Brief Definitive Report

Inflammatory Responses Induced by the Filarial Nematode *Brugia malayi* Are Mediated by Lipopolysaccharide-like Activity from Endosymbiotic *Wolbachia* Bacteria

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Abstract

The pathogenesis of filarial disease is characterized by acute and chronic inflammation. Inflammatory responses are thought to be generated by either the parasite, the immune response, or opportunistic infection. We show that soluble extracts of the human filarial parasite *Brugia malayi* can induce potent inflammatory responses, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and nitric oxide (NO) from macrophages. The active component is heat stable, reacts positively in the *Limulus* amebocyte lysate assay, and can be inhibited by polymyxin B. TNF-α, IL-1β, and NO responses were not induced in macrophages from lipopolysaccharide (LPS)-nonresponsive C3H/HeJ mice. The production of TNF-α after chemotherapy of microfilariae was also only detected in LPS-responsive C3H/HeN mice, suggesting that signaling through the Toll-like receptor 4 (TLR4) is necessary for these responses. We also show that CD14 is required for optimal TNF-α responses at low concentrations. Together, these results suggest that extracts of *B. malayi* contain bacterial LPS. Extracts from the rodent filaria, *Acanthocheilonema viteae*, which is not infected with the endosymbiotic *Wolbachia* bacteria found in the majority of filarial parasites, failed to induce any inflammatory responses from macrophages, suggesting that the source of bacterial LPS in extracts of *B. malayi* is the *Wolbachia* endosymbiont. *Wolbachia* extracts derived from a mosquito cell line induced similar LPS-dependent TNF-α and NO responses from C3H/HeN macrophages, which were eliminated after tetracycline treatment of the bacteria. Thus, *Wolbachia* LPS may be one of the major mediators of inflammatory pathogenesis in filarial nematode disease.

Key words: filariasis • endotoxin • pathogenesis • symbiosis • lipopolysaccharide

Introduction

Filariasis is a major cause of morbidity throughout the tropics. More than 140 million people are infected with the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus*, which are responsible for the majority of human filarial disease (1, 2). Pathology of filariasis is associated with a diverse range of inflammatory conditions. In lymphatic filariasis, inflammatory pathology can present as acute inflammation, characterized by recurrent attacks of adenolymphangitis with associated systemic febrile responses, or chronic inflammation, associated with hydrocele, lymphedema, and elephantiasis (1). In onchocerciasis,

pathogenesis is principally associated with the death of microfilariae and subsequent inflammation in the skin and eye (2, 3). Inflammatory responses are also a feature of the adverse reaction to filarial chemotherapy that is thought to occur after the release of large amounts of parasite material after treatment (4). The severity of adverse reaction to chemotherapy is related to the intensity of parasite burden and presents clinically as fever with local or systemic inflammation (4).

Several factors are thought to contribute to the inflammatory pathogenesis of filariasis, including the parasite, the immune response, and opportunistic infection (1–6). Infection of immunodeficient mice with *Brugia* species results in the development of lymphedema in the absence of T cells and opportunistic infection (7), and is associated with the local production of proinflammatory cytokines including IL-1, IL-6, TNF-α, and GM-CSF in parasitized lymphatics

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(8). Together with the development of inflammation after death of filarial parasites, this prompted us to investigate the role of the parasite in the induction of inflammatory responses.

The induction and regulation of inflammatory responses has been shown to be under the control of key proinflammatory cytokines including IL-1β and TNF-α (9). These cytokines are produced predominantly by macrophages and result in a cascade of inflammatory mediators and physiological responses that serve to amplify and regulate innate immunity (10). We have investigated the induction of these proinflammatory cytokines and nitric oxide (NO) from mouse macrophages by soluble extracts of *B. malayi* in order to characterize parasite derived inflammatory stimuli.

Materials and Methods

Parasites

The human filarial parasite *B. malayi* was obtained from TRS Laboratories and maintained in the peritoneal cavity of Mongolian jirds (*Meriones unguiculatus*). Adult male and female worms and microfilariae were collected under aseptic conditions. Microfilariae were passed through Sephadex PD-10 columns (Amersham Pharmacia Biotech) to remove host cells and serum proteins, and together with adult parasites were thoroughly washed (four times) in RPMI 1640 containing 5% FCS and 1% penicillin/streptomycin (GIBCO BRL). Parasites were cultured at 37°C in 5% CO_2 for 5 d to ensure the absence of contaminating microorganisms and to select viable motile parasites. Adult parasites were separated into male and female worms under sterile conditions and washed (four times) in sterile PBS. Extracts were prepared by finely mincing the worms followed by homogenization on ice. Microfilarial extracts were prepared after disruption by sonication on ice. All procedures were carried out under stringent sterile conditions. Adult *Acanthocheilonema viteae* were obtained from subcutaneous tissues of Mongolian jirds and prepared as for *B. malayi* adult worms. Extracts were centrifuged at 20,000 *g* for 30 min, and the supernatant was collected and stored at −20°C until required. Protein concentration of parasite extracts was determined by the Coomassie protein assay (Pierce Chemical Co.).

