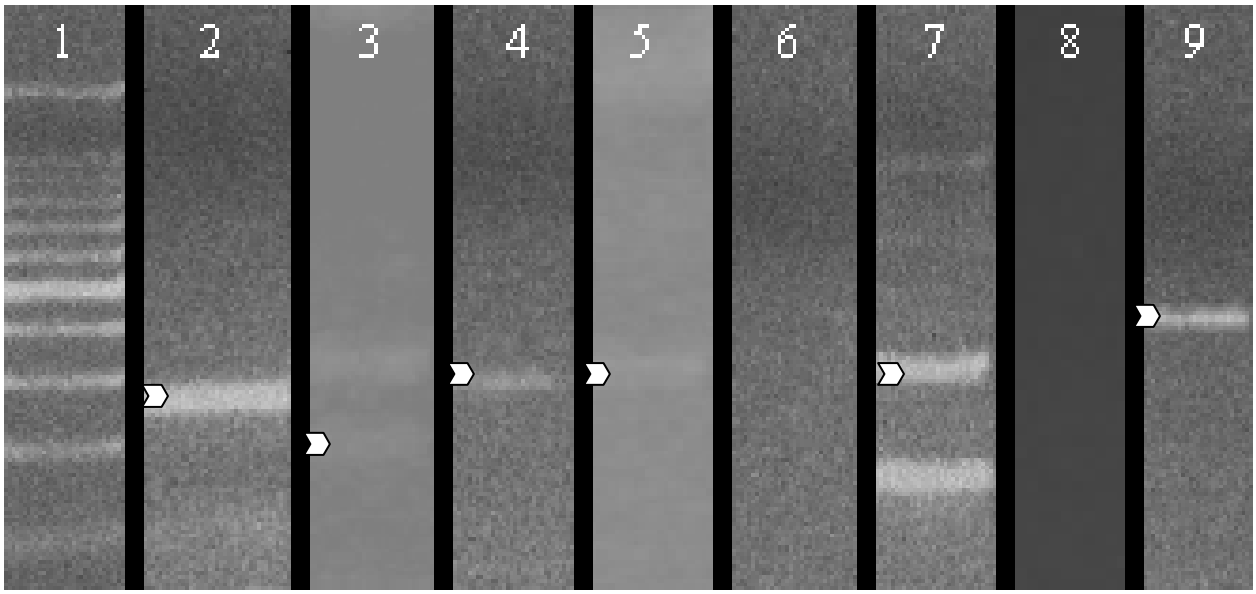

Figure.4.1. Examples of RT-PCR amplicon products of cytokines produced when heartworm positive and negative dogs were stimulated with rWSP. RT-PCR products were ran on a 1% agarose gel and stained with ethidium bromide. Lane 1, 100-bp molecular weight marker; lane 2, GAPDH amplicon; lane 3, IFN-γ amplicon; lane 4, TNF-α amplicon; lane 5, IL-4 amplicon; lane 6, IL-5 amplicon; lane 7, IL-6 amplicon; Lane 8, IL-10 amplicon; Lane 9, IL-12p40 amplicon.

