

Wolbachia bacteria in filarial immunity and disease

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SUMMARY

Lymphatic filarial nematodes are infected with endosymbiotic Wolbachia bacteria. Lipopolysaccharide from these bacteria is the major activator of innate inflammatory responses induced directly by the parasite. Here, we propose a mechanism by which Wolbachia initiates acute inflammatory responses associated with death of parasites, leading to acute filarial lymphangitis and adverse reactions to antifilarial chemotherapy. We also speculate that repeated exposure to acute inflammatory responses and the chronic release of bacteria, results in damage to infected lymphatics and desensitization of the innate immune system. These events will result in an increased susceptibility to opportunistic infections, which cause acute dermatolymphangitis associated with lymphoedema and elephantiasis. The recognition of the contribution of endosymbiotic bacteria to filarial disease could be exploited for clinical intervention by the targeting of bacteria with antibiotics in an attempt to reduce the development of filarial pathology.

Keywords *Wolbachia, pathogenesis, inflammation, symbiosis, filariasis*

INTRODUCTION

A recent and exciting breakthrough in filarial research is the discovery that endosymbiotic *Wolbachia* bacteria play an important role in the biology of filarial nematodes (1). Studies so far indicate that all lymphatic filarial parasites are infected with closely related bacteria, and that these occur throughout the geographical distribution of the species (2–4) (Figure 1). Phylogenetic analysis and the effects of antibiotic therapy on embryogenesis, development and viability show that *Wolbachia* appears to have evolved an essential mutualistic association with its filarial hosts (3,5). The pervasive presence of large numbers of endosymbionts throughout all stages of the pathogenic filariae of humans suggest that the host will be exposed to *Wolbachia* following death of the parasite or through the release of bacterial products. Here, therefore, we will consider the role of *Wolbachia* in the immune response to filariasis and in the pathogenesis of disease.

WOLBACHIA IN THE PATHOGENESIS OF ACUTE INFLAMMATORY PATHOLOGY

Our initial encounter with *Wolbachia* came from studies aimed at understanding the inflammatory pathogenesis of filarial disease. We focused on the role of parasite-derived mediators in the activation of innate inflammatory responses, based on the ability of *Brugia* sp. to cause lymphatic pathology in mice in the absence of T cells and opportunistic infection (6,7) and the association of inflammatory responses with the death of parasites. These studies showed that soluble extracts of the human filarial parasite *B. malayi* could induce potent innate inflammatory responses, including the release of tumour necrosis factor (TNF)- α , interleukin (IL)-1 β and nitric oxide (NO) from macrophages. The active component was heat-stable, reacted positively in the *Limulus* amoebocyte lysate (LAL) assay, and could be inhibited by Polymyxin B, characteristics which bear all the hallmarks of a bacterial endotoxin or lipopolysaccharide (LPS)-like molecule (8).