Wolbachia Mosquito Cell Line

The Asian tiger mosquito, *Aedes albopictus*, cell line Aa23, which is naturally infected with *Wolbachia*, was cultured as described (11). Soluble extracts of the cell line and bacteria were prepared by sonication after washing of cultured cells in sterile PBS. Similar extracts were prepared from cells cultured for 2 wk in tetracycline (10 μg/ml), which resulted in clearance of the bacteria as judged by microscopy.

Macrophage Cultures

Primary murine macrophage cultures were derived from peritoneal exudate cells 4 d after administration of 1% thioglycollate (Difco) as described previously (12). Female CD-1 (Charles River), C3H/HeN, and C3H/HeJ (The Jackson Laboratory) mice aged 6–10 wk were used. 2.5×10^5 cells were placed in flat-bottomed 96-well tissue culture plates (Nunc), and macrophages were isolated by adherence. Cells were maintained in RPMI 1640 containing 5% FCS, 1% penicillin/streptomycin, 1% genta-mycin, 1% l-glutamine, and 1% nonessential amino acids at 37°C in 5% CO_2. The macrophage cell line J774.1 and the mutant J7.DEF.3 (14), which does not express membrane CD14 (15), were cultured under similar conditions at a concentration of 1×10^5 cells/well. Cells were stimulated with optimal concentrations (1 μg/ml) or serial dilutions of *Escherichia coli* LPS (026:B6; Sigma Chemical Co.) and 50 U/ml IFN-γ (Genzyme), either singly or in combination, and soluble extracts from filarial parasites for 24 h. Culture supernatants were collected for analysis of cytokines and nitrite.

Analysis of Cytokines and NO

IL-1β and TNF-α were detected in culture supernatants or plasma by capture ELISA using paired antibodies supplied by Genzyme or Biosource. NO production was analyzed by the detection of nitrite using the Griess reaction as described previously (12).

Limulus Amebocyte Lysate Test

The E-TOXATE® kit (Sigma Chemical Co.) was used according to the manufacturer's instructions.

PCR Detection of Bacteria

Eubacterial 16S rDNA PCR. *B. malayi* and *A. viteae* were screened for bacterial 16S rDNA. 1 μl of DNA was amplified in a mixture that contained 1.25 U HotStarTaq DNA polymerase (Qiagen), 1× Qiagen PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer (13), and water to a final volume of 50 μl. Temperature cycling conditions were as follows: 15 min at 95°C, followed by 1.5 min at 94°C, 1 min at the annealing temperature (60, 55, and 50°C for 5 cycles each, then 45°C), extension at 72°C for 2.5 min (total of 35 cycles), and a final extension of 8 min. PCR products were cloned and sequenced by standard procedures.

Nested PCR. To reevaluate the distribution of *Wolbachia* in a population of 50 male and 50 female *B. malayi* (13), a nested PCR was developed. After amplification with eubacterial primers, 1 μl of product was amplified with 3.0 mM $MgCl_2$ and 0.5 μM of each *Brugia Wolbachia*–specific primer (13) with temperature cycling conditions of 95°C for 15 min, followed by 25 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min, and a final extension of 8 min at 72°C.

LPS-induced TNF-α Responses after Chemotherapy

C3H/HeN and C3H/HeJ mice were infected with 350,000 *B. malayi* microfilariae injected intravenously. 24 h later, animals were treated intraperitoneally with a combination of ivermectin phosphate (1 mg/kg, Merck Research Laboratories) and d-galactosamine (d-gal, 100 mg/kg; Sigma Chemical Co.). Blood samples were collected from the tail vein before treatment and at 3 and 6 h after treatment, and plasma was analyzed for TNF-α by ELISA. Control uninfected animals were treated in a similar fashion.

Statistics

Student's *t* test was used to compare mean values with a $P < 0.05$ being taken as significant.