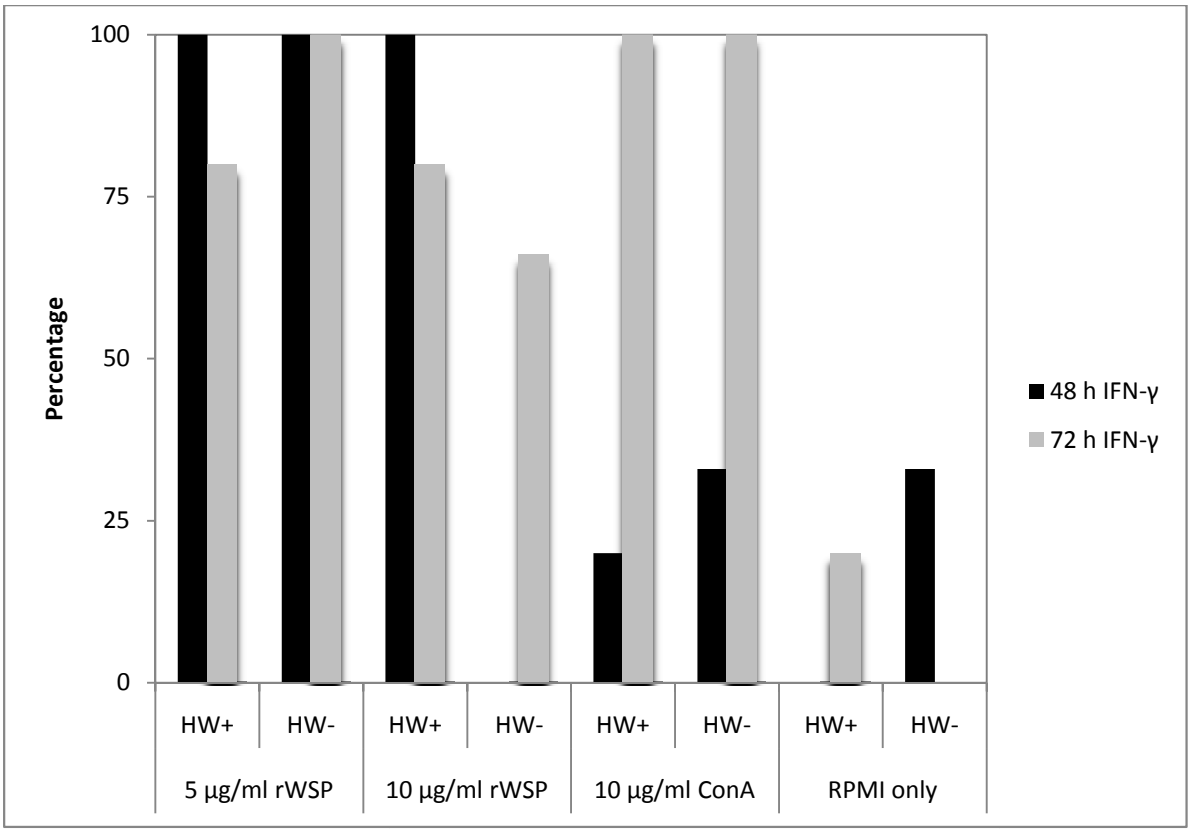

Figure 4.2. Percentage of heartworm positive (HW+) and heartworm negative (HW-) dogs producing IFN-γ when lymphocytes were incubated for 48 h or 72 h with either 5 µg/ml rWSP, 10 µg/ml rWSP, 10 µg/ml ConA, or RPMI media only.

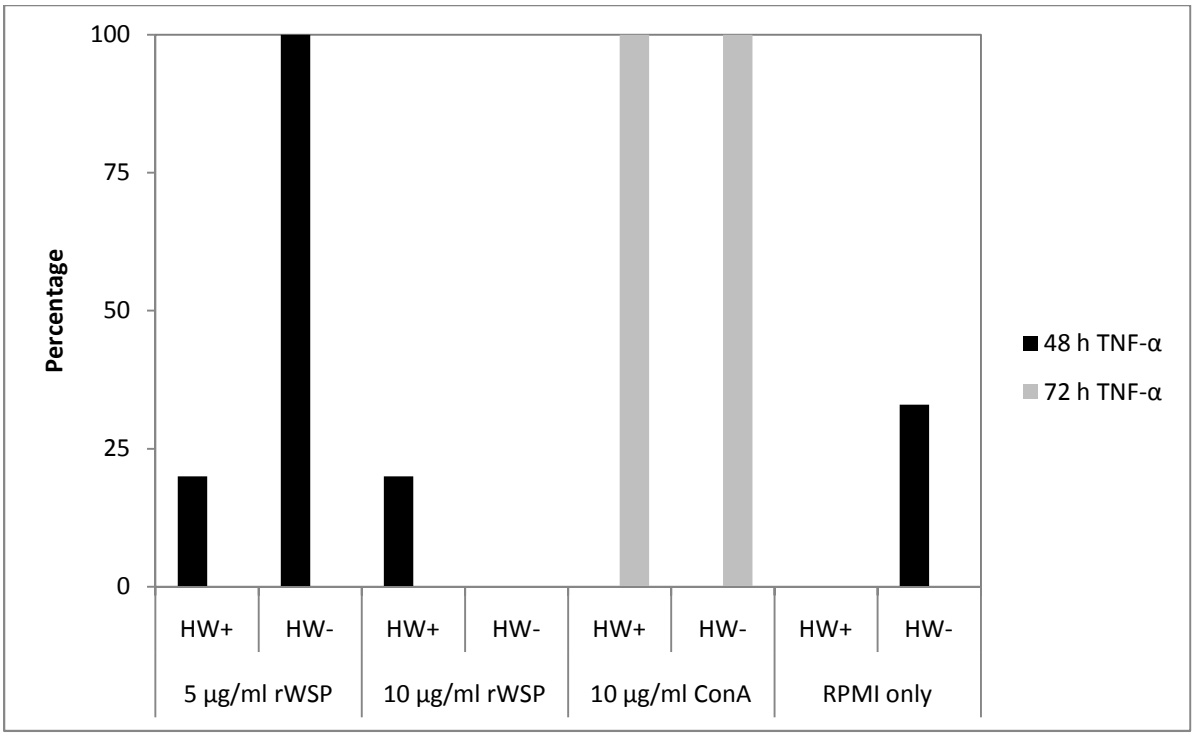

Figure 4.3. Percentage of heartworm positive (HW+) and heartworm negative (HW-) dogs producing TNF-α when lymphocytes were incubated for 48 h or 72 h with either 5 µg/ml rWSP, 10 µg/ml rWSP, 10 µg/ml ConA, or RPMI media only.

