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Research brief

Litomosoides sigmodontis: Vaccine-induced immune responses against *Wolbachia* surface protein can enhance the survival of filarial nematodes during primary infection

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Abstract

Wolbachia are bacteria present within the tissues of most filarial nematodes. Filarial nematode survival is known to be affected by immune responses generated during filarial nematode infection and immune responses to *Wolbachia* can be found in different species harbouring filarial nematode infections, including humans. Using the rodent filarial model *Litomosoides sigmodontis*, we show that pre-exposure to wolbachia surface protein in a Th1 context (but not in a Th2-context) enhances worm survival on subsequent challenge. This study suggests that despite abundant evidence that pro-inflammatory reactions to the endosymbiont have detrimental effects on the both the nematode and mammalian host, they may under some circumstances be beneficial to the nematode.

Index Descriptors and Abbreviations: *Wolbachia*; *Wolbachia* surface protein; Filarial nematode; *Litomosoides sigmodontis*; Complete Freund's Adjuvant; Alum; Male; Female; Antibody; Type 1 response; Filariasis; Nematode; Th1/Th2; Endosymbiont; Helminth; Antibody isotype; *Ls*WSP, *Litomosoides sigmodontis Wolbachia* surface protein; CFA, Complete Freund's Adjuvant

Wolbachia are intracellular bacteria found in the tissues of most species of filarial nematodes. Within individual filarial nematode species *Wolbachia* appear to be ubiquitously carried (Casiraghi et al., 2004) and the phylogeny of both the bacteria and their nematode host are closely matched (Bandi et al., 1998). Elimination of *Wolbachia* from nematode tissues using the antibiotic tetracycline has been shown to be an effective treatment against both onchocerciasis and lymphatic filariasis in humans, primarily via effects on nematode fertility (Hoerauf, 2006). Based on this information, *Wolbachia* and filarial nematodes are assumed to have co-evolved to the point where *Wolbachia* are now essential symbionts of the filarial nematodes that harbour them.

The manipulation of the host immune system by filarial nematodes is a key determinant of their survival within the mammalian host. Immune responses to *Wolbachia* can occur in human filarial infection (Lamb et al., 2004; Punkosdy et al., 2003; Suba et al., 2007) and appear to be generated, at least in part, from dying filarial nematodes, in particular incoming L3 stages (Lamb et al., 2004). Previous studies indicate that immune responses elicited by *Wolbachia* are required for granuloma formation around established adult filarial nematodes (Brattig et al., 2001) which are generally associated with dying nematodes. Innate pro-inflammatory responses to *Wolbachia* extracts involve Toll receptor 2 (TLR2) and TLR6 (Hise et al., 2007) and the *Wolbachia* surface protein (WSP) can signal through Toll receptor 4 (TLR4) (Brattig et al., 2004). TLR4-dependent immune responses have been linked to impaired fertility in *Litomosoides sigmodontis* (Pfarr et al.,

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2003). Therefore anti-*Wolbachia* immune responses have thus far been associated with traits unfavourable to filarial nematode fitness. This prompted us to investigate whether vaccine-induced anti-*Wolbachia* immune responses would have any protective effect against filarial nematode infection.

To test this hypothesis, we generated anti-*Wolbachia* surface protein (WSP) immune responses in male BALB/c mice 4–6 weeks of age (Harlan). WSP from the *Wolbachia* of *L. sigmodontis* (*Ls*WSP) was cloned and purified as described previously (Lamb et al., 2004). Since it is currently unclear whether in the context of filarial infection anti-*Wolbachia* immune responses occur under type 1 conditions that are normally evoked by bacteria, or type 2 conditions that normally prevail in filariasis, we generated anti-WSP immune responses under predominantly type 2 conditions using the adjuvant Alum, and under predominantly type 1 conditions using complete Freund's adjuvant (CFA). For Alum vaccinations, *Ls*WSP was precipitated in 9% Aluminium potassium sulphate solution (Sigma). Mice were given 3 doses of 50 μg of *Ls*WSP in 100 μl sterile PBS subcutaneously at 4 week intervals. Control animals were vaccinated with hen egg lysozyme (HEL) precipitated in Alum. HEL was included as a protein control as alum contains no protein. For CFA vaccinations, *Ls*WSP was emulsified with adjuvant (Sigma) by sonication on ice. Each mouse was given 3 doses of 20 μg of *Ls*WSP in a 100ul emulsion, subcutaneously, again at 4-week intervals. CFA was used for the first dose, with the remaining two doses given in incomplete Freund's adjuvant (IFA). Control animals were vaccinated with PBS in adjuvant alone.