Results and Discussion

To determine the ability of parasites to directly induce inflammatory responses, we prepared soluble extracts from

male and female adult worms and microfilariae of the human lymphatic filarial parasite, *B. malayi*, which were used to stimulate murine macrophages. Extracts from all developmental stages induced the production of IL-1β, TNF-α, and NO in a dose-dependent fashion (Fig. 1). Treatment of soluble extracts by boiling (100°C, 5 min) had no effect on the induction of inflammatory responses, whereas incubation of extracts with polymyxin B inhibited these responses (Fig. 1 C). Extracts were also shown to react positively in the *Limulus* amebocyte lysate (LAL) test, in which the lysate coagulates in the presence of bacterial endotoxin. Together these results suggest that the inflammatory stimulus in parasite extracts has similarities to bacterial endotoxin or LPS.

Next we tested whether the presence of the LPS receptor, CD14, was required for the induction of inflammatory responses. Using the cell line J7.DEF.3, which has been se-

lected from a parent J774.1 macrophage cell line to be free of surface CD14 (14, 15), we show that at low concentrations of parasite extract and LPS, CD14 is required for TNF-α responses (Fig. 2). At higher concentrations, CD14-independent stimulation of TNF-α occurs (Fig. 2), in accordance with previous studies on LPS (10). To confirm that the mutation in J7.DEF.3 cells is restricted to CD14 expression, we attempted to restore activity by the addition of soluble CD14 (sCD14), which was produced as described previously (16). Suboptimal concentrations of LPS and *B. malayi* microfilarial extract were incubated on J7.DEF.3 cells in the presence of 1–10 μg/ml of sCD14. The addition of sCD14 to cultures restored the ability of J7.DEF.3 cells to produce TNF-α, in response to both LPS and parasite extracts (Fig. 2 B). The restoration of responses to parasite extracts was equivalent to that observed from parent J774.1 cells (Fig. 2 A).
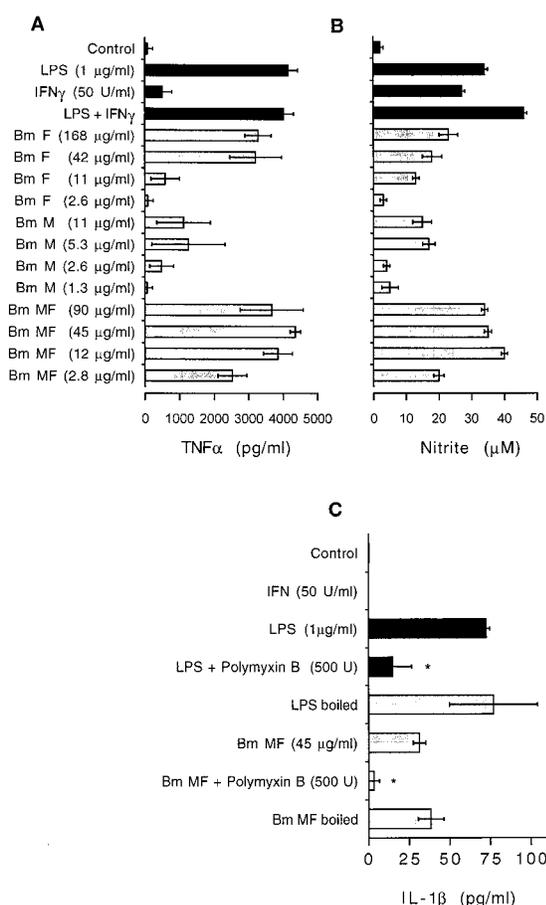
Figure 1. Production of TNF-α, NO, and IL-1β from CD-1 adherent peritoneal exudate cells after exposure to soluble extracts of *B. malayi* (Bm) adults and microfilariae for 24 h (gray bars). Bm F, adult female worms; Bm M, adult male worms; Bm MF, microfilariae. Black bars represent control cultures incorporating *E. coli* LPS and IFN-γ, singly or in combination. (A) TNF-α; (B) nitrite; and (C) IL-1β. Concentration of filarial extract refers to the total protein concentration of the extract. Values represent the mean of three replicate wells (±SD). *Statistical significance of *P* < 0.05. Results are representative of at least three similar experiments.
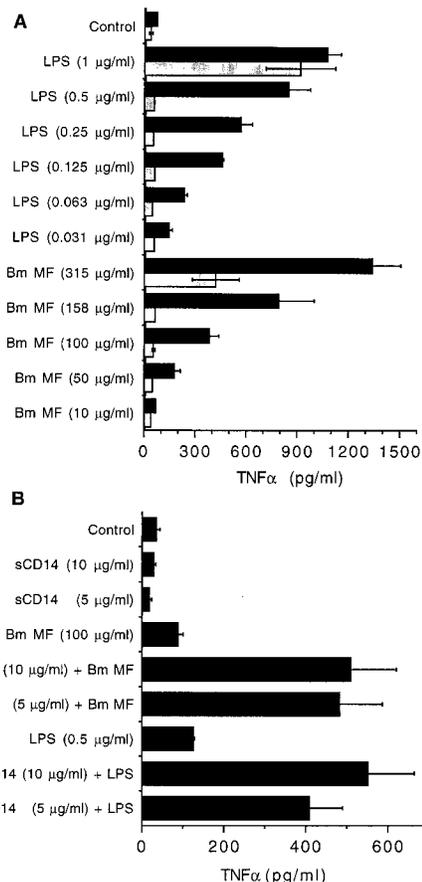