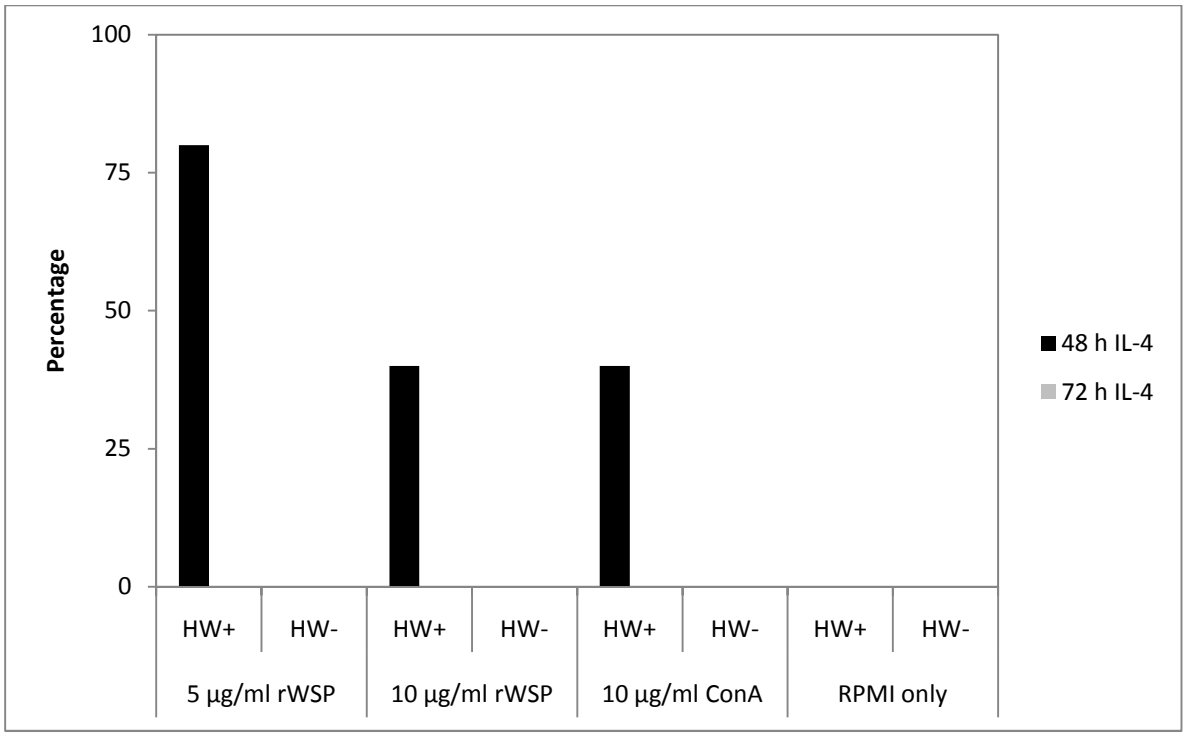

Figure 4.4. Percentage of heartworm positive (HW+) and heartworm negative (HW-) dogs producing IL-4 when lymphocytes were incubated for 48 h or 72 h with either 5 µg/ml rWSP, 10 µg/ml rWSP, 10 µg/ml ConA, or RPMI media only.

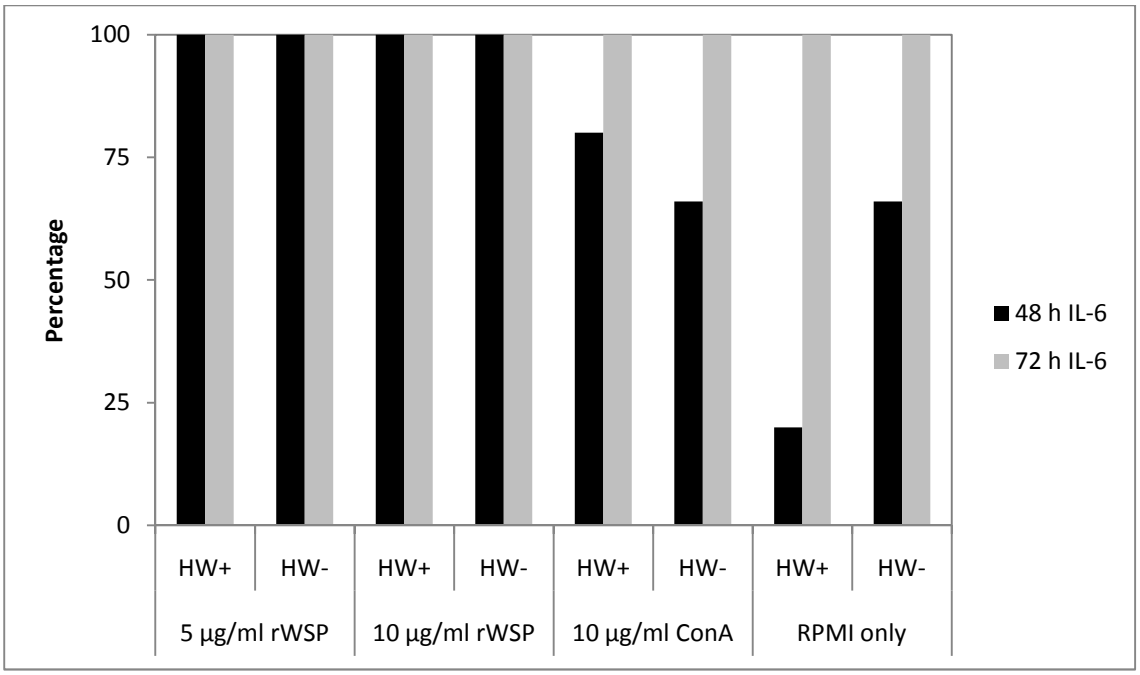

Figure. 4.5. Percentage of heartworm positive (HW+) and heartworm negative (HW-) dogs producing IL-6 when lymphocytes were incubated for 48 h or 72 h with either 5 µg/ml rWSP, 10 µg/ml rWSP, 10 µg/ml ConA, or RPMI media only.