Serum antibody isotypes were assessed to determine the magnitude and nature of the immune response to vaccination. IgG2a is the main isotype produced by B cells in response to IFN-γ in mice whereas the type 2 cytokine IL-4 switches B cells to produce IgG1 (Snapper and Paul, 1987). Whilst switching of B cells *in vitro* to produce IgG2a has been shown to be dependent on IFN-γ, switching to produce IgG1 is less strictly dependent on IL-4 to the extent that it can be triggered by cloned T cell lines displaying a type 1 phenotype (DeKruyff et al., 1990). Therefore IgG2a can be considered a reliable marker for a type 1 response whilst the generation of IgG1 as a marker for type 2 is less definitive.

Antibodies to *Ls*WSP in the vaccinated animals were detected by ELISA as described previously (Lamb et al., 2004). There was a good total IgG response to vaccination in both Alum-vaccinated and CFA-vaccinated animals (Fig. 1a). Alum vaccination generated anti-*Ls*WSP IgG1 but not IgG2a, whereas CFA vaccination induced an anti-*Ls*WSP response of both isotypes (Fig. 1b). A correlation of IgG1 vs IgG2a responses showed that some animals vaccinated with CFA did not produce any IgG1 or IgG2a above control vaccinations, whilst animals that did respond with these isotypes generally made antibodies against *Ls*WSP of both isotypes (Fig. 1c). This data is consistent with other studies that have found that the generation of immune responses with CFA induces both IgG2a and IgG1 whilst only IgG1 can be measured in animals injected with Alum (Brewer et al., 1996). Cytokine responses measured by Brewer et al. (1996) determined that the mixed IgG1/IgG2a generated in animals injected with CFA was associated with cultured splenocyte IFN-γ secretion and a low level of IL-5 secretion. On the other hand generation of immune responses by Alum was associated with little or no IFN-γ, and high production of either IL-5 or IL-4 (Brewer et al., 1996). This data demonstrates that CFA induces immune responses that are polarised towards type 1 whilst Alum induces immune responses that are polarised towards type 2.