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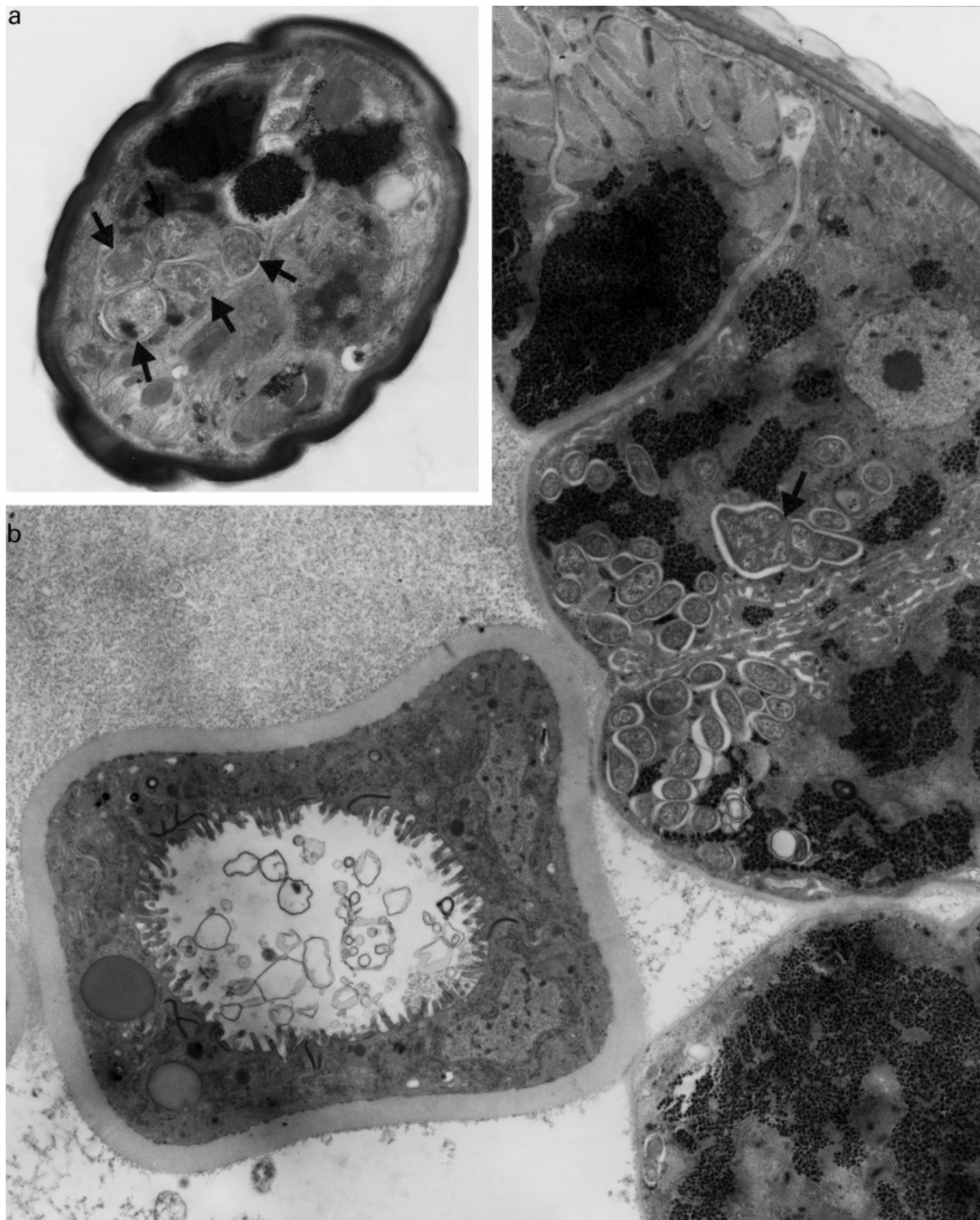


Figure 1 *Wolbachia* in human lymphatic filarial nematodes. (a) Bacteria (arrows) in a microfilaria of *Wuchereria bancrofti*. (b) Bacteria (arrows) in the lateral cord of *Brugia malayi*.