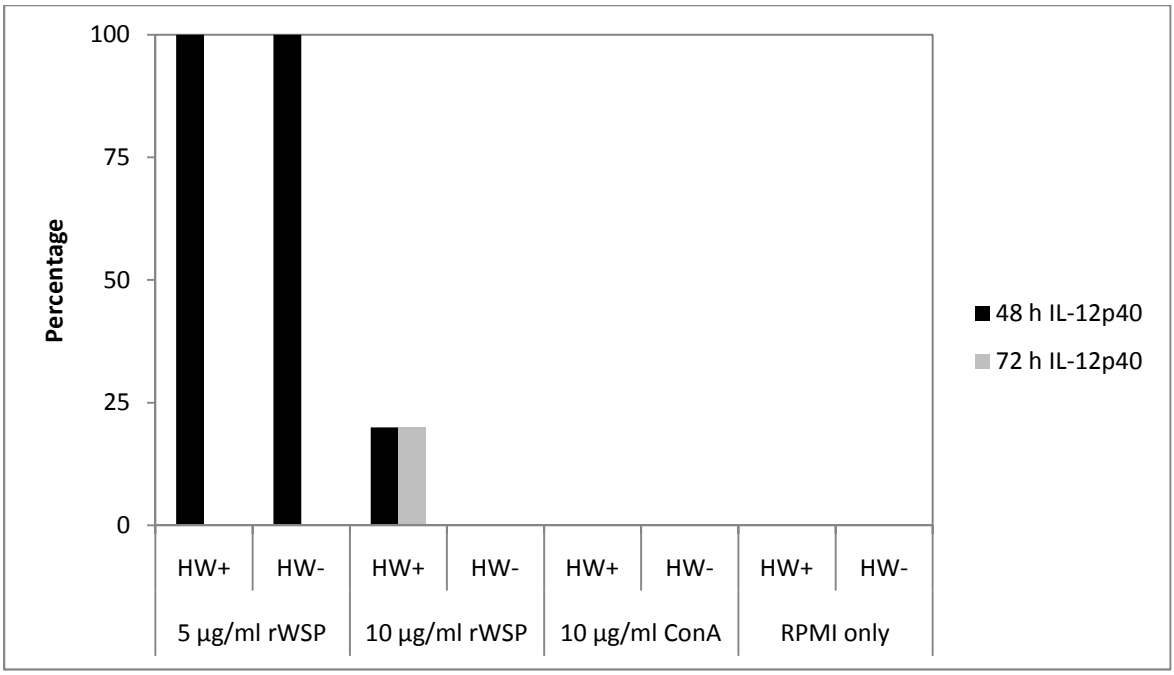

Figure 4.6. Percentage of heartworm positive (HW+) and heartworm negative (HW-) dogs producing IL-12p40 when lymphocytes were incubated for 48 h or 72 h with either 5 µg/ml rWSP, 10 µg/ml rWSP, 10 µg/ml ConA, or RPMI media only.

Discussion

Canine lymphocyte production of seven cytokines was analyzed using reverse transcription polymerase chain reaction (RT-PCR) after lymphocytes were stimulated with either 5 μg/ml recombinant *Wolbachia* surface protein (rWSP), 10 μg/ml rWSP, 10 μg/ml concanavalin A (ConA), or RPMI media during an incubation of either 48 h or 72 h. The house keeping gene, GAPDH, was detected in all samples. Therefore, mRNA was present in all processed samples that were not damaged. Canine lymphocytes were stimulated with ConA to indicate that lymphocytes were able to be immunologically stimulated. Although there was a time lag, ConA was able to incite canine lymphocytes to produce TNF-α and IFN-γ by 72 h (Figure 4.2, Figure 4.3).

There are no known published data to date that exposes canine lymphocytes to rWSP. Due to the limited availability of rWSP, the present study utilized rWSP at 5 μg/ml and 10 μg/ml which corresponded to the same dose and 50% of the dose of ConA normally used in lymphoblastogenesis. It is understood that these doses may not be ideal and further work employing dose titration responses could be studied should ample amounts of rWSP become available. Furthermore, natural concentrations of *Wolbachia* within a host of *Dirofilaria immitis* are unknown; however, the concentrations chosen for this study were able to provide preliminary data indicating in vitro responses to rWSP.

Samples showed an indication that *Wolbachia* promotes the initial production of IFN-γ, a pro-inflammatory cytokine produced mainly by T cells and natural killer cells regardless of heartworm infection status (Figure 4.2). IFN-γ is an important cytokine in the development of a Th1 response, as IFN- γ inhibits proliferation of Th2 cells. Other functions of IFN- γ include activation of macrophages and cytotoxic T cells, antiviral activity promotion, and elimination of

intracellular bacteria. As *Wolbachia* is an intracellular pathogen, production of IFN- γ could be used to remove *Wolbachia*. However, there is more response from heartworm positive dogs when exposed to 10 μg/ml rWSP compared to heartworm negative dogs (Figure 4.2). Heartworm positive animals may be producing IFN- γ as they are more primed than heartworm negative animals due to chronic infection. Canine lymphocyte production of IFN-γ when stimulated with rWSP partially coincides with previously published results implicating rWSP in production of IgG2 and IgG1 in ELISAs using heartworm positive dogs (Kramer, et al., 2005). A higher IgG2:IG1 compared to clinical disease is characteristic of a Th1 response (Quinnell, et al., 2003, Kramer, et al., 2005, Simón, et al., 2007).