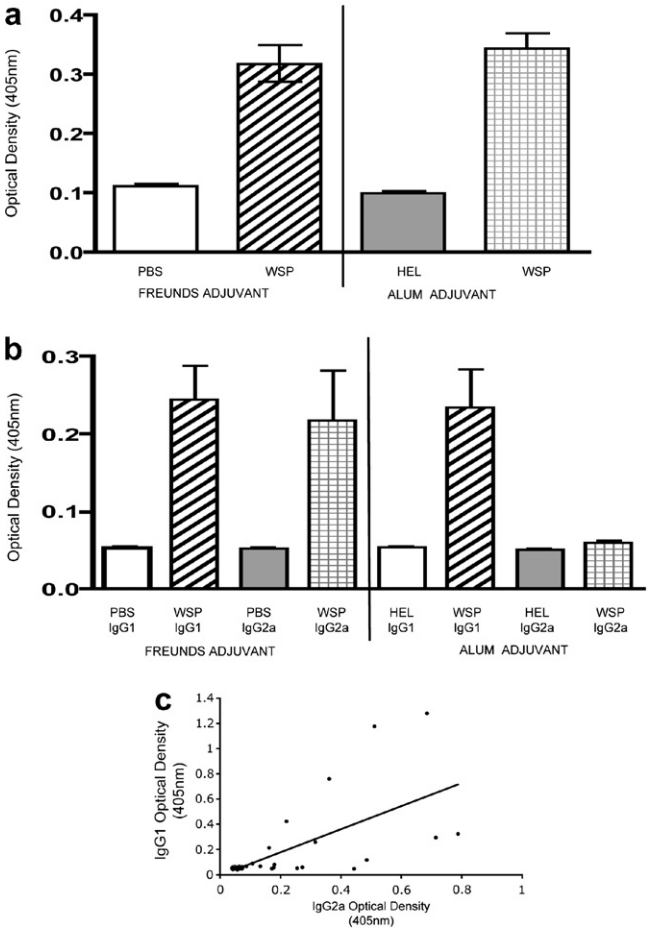

Fig. 1. Animals immunised with *Ls*WSP produced antibody responses to *Ls*WSP. Total IgG responses against *Ls*WSP are shown in (a). IgG1 and IgG2a responses against *Ls*WSP are shown in (b). A scatterplot representing of IgG1 and IgG2a responses from individual animals is shown in (c). Each symbol represents a single mouse. Sera was diluted 1:200 in all cases and 50 μl of sera samples diluted 1:200 in PBS with 0.5% Tween (PBST) was loaded into each duplicate well. Antibodies were detected with 50 μl of peroxidase-conjugated goat anti-mouse total IgG (1:1000, Bio-Rad), peroxidase-conjugated goat anti-mouse IgG1 (1:6000, Southern Biotech Associates, Inc.) or peroxidase-conjugated goat anti-mouse IgG2a (1:4000, Southern Biotech Associates, Inc.) diluted in PBST.

Having established the success of the anti-*Ls*WSP vaccinations, we next infected these animals with the rodent

filarial nematode *L. sigmodontis*. The life cycle of *L. sigmodontis* was maintained by cyclical passage of the parasites between *Meriones unguiculatus* jirds, and the mite species *Ornothonyssus bacoti*. Each experimental mouse was injected with 25 L3s subcutaneously in the left lumber area and at 60 days post-infection the number and sex of the surviving adult parasites in the pleural cavity was recorded. To eliminate investigator bias, nematode recovery and sex determination were performed blind. The mice were maintained in individual ventilated cages and given food and water *ad libitum* at all times. Two experiments were conducted for each adjuvant.

Twenty-eight animals vaccinated with *Ls*WSP precipitated in Alum (13 animals in the first experiment and 15 animals in the second experiment) and 27 control animals vaccinated with HEL precipitated in Alum (13 animals in the first experiment and 14 in the second experiment) were included in the analyses for *Ls*WSP responses induced with Alum adjuvant. A further 27 animals were vaccinated with *Ls*WSP in CFA (8 animals in the first experiment and 19 animals in the second experiment) and 24 animals were vaccinated with CFA emulsified with PBS alone (10 animals in the first experiment and 14 animals in the second experiment). Mice were challenged 4–6 weeks following the final immunization.

A number of animals in each group were able to clear their *L. sigmodontis* infection completely (3 of 27 animals for WSP in CFA, 5 of 24 PBS/ CFA control animals, 8 of 28 animals injected with WSP in Alum and 5 of 27 HEL in Alum control animals). Therefore, whilst 18.5% (HEL in Alum) and 20.8% (PBS/ CFA) of control animals were able to clear infection, fewer animals vaccinated with *Ls*WSP in type-1 inducing CFA were able to clear their infection (11.1%), and more animals vaccinated with *Ls*WSP in type-2 only inducing Alum did so (28.6%), although neither of these trends were significant (CFA vaccinations Fishers exact test $P = 0.4$; Alum vaccinations Fishers exact test $P = 0.5$).