Figure 2. The requirement for CD14 for TNF-α production from macrophages stimulated with *B. malayi* microfilarial extract or LPS. (A) J774.1 macrophages (black bars) or J7.DEF.3 mutant cells (gray bars, deficient in surface CD14) were stimulated with LPS or *B. malayi* microfilarial extract (Bm MF) for 24 h. (B) Suboptimal concentrations of LPS or *B. malayi* microfilarial extract (Bm MF) were cultured on J7.DEF.3 cells in the presence of sCD14 leading to a restoration of TNF-α responses. Concentration of filarial extract refers to the total protein concentration of the extract. Values represent the mean of three replicate wells (±SD). Results are representative of three similar experiments.

Recently, LPS has been shown to induce signal transduction across the plasma membrane via Toll-like receptors (TLRs, 17–23). A mutation in the *lps* gene, which renders C3H/HeJ mice unresponsive to LPS, has been mapped to a mutation in TLR4 (17–20). Therefore, we tested whether TLR4 is required for the induction of inflammatory responses from *B. malayi* extracts. Macrophages derived from C3H/HeJ mice failed to produce IL-1β, TNF-α, and NO in response to adult female and microfilarial *B. malayi* extracts or LPS, but were able to respond to IFN-γ (Fig. 3 A). This suggests that these inflammatory responses induced by *B. malayi* extracts are dependent on signaling through the TLR4 LPS receptors.

We and others have shown that the majority of filarial parasites are infected with symbiotic intracellular bacteria belonging to the *Wolbachia* complex (13, 24–26). One excep-