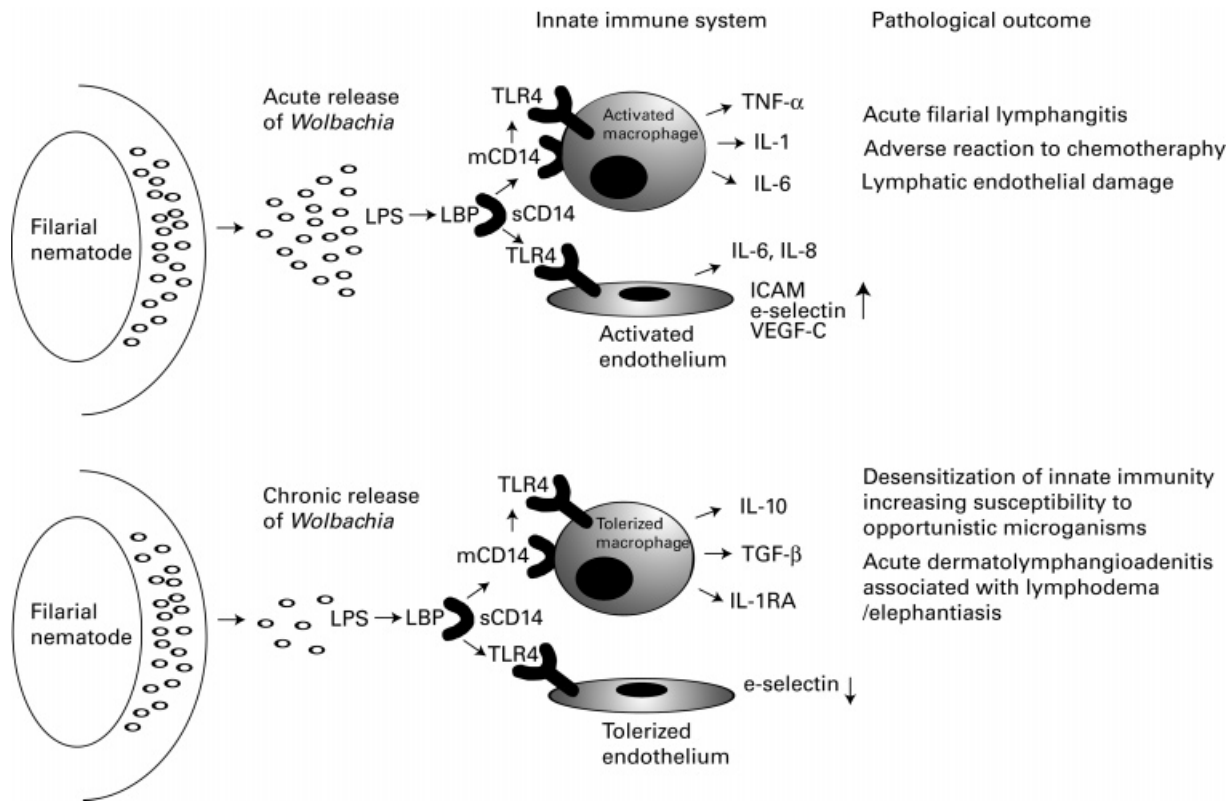


Figure 2 An overview of the proposed mechanisms by which *Wolbachia* contributes to the pathogenesis of lymphatic filarial disease.

LPS is one of the most potent and well-studied mediators of inflammation and is thought to play a fundamental role in the development of Gram negative bacterial sepsis (9). A number of recent advances have been made in the understanding of how LPS generates inflammatory responses via activation of the innate immune system (10,11). Recognition of LPS and the activation of innate immune responses play a major role in the control of bacterial infection (12). Pathogenesis results from the overwhelming stimulation of systemic inflammatory responses, which can lead to tissue damage, septic shock and multiple organ failure (13). The sequence of events leading to activation of innate inflammatory responses by LPS begins with binding to the serum protein, LPS binding protein (LBP), which facilitates the transfer of LPS to CD14 (14). Membrane-bound CD14 (mCD14) is a pattern recognition receptor expressed predominantly on monocytes, macrophages and neutrophils, and can also function as a soluble receptor presenting LPS to mCD14 negative cells including endothelial and epithelial cells, smooth-muscle cells and dendritic cells (14). The key LPS receptor involved in signal transduction of inflammatory response genes has recently been shown to be the Toll-like 4 receptor (TLR4), one of an ancient family of receptors central to the defence system of mammals, insects and

plants (10,11,15). The activation of TLR4 by the LPS-CD14 complex leads to a signalling cascade that results in the activation of NF-κB and transcription of several inflammatory response genes including TNF-α, IL-1β and IL-6 (10,11,16). We have shown that the production of TNF-α, IL-1β and NO from macrophages by LPS-like molecules in soluble extracts of *B. malayi* also requires CD14 and TLR4 (Figure 2) (8) and is enhanced in the presence of serum as a source of LBP (unpublished observations). A recent study by Brattig *et al.* (17) has also shown that LPS-like molecules in extracts of *Onchocerca volvulus* activate human monocytes to produce TNF-α, through binding to CD14.

Evidence to suggest that the LPS-like activity is derived from *Wolbachia* came from experiments on *Acanthocheilonema viteae*, one of only a few filarial parasites free of *Wolbachia* infection (1,3,8). Soluble extracts derived from *A. viteae* failed to induce any inflammatory responses from macrophages and were negative in the endotoxin LAL assay (8,17). Not only does this suggest that *Wolbachia* are the source of LPS-like molecules, but it also shows that soluble extracts prepared in this way contain no additional mediators of inflammatory responses derived from the nematode. Extracts prepared from insect *Wolbachia*,

derived from a mosquito cell-line, also induced LPS-like responses, which were dependent on TLR4 and eliminated following antibiotic clearance of the bacteria (8). Taken together, these data support the idea that *Wolbachia* LPS is the major mediator of inflammatory responses induced directly by the parasite.