For nematode survival, ANOVA was carried out after square-root transformation of the number of adult nematodes recovered from each mouse. Statistical analyses were carried out on data combined from both repeat experiments using parametric analysis of variance (ANOVA, Minitab, Inc.) to remove experimental effects for analyses of data from more than one experiment. For more detailed explanations of the statistical methods used see Grafen & Halls (Grafen and Halls, 2002). There were no significant experiment-by-treatment interactions observed in this analysis and quoted *P*-values refer to analyses with all non-significant terms removed. The model used to assess nematode recovery was adjuvant + vaccination treatment + adjuvant *vaccination treatment. The data are reported as the arithmetic mean ± standard error of the mean (SEM) and this analysis included animals which cleared the infection with *L. sigmodontis* completely.

Significantly more adult parasites were recovered from animals vaccinated with *Ls*WSP in CFA (5.11 ± 0.65 nematodes) compared to animals vaccinated with CFA alone (2.75 ± 0.47 nematodes) whilst vaccination with Alum-precipitated *Ls*WSP and Alum-precipitated HEL resulted in similar nematode recovery (3.0 ± 0.46 nematodes and 2.39 ± 0.49 nematodes, respectively) (interaction term between adjuvant and vaccination, ANOVA $F_{1,102} = 4.95$, $P = 0.028$) (Fig. 2). Therefore type 1 anti-*Ls*WSP immune responses, but not type 2 anti-*Ls*WSP immune responses, were associated with a greater survival of *L. sigmodontis* nematodes.

Although we measured anti-*Ls*WSP antibody responses as a readout for the success of our vaccination protocol, we do not know which component of the immune response mediated the observed positive effect on worm recovery. Antibody-mediated mechanism could be involved since the anti-*Ls*WSP IgG2a antibodies generated in vaccination with CFA, but not alum (Fig. 1b), were positively correlated with female nematode recovery (square root transformed females, $F_{1,24} = 5.31$, $P = 0.03$) but not male nematode recovery ($F_{1,25} = 2.79$ $P = 0.107$) in the *Ls*WSP/CFA vaccinated animals (data not shown). However the correlation with female nematode recovery was relatively weak and unduly affected by some outliers (with outliers removed $F_{1,22} = 0.00$, $P = 0.967$). Further, IgG2a responses are likely to be a reflection of the Th1 bias of the CFA immunisation protocol rather than a direct cause of altered worm recovery.

We have previously shown that a major component of the immune responses against *Wolbachia* (as measured by anti-WSP antibodies) is generated by the L3 stage (Lamb et al., 2004). Since most L3 stages infect hosts in endemic areas that already have immune responses to WSP (Lamb et al., 2004; Punkosdy et al., 2003), the *L. sigmodontis* model allowed us to ask what effects anti-*Wolbachia* immune responses have on filarial nematode infection which has been established subcutaneously with the L3 stage. We have shown that pre-existing anti-WSP responses generated in CFA can almost double the number of adult nematodes

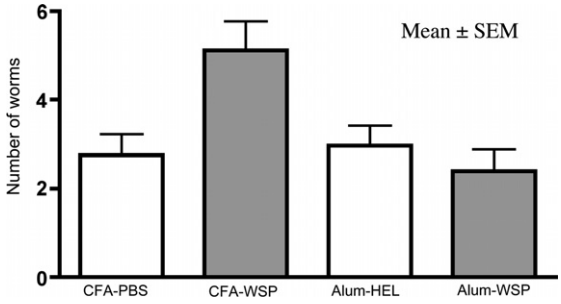

Fig. 2. The recovery of filarial nematodes is greater in animals with pre-existing anti-WSP responses generated with Freud's adjuvant but not Alum. The number of nematodes from the infective larvae innoculum recovered at 60 days post-infection is shown. The white bars represent the control animals immunised with PBS in CFA or HEL in Alum. The shaded bars shows animals immunised with *Ls*WSP in CFA or the immunised with *Ls*WSP in Alum. The error bars represent the standard error of the mean for each group of animals.

recovered at the end of 60-day primary infections of BALB/c mice (Fig. 2). It is unclear whether pre-existing CFA-induced anti-WSP responses acted to increase the establishment of the L3 stage, or whether these responses helped later stages to survive. However the previous data by Brattig et al. (2001) and Pfarr et al. (2003) indicate that pro-inflammatory responses induced by *Wolbachia* are detrimental to the adult stage suggesting the former may be more likely. In either case, one must consider the mechanisms by which immune responses generated against a target that is contained within nematode tissues (presumably released upon nematode death) could be of benefit to live nematodes.