TNF-α is a pro-inflammatory cytokine produced by macrophages and lymphocytes involved with systemic inflammation, apoptosis, and inhibition of viral replication. Although collection of lymphocytes contained some monocytes, production of TNF-α was based primarily on stimulation of lymphocytes. In this study, heartworm negative dogs produced TNF-α when stimulated with 5 μg/ml rWSP; however, heartworm negative dogs appeared to demonstrate immunosuppression when stimulated with increased concentrations of rWSP (Figure 4.3). In addition, heartworm positive dogs were not stimulated to produce TNF-α (Figure 4.3). Lack of TNF-α production possibly indicates an anti-inflammatory response from heartworm positive dogs as a means of survival for *D. immitis* and/or *Wolbachia*. Heartworm positive dogs have been documented to produce anti-inflammatory cytokines, a means for immunosuppression for survival of *D. immitis* (Brattig, et al., 2002, Morchón, et al., 2007b). However, few intracellular bacteria have been reported to induce immunosuppression (for example, *Mycobacterium* spp.).

Overall, IL-4 appeared to be upregulated in only heartworm positive dogs (Figure 4.4). Interleukin 4 is an anti-inflammatory cytokine used to differentiate naïve T cells into Th2

lineage, induce B cells to produce IgE, and decrease production of Th1 cells. Production of IL-4 coincides with immunosuppression by *D. immitis* and other filarial parasites (MacDonald, et al., 1998, Morchón, et al., 2007b). In addition, there have been previous reports of immunosuppression by other intracellular bacteria through the production of IL-4 (Sieling, et al., 1993). Upregulation by heartworm positive dogs in production of IL-4 indicates a skew towards an anti-inflammatory response by *Wolbachia* and/or *D. immitis*. An anti-inflammatory response could be caused by the parasite and/or bacteria to ensure its survival within the canine host.

Interleukin 5 is an anti-inflammatory cytokine produced mainly by Th2 cells. A major function of IL-5 is upregulation of B cells. Human asthmatic patients with greater airflow obstruction have a greater chance to develop IL-5 within the bronchial mucosa than asthmatic patients with less severe disease (Hamid, et al., 1991). Oddly, heartworm infected cats that often express asthmatic symptoms do not upregulate IL-5 in bronchoalveolar lavages (Spencer, et al., 2006). In this study, canine lymphocytes were not stimulated to produce IL-5.

As a pro-inflammatory cytokine, IL-6 is produced by macrophages, T cells, and endothelial cells. A major function of IL-6 includes fever induction and recruitment of cells from bone marrow. Results indicated that all groups of dogs produced IL-6 regardless of stimulation (Figure 4.5). Therefore, *Wolbachia* may not specifically stimulate canine lymphocytes to produce IL-6.

Stimulation of canine lymphocytes did not indicate production of IL-10. Interleukin-10 is an anti-inflammatory cytokine responsible for inhibition of IFN-γ production by T cells, decreased secretion of TNF-α, and downregulation of MHC class II of macrophages (Moore, et al., 2001). Lack of IL-10 expression by canine lymphocytes corresponds with expression of IFN-γ due to an inverse relationship.

Interleukin 12 is a heterodimeric cytokine produced by the formation of p35 and p40 units. In this study, production of IL-12p40 was analyzed as a means of observing IL-12. Produced by T cells, major functions of IL-12 include differentiation of naïve T cells into Th0 cells and stimulation of T cell production of IFN-γ and TNF-α. Canine production of IL-12p40 was detected when lymphocytes were stimulated with lower concentrations of rWSP regardless of heartworm infection status (Figure 4.6). Increased stimulation of lymphocytes with an increased concentration of rWSP hindered production of IL-12p40, possibly indicating that increased levels of rWSP leads to immune paralysis by too high a dose of antigen. Although increased concentrations of rWSP did not produce IL-12, IFN-γ continued to be detected, though possibly at a lower yield.

The results from the heartworm positive, amicrofilaremic dog were combined with results from microfilaremic dogs because of similarity. Initially, these two groups were separated due to previously published accounts that microfilaremia affected immune response to *Wolbachia* and *D. immitis* (Kramer, et al., 2005, Morchón, et al., 2007b). Previous published data has shown that microfilaremia has coincided with increased immune suppression (Brattig, et al., 2002, Morchón, et al., 2007b, Simón, et al., 2007).

I hypothesized that rWSP would stimulate heartworm positive dogs to produce pro-inflammatory cytokines. Although IFN-γ and IL-12p40 were detected, rWSP failed to produce TNF-α and IL-12p40 at a greater concentration of rWSP. Therefore, rWSP may not always induce an inflammatory response. However, chronic heartworm infection may prime a dog's immune response to be suppressed. Previous research analyzing helminthes have indicated host immunosuppression as a means for the worm to survive (Brattig, et al., 2002, Morchón, et al., 2007b).