Studies using living parasites or culture supernatants failed to stimulate inflammatory responses from macrophages (8), suggesting that the release of *Wolbachia* and/or LPS in sufficient amounts to induce inflammatory responses is only likely to occur following the death of parasites. This is consistent with the association of acute inflammatory episodes with the death of adult parasites and inflammatory adverse reactions following chemotherapy. In animal models, the sensitization of rodents to the toxic effects of LPS with D-galactosamine led to lethal shock-like adverse reactions following antifilarial chemotherapy (18). These adverse reactions could be prevented by inhibitors of TNF- α and NO and are consistent with the release of LPS following parasite death. Similar lethal shock reactions are observed in dogs injected with extracts of *Dirofilaria immitis* or following chemotherapy (19–21). In order to determine whether LPS is responsible for the induction of inflammatory responses post-treatment, we infected LPS responsive (C3H/HeN) and nonresponsive (C3H/HeJ) mice with *B. malayi* microfilariae and treated them with ivermectin. TNF- α production was only observed following chemotherapy in LPS-responsive C3H/HeN mice, and not C3H/HeJ mice which carry a mutation in the TLR4 receptor (8), supporting the hypothesis that *Wolbachia* LPS released from dead microfilariae mediates inflammatory responses post-treatment.

Ongoing studies in our laboratory have also shown that in humans infected with *B. malayi*, *Wolbachia* are released into the blood following DEC chemotherapy in individuals presenting with severe inflammatory adverse reactions (Cross *et al.*, unpublished data). Adverse reactions in these individuals is accompanied by the release of pro-inflammatory cytokines and inflammatory mediators including IL-1 β , IL-6, interferon (IFN)- γ , TNF- α , NO and LBP and occurs predominantly in individuals with high microfilarial loads (22–28). Localized inflammation following chemotherapy is also associated with the death of adult worms in the lymphatics, an event which is also thought to account for the pathogenesis of acute filarial lymphangitis (29). The severity and presentation of fever associated with acute lymphatic filarial disease also correlates with the systemic production of TNF- α (30). These studies support the idea that the release of *Wolbachia* following the death of parasites activates inflammatory responses, leading to acute inflammatory pathology associated with death of adult worms and adverse reactions to chemotherapy.

WOLBACHIA IN THE PATHOGENESIS OF CHRONIC INFLAMMATORY PATHOLOGY

The presentation of chronic pathology in lymphatic filariasis develops after several years exposure to infection and is characterized by hydrocoele, lymphoedema and elephantiasis. The events that lead to the development of chronic pathology are poorly understood, but the risk of developing chronic disease is associated with an increased frequency of acute filarial lymphangitis (31,32). Evidence for the role of inflammatory responses in the pathogenesis of chronic pathology is shown by the presence of high levels of inflammatory cytokines including IL-1 β , IL-6, IL-8, TNF- α and granulocyte-macrophage colony-stimulating-factor in fluid from limb lymphoedema and hydrocoele (33) and from the parasitized lymphatics of immunodeficient mice (34). Systemic inflammatory cytokines and receptors including IL-6, IL-8 and sTNF-R75 have also been shown to be elevated in individuals with elephantiasis (26) and hydrocoele (our unpublished observations), indicating an active and ongoing inflammatory response in some individuals with chronic pathology. How might the release of *Wolbachia* lead to the development of chronic pathology?

In addition to the release of large numbers of *Wolbachia*, which accompany acute inflammatory episodes, chronic exposure to *Wolbachia* is likely to occur following the natural attrition of parasites. This will include the constant turnover of microfilariae and exposure to L3–L4 larvae, which fail to achieve complete development. An additional source of endosymbiont release may occur during the birth of microfilariae as part of the debris from the uteri or through excretory or secretory processes.