First, in this study we have generated Th1-type anti-WSP responses through vaccination but the nature of the anti-*Wolbachia* responses in the context of filarial infection is not fully understood. Immunisation with filarial extracts induce a predominantly Th2 response in mice, despite the presence of *Wolbachia* (Allen et al., 1995). However antibody responses against *Wolbachia* proteins in areas endemic for *Onchocerca volvulus*, *Wuchereria bancrofti* and *B. malayi* infection are of the IgG1 isotype (similar to murine IgG2a) (Brattig et al., 2004; Fischer et al., 2003; Suba et al., 2007) supporting the case that *Wolbachia* may be seen by the immune system in a pro-inflammatory context. Even in the most permissive host, a proportion of inoculated L3 will die within the first few hours. Therefore, in mice immunised with *Ls*WSP in CFA the dying L3 may release *Wolbachia* that boost the pro-inflammatory IFN-γ response. Thus early local production of IFN-γ may promote the longer-term survival of the remaining L3 by down-regulating the development of Th2 cells that mediate resistance (Le Goff et al., 2002; Volkmann et al., 2003). In addition, Ravindaran (Ravindran, 2001) has hypothesised that type 1 cytokines actually assist the growth and development of filarial parasites. This hypothesis is consistent with evidence that NK cells, which produce IFN-γ, are required for the development of *B. malayi* larvae (Babu et al., 1998). Thus, on a proximate level, we hypothesise that pre-existing anti-WSP responses induced by CFA vaccination may promote L3 survival through increased IFN-γ production in response to infection. Pre-existing Th1 responses may promote infection in a susceptible host (such as the BALB/c mouse used here) but would not be sufficient to overcome resistance in a non-permissive host or fully immune host.

The ultimate influences contributing to our observation are hard to visualise if the only source of *Wolbachia* is from dead and dying pre-patent larvae. Filarial *Wolbachia* are obligate intracellular bacteria. Therefore initiation of immune responses from *Wolbachia* released from dead and dying nematodes would offer no selective advantage because they will die in the absence of a reproducing filarial nematode host. Although evidence for the release of *Wolbachia* from live filariae is currently lacking, it is possible that *Wolbachia* may be released from pre-patent stages via the excretory-secretory products and/or from the material released during moulting of filarial nematodes.

The original papers of McLaren et al. (1975) demonstrated that numbers of *Wolbachia* are not homogenous between nematodes, an observation which has recently been verified within species, sex and life cycle stages by quantitative PCR (Fenn and Blaxter, 2004a; McGarry et al., 2004). If *Wolbachia* do indeed form an obligate mutualist relationship with filarial nematodes then this variation may result in differences between the fitness of individual filarial nematodes. The observation by McLaren et al. (1975) that heavily infected larvae have arrested development indicate that the bacteria are not merely silent passengers of filarial nematodes, and the observed variation in *Wolbachia* load between nematodes may provide an opportunity for selection to operate.

Wolbachia are dependent on the survival of their filarial nematode host and, as transmission is exclusively vertical, they are also dependent on the successful mating of the nematodes within the mammalian host and the subsequent transmission of the microfilaria to mosquitoes. By promoting some, or all of these aspects in their filarial host, *Wolbachia* would maximise their own chances of transmission. Thus, as suggested by the numerous experiments showing that reduced nematode fitness results from removing *Wolbachia* via chemotherapeutic means, *Wolbachia* should bring benefits to its nematode host.

In conclusion we have observed that, surprisingly, the presence of anti-WSP immune responses can enhance filarial nematode infection. Further characterisation of anti-WSP responses, the conditions in which they naturally occur in filarial nematode infection, and the mechanism by which these responses promote nematode infection are needed to interpret the indirect effects *Wolbachia* exert on filarial nematode biology via the mammalian immune system.

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