With some exceptions, there appeared to be a lack of consistent trends in the data to make valid conclusions regarding cytokine production due to incubation of canine lymphocytes with rWPS. Although some trends could be detected, correlations could not be made based on all cytokine productions in regards to heartworm infection status. Results are likely due to canine model and either prior exposure to antigens or the immunogenic influences of inter-current antigens. Dogs were housed in a facility that may have exposed them to several antigens, priming their immune systems.

Future work includes analyzing cytokine levels quantitatively. As IL-6 was produced regardless of stimulation, only quantitative PCR will determine if rWSP induces canine lymphocytes to make increased or decreased IL-6. The cytokines IFN-γ, TNF-α, IL-10, and IL-12 affect each other, whereby IFN-γ, TNF-α, and IL-12 share an inverse relationship with IL-10. Although IFN-γ was detected throughout all conditions of lymphocyte exposure to rWSP, TNF-α and IL-12 were not always produced. A quantitative understanding of cytokine production may give a better relationship of how *Wolbachia* possibly affects cytokine profile of dogs. As rWSP is not the only antigen of *Wolbachia*, the immune response elicited by canine lymphocyte exposure to pure *Wolbachia* should be studied as soon as a nematode-derived culture has been developed. Use of gnotobiotes may provide a true immune response to rWSP, as the canines used in my study have been exposed to several antigens.

Treatment of *D. immitis* induces an inflammatory response (Kramer, et al., 2008). However, the results from this study cannot indict *Wolbachia* surface protein as the pure stimulant of inflammation. Because the cytokine profile was to better understand the inflammatory affects of heartworm treatment, canine lymphocytes should be incubated with pure antigens of *D. immitis* free of *Wolbachia*. Pro-inflammatory cytokines may be produced when

stimulated with *D. immitis* and explain inflammation following injections of melarsomine dihydrochloride, the treatment of canine heartworms.

Chapter V.

Overall Conclusions

Dirofilaria immitis is the causative agent of heartworm disease in canines, cats, and other mammals (Bowman and Atkins, 2009). Symptoms of canine heartworm infection include exercise intolerance, sudden onset of lethargy, enlarged pulmonary arteries, and decreased blood flow to the lungs (Bowman and Atkins, 2009). Currently, melarsomine dihydrochloride (Immiticide®, Merial) is the only FDA approved treatment of canine heartworm infection (Bowman and Atkins, 2009, McCall, et al., 2008). At treatment, dogs often experience pulmonary thrombosis and an inflammatory response (Kramer, et al., 2008, Saint André, et al., 2002). Like other filarial parasites, *D. immitis* harbors a strain of *Wolbachia*, an obligate, intracellular, Gram-negative bacteria (Kozek, 2005, Taylor, et al., 2005). Upon death of *D. immitis*, *Wolbachia* is released and may contribute to the inflammatory response (Keiser, et al., 2002).

In order to gain a better understanding of canine immune response to *Wolbachia*, an attempt was made to establish a cytokine profile of heartworm positive dogs when exposed to recombinant *Wolbachia* surface protein (rWSP). I hypothesized that heartworm positive dogs would elicit a detectable immune response to rWSP, producing pro-inflammatory cytokines. This study analyzed results from five heartworm positive dogs and three heartworm negative dogs. All dogs used were female beagles, gang-housed in indoor facilities at Auburn University College of Veterinary Medicine. One heartworm positive dog was amicrofilaremic. The other

four heartworm positive dogs had microfilaria counts between 6000 to 33,000 microfilaria/ml. Heartworm status was determined using SNAP semi-quantitative heartworm antigen test (Idexx, Westbrook, ME) and microfilaremia was determined using modified Knott's tests (MKT). Dogs were grouped because of anticipated differences in immune response due to variances in exposure to *Wolbachia* and immunity changes associated with microfilaria (Brattig, et al., 2002, Morchón, et al., 2007b, Simón, et al., 2007).

After establishment of heartworm infection status of dogs, microfilaria and canine blood cells were analyzed using polymerase chain reaction (PCR) to detect DNA from *Wolbachia* surface protein (*wsp*). Four heartworm positive, microfilaremic dogs and one heartworm negative dog were used. Microfilaria were separated using methods derived by Grieve, et al. (1984), co-aggregated with some blood cells. In the case of the heartworm negative dog, the aggregate consisted entirely of blood cells. Results indicated presence of *wsp* in the four heartworm positive, microfilaremic dogs, not the heartworm negative dog (Figure 2.1). Because all stages of *D. immitis* contain *Wolbachia*, detection of *wsp* was anticipated (Kozek, 2005). Although the heartworm positive dog did not have microfilaria, the dog may still have been exposed to *Wolbachia*. However, *wsp* was not detected in the blood cells of the heartworm negative dog.