Following exposure to LPS, the production of pro-inflammatory responses is regulated by anti-inflammatory mediators including IL-4, IL-10, IL-13, transforming growth factor (TGF)- β , IL-1Ra, glucocorticoids, prostaglandin E₂ and pro-inflammatory cytokine soluble receptors (13). This is thought to provide protection from the uncontrolled immunological activation of acute endotoxic shock, but can lead to an inability to respond appropriately to secondary infections in survivors of endotoxic shock. At the cellular level, repeated exposure to LPS, or exposure to low doses, results in the development of a state known as 'LPS tolerance' in which cells show a reduced sensitivity to subsequent exposure to LPS through differential regulation of pro- and anti-inflammatory cytokines (35–37). Although the mechanisms leading to LPS tolerance have yet to be defined, it is thought to be due to alterations in signalling pathways and is associated with the downregulation of cytokine, chemokine and TLR4 expression (38,39). LPS tolerance has been mostly studied in monocytes and

macrophages but similar phenomena have been described in endothelial cells (40) and neutrophils (41).

The chronic release of *Wolbachia* may therefore result in the desensitization of cells of the innate immune system. This, together with the damage inflicted by acute inflammatory episodes on the structure and function of parasitized lymphatics, would promote the establishment of opportunistic infections acquired from the environment as occurs during acute dermatolymphangioadenitis (ADLA) associated with chronic lymphodema and elephantiasis (29,32,42).

THE INFLUENCE OF *WOLBACHIA* INFLAMMATORY MEDIATORS ON OTHER CELLS

Although monocytes and macrophages are the principal cells responsible for the activation and regulation of innate inflammatory responses, LPS can influence the function of a wide variety of other cells, including endothelial and epithelial cells, fibroblasts, lymphocytes, granulocytes, smooth muscle cells and adipocytes (43,44). Of direct relevance to the blood- and lymph-dwelling lymphatic filariae are the endothelial lining of the vascular and lymphatic vessels, the reticulo-endothelial system of organs, such as the lung, liver and spleen, and blood leucocytes.

LPS can directly activate endothelial cells to produce cytokines, chemokines and adhesion molecules, which promote the recruitment and activation of blood leucocytes (13). Endotoxin can also cause endothelial cell damage through loss of barrier function and integrity (45). Activation of endothelial cells by LPS requires the participation of LBP and soluble CD14 (46) which, through engagement of TLR4, activates signalling pathways leading to the activation of NF κ B in a similar manner to monocytes/macrophages (47,48). In the context of lymphatic endothelium, recent studies have shown that vascular endothelial growth factor VEGF-C and its receptor VEGFR-3, are specific regulators of lymphatic endothelial activation and angiogenesis (49). Overexpression of VEGF-C in the skin of transgenic mice resulted in lymphatic endothelial proliferation and dilation of vessels (50) with a striking resemblance to lymphatics infected with filarial parasites. Pro-inflammatory cytokines, including IL-1 β and TNF- α , have been shown to upregulate the expression of VEGF-C, raising the possibility that pro-inflammatory cytokines affect the lymphatic vessels via VEGF-C (51). Lymphatic endothelium can also respond to LPS with the production of NO through activation of inducible nitric oxide (52).

Studies on the morphology of infected lymphatics in animal models show that the endothelium appears activated

with associated adherent mononuclear cells (34). Pro-inflammatory cytokines that can stimulate the proliferation of lymphatic endothelia (53) are elevated in lymph from parasitized lymphatics (54). The activation of lymphatic endothelium may be important in controlling the composition and pressure of interstitial fluid and in facilitating lymphocyte trafficking and thus have an important role in inflammatory processes in filarial pathology. Furthermore, activation of endothelial cells and the promotion of lymphangiogenesis and hyperplasia leading to vessel dilation may even be necessary for the survival of adult worms within the lymphatics.

Activation of innate immune cells leads to the release of several chemokines, which recruit leucocytes to the site of inflammation and contribute to tissue damage (13). Neutrophils and eosinophils can be directly activated by LPS to produce pro-inflammatory cytokines and NO (55), which can lead to a delay in apoptosis (56). Evidence to link *Wolbachia* with the presence of neutrophils comes from a recent study by Brattig *et al.* (57) on the granulocyte responses in nodules of adult *Onchocerca* species. The study showed that neutrophils occur only in nodules from species with *Wolbachia*, but are absent from nodules of *O. volvulus* depleted of *Wolbachia* by antibiotics, and in cellular responses to *Wolbachia*-free *Onchocerca flexuosa*. Chemotactic factors for neutrophils have also been demonstrated from extracts of *O. volvulus* (58). Neutrophils and eosinophils are activated following chemotherapy and are thought to contribute to the Mazzoti reaction (59,60). Interestingly, in addition to neutrophils, eosinophils are also recruited to tissues following exposure to LPS (61).

LPS is well known as an activator of B cell proliferation and polyclonal antibody production and can be regarded as a classical T-independent antigen (62). Recent studies have also shown that LPS can have potent effects on T cells. Injection of LPS results in the activation of both naïve and memory CD4⁺ and CD8⁺ T cells, with proliferation of memory CD8⁺ T cells (63). Further studies have shown that similar LPS activation of T cells leads to profound apoptosis of T cells (64). Following injection of humans with LPS, stimulation of peripheral T cells results in a reduced production of IFN- γ and IL-2, whilst IL-4 and IL-5 responses were either unaffected or slightly increased, implying a shift towards Th2 cytokine responses (65). The influence of LPS on T cells is thought to occur indirectly through effects on antigen presenting cells (APC) as shown by the ability of APC from LPS responsive mice to restore T cell activation in LPS nonresponsive animals (63). LPS has potent effects on the activation, differentiation and migration of dendritic cells (DC), influencing their ability to process and present antigen to T cells (66). Although LPS stimulates the upregulation of major histocompatibility

complex (MHC) Class II and costimulatory molecules in DCs (66), it can have the opposite effect on monocytes, macrophages and liver sinusoidal endothelial cells, which leads to a downregulation of T cell activation (17,67). IL-12 derived from DCs can also regulate Th1 development by activation of B cells to produce IL-10 and IL-6 which both promote Th2 development and downregulate Th1 differentiation (68). Clearly the activation of innate immune responses are critical in defining the regulation of acquired immune response through expression of costimulatory molecules and effector cytokines (69).

WOLBACHIA AND ACQUIRED IMMUNE RESPONSES

The analysis of acquired immune responses to filarial parasites with native worm preparations will inevitably include antibody and cellular responses to *Wolbachia* antigens. These responses may be to cross-reactive conserved bacterial antigens or those specific to *Wolbachia*. Several *Wolbachia* antigens have already been shown to be immunogenic, with the detection of antibodies from infected individuals to *Wolbachia* hsp60, catalase and the outer membrane protein *wsp* [Koszarski, cited in (17), our unpublished observation, 70]. Further studies with purified *Wolbachia* and nematodes depleted of bacteria by antibiotics, together with recombinant *Wolbachia* proteins, will be useful to characterize other antigens derived from the bacteria and determine their association with clinical presentation.

Many studies have investigated the concept of 'tolerance' to T cell responses during filarial infection. Several mechanisms to account for this phenomenon have been proposed with varying support from experimental and clinical studies (71). In particular, it is not clear what the functional significance of T cell tolerance is, if any, to immunity or disease. Evidence from a study on LPS-like molecules from *Onchocerca volvulus* *Wolbachia* show that the induction of TNF- α from purified human monocytes leads to the production of IL-10, resulting in the downregulation of HLA-DR and costimulatory molecules B7-1 and B7-2 (17). The combination of IL-10 activity inhibiting Th1-like responses and the downregulation of HLA and costimulatory receptors would be likely to have a profound effect on the expression of T cell tolerance. Studies in human bancroftian filariasis and experimental animal models lend support to the role of IL-10 and downregulation of costimulatory molecules in the regulation of T cell responses (72–74). In onchocerciasis, the production of IL-10 and TGF- β from Th3 cells has been suggested to mediate cellular hyporesponsiveness (75). The lack of downregulation of responses to other nematode antigens

from *Ascaris* sp. was interpreted as evidence for an *O. volvulus* antigen specific effect (75). Although this may be the case, an alternative interpretation could be that hyporesponsiveness was only induced with antigens from a nematode containing *Wolbachia* and LPS (17,76).

Taken from the view presented here that the natural history of filariasis is associated with acute and chronic exposure to inflammatory stimuli from *Wolbachia*, the development of an anti-inflammatory immune response may be generated by the host predominantly to regulate this inflammation. This would be consistent with the rapid onset of acute filarial inflammation and pathology in people not previously exposed to infection (77,78) and in early prepatent infections in animals (79).