An ELISA was performed to determine if dogs developed IgG specific to rWSP. As nematode-derived *Wolbachia* cannot be cultured, rWSP was developed by Bazzocchi, et al. (2000a). Results indicated that heartworm positive dogs had developed antibody specific to rWSP. Therefore, heartworm positive dogs have been exposed to *Wolbachia* due to their chronic infections. One heartworm positive dog was amicrofilaremic. Although the antibody amount produced by the amicrofilaremic dog was similar to the results from microfilaremic dogs, the

antibody amount of the amicrofilaremic dog was slightly elevated. This result is in contradiction with a previous study that indicated amicrofilaremic dogs produced less IgG specific to rWSP than microfilaremic dogs (Kramer, et al., 2005). However, past studies have indicated microfilaria may induce immunosuppression (Brattig, et al., 2002, Morchón, et al., 2007b, Simón, et al., 2007). Because the study only analyzed one amicrofilaremic dog, future work should analyze more experimentally infected dogs that are amicrofilaremic due to naivety of heartworm infection.

To develop a cytokine profile, canine lymphocytes were incubated with rWSP to induce cytokine production. Canine lymphocytes were isolated and exposed to either 5 μg/ml rWSP, 10 μg/ml rWSP, 10 μg/ml ConA, or RPMI media only. Lymphocytes were incubated with mitogens for 48 h or 72 h at 37°C and 5% CO_2. Following incubation, lymphocyte mRNA was isolated to be analyzed for the presence of seven cytokines using reverse transcriptase PCR (RT-PCR). Cytokines analyzed were IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10, and IL-12p40, along with the housekeeping gene, GAPDH.

All samples showed presence of GAPDH, indicating that mRNA was isolated. In addition, production of IFN-γ and TNF-α after lymphocytes were stimulated with ConA indicated that lymphocytes were immunologically viable (Figure 4.2, Figure 4.3). Samples produced IFN-γ when stimulated with rWSP (Figure 4.2). Because IFN-γ is a pro-inflammatory cytokine produced to eliminate intracellular bacteria, production of IFN-γ is a logical response when stimulated with rWSP. Regardless of stimulation, all samples indicated production of pro-inflammatory cytokine, IL-6 (Figure 4.6). However, TNF-α was only produced by heartworm negative dogs when exposed to 5 μg/ml rWSP (Figure 4.3). When incubated with 10 μg/ml rWSP, neither heartworm negative or positive dogs produced TNF-α (Figure 4.3). Perhaps

heartworm positive dogs do not produce TNF-α as an anti-inflammatory response relating to survival of *D. immitis* and/or *Wolbachia*. In addition, greater concentrations of *Wolbachia* seemed to induce immunosuppression by failure of lymphocytes to make TNF-α. Like TNF-α, production of IL-12p40, a pro-inflammatory cytokine, was downregulated when lymphocytes were stimulated with a greater concentration of rWSP (Figure 4.8). At a decreased concentration, rWSP was able to stimulate production of IL-12p40 (Figure 4.8). Canine lymphocytes may not produce cytokines when exposed to increased antigen as a form of immune paralysis. The anti-inflammatory cytokine, IL-4, was upregulated only by heartworm positive dogs (Figure 4.4). Therefore, *D. immitis* and/or *Wolbachia* may induce IL-4 in heartworm positive dogs as a means of evading host immunity. Anti-inflammatory cytokines, IL-5 and IL-10, were not produced (Figure 4.5, Figure 4.7).

Overall, cytokine profile produced through the stimulation of lymphocytes from heartworm positive dogs with rWSP was not representative of a pro-inflammatory response. Although IFN-γ was produced, TNF-α and IL-12p40 were not always produced. *Wolbachia* may induce immunosuppression by stimulating production of the anti-inflammatory cytokine IL-4 and downregulating TNF-α and IL-12p40 when lymphocytes were stimulated with increased concentration of rWSP.

Overall, there was an apparent lack of trends in the data to make substantial claims concerning canine immune response following exposure to rWSP as it pertained to heartworm infection status. In addition, increased incubation time failed to produce results consistent with less incubation time. Although I am unsure why results differed depending on incubation time, some cytokines (such as IL-12) may have only been produced early in response to rWSP.

Results were probably inconclusive due to the dogs used in my study. All dogs were housed in a facility that may expose them to antigen, priming their immune systems.

A dose titration should be done in the future to determine optimal concentration of rWSP for lymphoblastogenesis. In my study, immune paralysis may have occurred several times when increased concentration of rWSP failed to stimulate production of TNF-α, IL-4, and IL-12p40 (Figure 4.3, Figure 4.4, and Figure 4.6). I was unable to do a dose titration because of limited rWSP.

Quantitative PCR should be done in the future to determine if cytokine levels change depending on incubation time and concentration of rWSP. Interleukin 6 was produced regardless of stimulation. Therefore, only a quantitative understanding would determine if rWSP stimulated IL-6 production. Although IFN-γ was always detected, Il-12p40 was not produced when canine lymphocytes were stimulated using greater concentration of rWSP, indicating stimulation with more rWSP leads to immunosuppression. As IFN-γ production relies on IL-12, a quantitative account may indicate that IFN-γ levels decreased with decreasing IL-12. Use of gnotobiotes may give a more realistic account of how rWSP affects the immune system as these animals have never experienced any antigen. In addition, the immune response of mice infected with *Brugia malayi*, can be studied as it pertains to exposure to rWSP. Although mice cannot be infected with *D. immitis*, mice can be infected with *B. malayi*, a filarial symbiont of *Wolbachia*. When nematode strains of *Wolbachia* can be cultured, canine lymphocytes should be exposed to pure *Wolbachia*. As this study only analyzed one protein, a better understanding of canine immune response would come from exposure to the entire bacteria.

Finally, canine lymphocytes should be stimulated with pure antigens from *D. immitis* free of *Wolbachia*. Although this study does indicate some inflammation produced due to rWSP

exposure, dogs experience a profound inflammatory response during the treatment of heartworms (Kramer, et al., 2008). A study analyzing response of lymphocytes from heartworm positive dogs to pure antigens from *D. immitis* may implicate *D. immitis* as the main source of inflammation.

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Appendix A.

Data from Detection of Canine Immunoglobulin G Specific to Recombinant *Wolbachia* Surface Protein

Canine	Group	Mean absorbance at 450 nm
1	HW+mf+	0.214
2	HW+mf+	0.204
3	HW+mf+	0.215
4	HW+mf+	0.190
5	HW+mf-	0.218
6	HW-	0.070
7	HW-	0.093
8	HW-	0.047

Appendix B.

Data from Cytokine Analysis using Reverse Transcriptase Polymerase Chain Reaction

Stimulant	Canine	Group	GAPDH 48 h	GAPDH 72 h	TNF-α 48 h	TNF-α 72 h	IFN-γ 48 h	IFN-γ 72 h	IL-4 48 h	IL-4 72 h	IL-5 48 h	IL-5 72 h	IL-6 48 h	IL-6 72 h	IL-10 48 h	IL-10 72 h	IL-12p40 48 h	IL-12p40 72 h
5 µg/ml rWSP	1	HW+ mf+	yes	yes	yes	no	yes	yes	yes	no	no	no	yes	yes	no	no	yes	no
	2	HW+ mf+	yes	yes	no	no	yes	yes	yes	no	no	no	yes	yes	no	no	yes	no
	3	HW+ mf+	yes	yes	no	no	yes	yes	no	no	no	no	yes	yes	no	no	yes	no
	4	HW+ mf+	yes	yes	no	no	yes	no	yes	no	no	no	yes	yes	no	no	yes	no
	5	HW+mf-	yes	yes	no	no	yes	yes	yes	no	no	no	yes	yes	no	no	yes	no
	6	HW-	yes	yes	yes	no	yes	yes	no	no	no	no	yes	yes	no	no	yes	no
	7	HW-	yes	yes	yes	no	yes	yes	no	no	no	no	yes	yes	no	no	yes	no
	8	HW-	yes	yes	yes	no	yes	yes	no	no	no	no	yes	yes	no	no	yes	no
10 µg/ml rWSP	1	HW+ mf+	yes	yes	yes	no	yes	yes	no	no	no	no	yes	yes	no	no	yes	yes
	2	HW+ mf+	yes	yes	no	no	yes	yes	no	no	no	no	yes	yes	no	no	no	no
	3	HW+ mf+	yes	yes	no	no	yes	yes	yes	no	no	no	yes	yes	no	no	no	no
	4	HW+ mf+	yes	yes	no	no	yes	yes	no	no	no	no	yes	yes	no	no	no	no
	5	HW+mf-	yes	yes	no	no	yes	no	yes	no	no	no	yes	yes	no	no	no	no
	6	HW-	yes	yes	no	no	no	yes	no	no	no	no	yes	yes	no	no	no	no
	7	HW-	yes	yes	no	no	no	yes	no	no	no	no	yes	yes	no	no	no	no
	8	HW-	yes	yes	no	no	no	no	no	no	no	no	yes	yes	no	no	no	no
10 µg/ml ConA	1	HW+ mf+	yes	yes	no	yes	yes	yes	yes	no	no	no	yes	yes	no	no	no	no
	2	HW+ mf+	yes	yes	no	yes	no	yes	yes	no	no	no	yes	yes	no	no	no	no
	3	HW+ mf+	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	no	no	no	no
	4	HW+ mf+	yes	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no
	5	HW+mf-	yes	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no
	6	HW-	yes	yes	no	yes	yes	yes	no	no	no	no	yes	yes	no	no	no	no
	7	HW-	yes	yes	no	yes	no	yes	no	no	no	no	yes	yes	no	no	no	no
	8	HW-	yes	yes	no	yes	no	yes	no	no	no	no	no	yes	no	no	no	no
RPMI only	1	HW+ mf+	yes	yes	no	no	no	no	no	no	no	no	yes	yes	no	no	no	no
	2	HW+ mf+	yes	yes	no	no	no	yes	no	no	no	no	no	yes	no	no	no	no
	3	HW+ mf+	yes	yes	no	no	no	no	no	no	no	no	no	yes	no	no	no	no
	4	HW+ mf+	yes	yes	no	no	no	no	no	no	no	no	no	yes	no	no	no	no
	5	HW+mf-	yes	yes	no	no	no	no	no	no	no	no	no	yes	no	no	no	no
	6	HW-	yes	yes	no	no	no	no	no	no	no	no	no	yes	no	no	no	no
	7	HW-	yes	yes	yes	no	no	no	no	no	no	no	yes	yes	no	no	no	no
	8	HW-	yes	yes	no	no	yes	no	no	no	no	no	yes	yes	no	no	no	no