OTHER WOLBACHIA INFLAMMATORY MEDIATORS

Although LPS is considered to be the major mediator of inflammatory responses in Gram-negative bacteria, other mediators have been identified as key molecules in the pathogenesis of bacterial disease, including heat shock proteins, CpG motifs in DNA, lipoproteins and peptidoglycan (80–82). Although *Wolbachia* LPS appears to be the major inducer of IL-1 β , TNF- α and NO responses from macrophages, other inflammatory stimuli may play a role in the activation of alternative inflammatory responses or contribute additively or synergistically with LPS.

Heat-shock protein 60 or Chaperonin 60 (hsp60) is one of an abundant and conserved family of proteins which play a fundamental role in the post-translational folding, assembly and targeting of proteins within prokaryotic and eukaryotic cells (83). Microbial hsp60 is well characterized as a major antigen of immune protection or pathogenesis of bacterial infection in the stimulation of both antibody and T cell responses (84). Mammalian hsp60 can also function as an autoantigen during chronic inflammation (85). Recently, bacterial and human hsp60 have been shown to activate potent innate immune responses from macrophages and endothelial cells and so provide a 'danger' signal to antigen presenting cells and contribute to bacterial inflammation (80,86,87). Surprisingly, hsp60 activation of macrophages is dependent on CD14 and TLR4, showing that hsp60 stimulation of innate immunity uses the same pathways as LPS (88,89).

Preliminary studies in our laboratory show that recombinant *Wolbachia* hsp60 can stimulate potent TNF- α and IL-6 production from macrophages. These responses are dependent on CD14 and TLR4 but, in contrast to LPS, are unaffected by polymyxin B and serum LBP. The possible upregulation of *Wolbachia* hsp60 in response to stressors, including antifilarial and antibiotic treatment, exposure to

immunological effector molecules or fever, may influence the contribution of hsp60 to inflammatory responses.

Another recently discovered mediator of bacterial inflammation is bacterial DNA (82). Pattern recognition receptors of the innate immune system recognize unmethylated CpG motifs of bacterial DNA. Macrophages, dendritic cells and NK cells are activated to produce cytokines including IFN- γ , IL-12, IL-18, TNF- α and IL-6, which promote Th1 cell development. B cells can also be activated to proliferate and produce polyclonal immunoglobulin (90). The immunostimulatory action of CpG motifs on dendritic cells and the enhancement of antigen-specific immune responses are thought to provide adjuvancy crucial to the success of DNA vaccination (82). Although dendritic cells show a marked expression of MHC class II and costimulatory molecules in response to CpG motifs, macrophages respond by downregulation of the same molecules and production of IL-10, which counteract the Th1 stimulatory cytokines (67). Exposure to bacterial DNA or CpG motifs can lead to inflammatory responses in exposed lung and synovial tissues and induce toxic shock in mice (82). In contrast to LPS and hsp60, CpG motifs induce TNF- α independently of TLR4 (89).

CONCLUSIONS

Stimulated by the discovery of *Wolbachia* LPS as the major cause of inflammatory responses induced by *B. malayi*, we propose mechanisms whereby *Wolbachia* mediates the acute inflammatory pathogenesis associated with acute filarial lymphangitis and adverse reactions to chemotherapy. We also speculate that repeated episodes of acute inflammatory pathology and chronic exposure to *Wolbachia* inflammatory mediators leads to lymphatic dysfunction and the desensitization of innate immunity. This may lead to an increased susceptibility to infection and establishment of opportunistic microorganisms associated with ADLA in lymphoedema and elephantiasis.

Does the recognition of *Wolbachia* as a mediator of filarial disease offer any prospects for clinical intervention? Many studies in animal models have shown that antibiotic treatment can lead to the clearance of bacteria from worms, resulting in embryotoxicity, inhibition of moulting and development and eventually, the death of adult parasites (5,91,92). Treatment with doxycycline in human onchocerciasis leads to a profound embryotoxicity and sustained clearance of bacteria for several months (91). In addition to the antiparasitic effects of antibiotic therapy, the clearance of bacteria may also lead to a reduction in inflammatory pathogenesis. Studies to determine the effect of antibiotic treatment on acute filarial lymphangitis, adverse reactions

to filarial chemotherapy and the onset of chronic pathology would appear to be warranted.

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