tion to this is the rodent filaria, *A. viteae*, which are free of bacteria as judged by electron microscopy, PCR, and lack of sensitivity to tetracycline therapy (24, 26–28). Therefore, we compared the ability of extracts derived from filaria with (*B. malayi*) and without (*A. viteae*) *Wolbachia* infection to induce inflammatory responses from macrophages. Fig. 3, B and C, shows that inflammatory responses are only induced from *B. malayi*. Extracts of adult *A. viteae* parasites failed to induce any inflammatory response from macrophages. In addition, extracts of *A. viteae* did not react positively with the LAL assay, supporting the notion that the endosymbiotic bacteria of *B. malayi* are the source of LPS. To determine whether *A. viteae* parasites contained endogenous inhibitors of LPS-induced inflammatory responses, we cocultured extracts of *A. viteae* and *B. malayi*. No evidence for the presence of inhibitory activity was detected in *A. viteae* (data not shown).
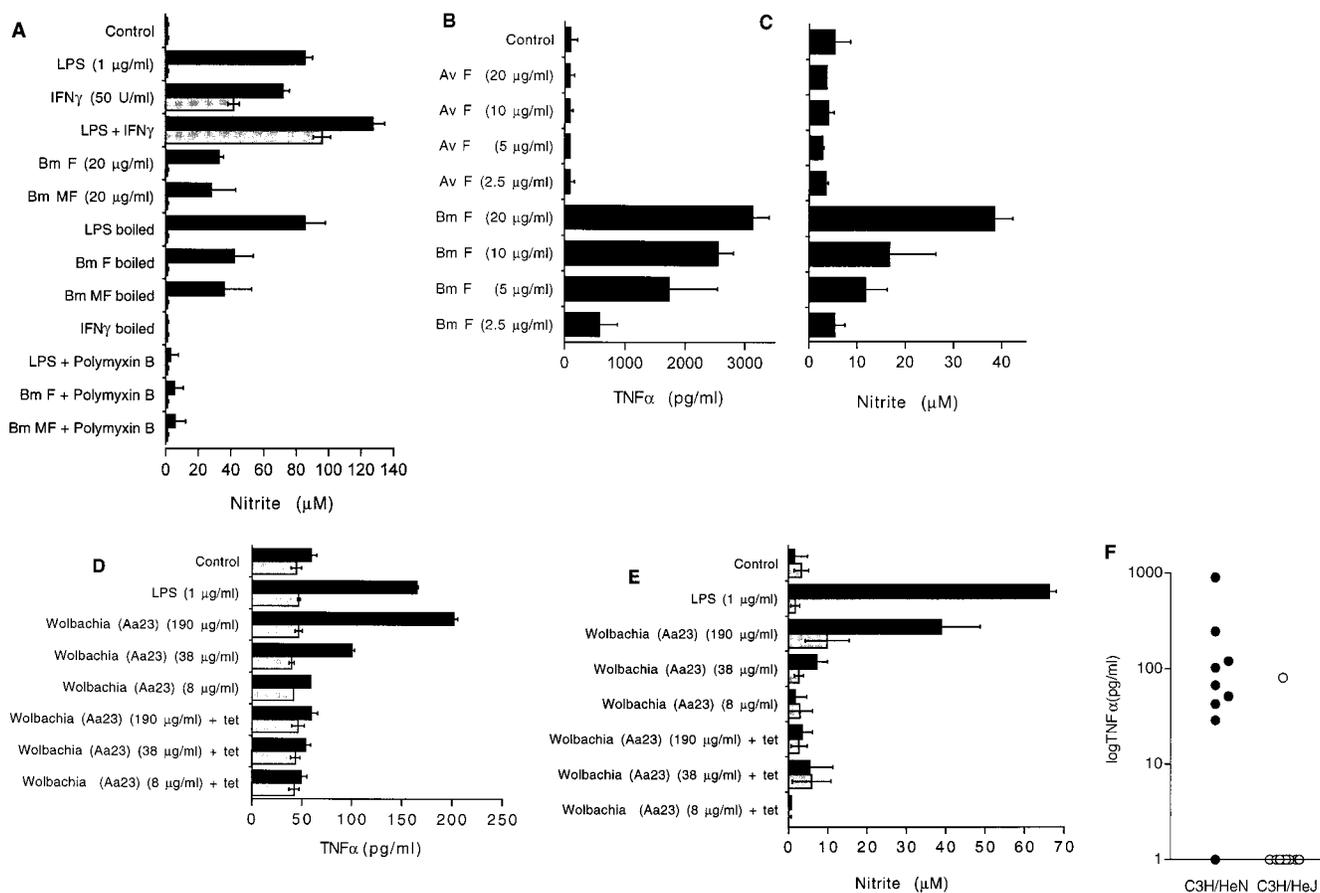
Figure 3. Induction of NO and TNF-α in C3H/HeN (LPS-responsive) and C3H/HeJ (LPS-nonresponsive) mice. (A) Production of NO from adherent peritoneal exudate cells derived from C3H/HeN (black bars) or C3H/HeJ mice (gray bars) after exposure to soluble extracts of *B. malayi* for 24 h. Extracts of *B. malayi* adult female worms (Bm F) or microfilaria (Bm MF) and *E. coli* LPS induce NO from macrophages from C3H/HeN mice but not C3H/HeJ mice. The induction of NO is unaffected by boiling the filarial extract but can be inhibited with polymyxin B. IFN-γ responses are induced from both strains of mice and are destroyed by boiling. (B) Production of TNF-α and (C) NO from C3H/HeN adherent peritoneal exudate cells after exposure to soluble extracts of *B. malayi* adult female (Bm F) and *A. viteae* adult female (Av F) extracts for 24 h. TNF-α and NO are produced only in response to *B. malayi*. (D) Production of TNF-α and (E) NO from C3H/HeN (black bars) and C3H/HeJ (gray bars), induced by soluble extracts derived from a *Wolbachia*-infected mosquito cell line (Aa23) or after tetracycline (tet) treatment of the cell line. Concentration of the extract refers to the total protein concentration. Values represent the mean of three replicate wells (±SD). Results are representative of three to six similar experiments. (F) Plasma TNF-α levels 6 h after treatment of C3H/HeN (filled circles) and C3H/HeJ mice (open circles) infected with *B. malayi* microfilariae and treated with ivermectin (1 mg/kg) and d-gal (100 mg/kg) (*n* = 9 per group). Representative of two repeat experiments.

Attempts to "cure" filarial nematodes of *Wolbachia* with antibiotics and retain viability have so far proved impossible, suggesting a mutualistic association between nematodes and their symbionts (24). In contrast, arthropod *Wolbachia* can be eliminated from hosts using tetracycline. Therefore, we used a mosquito cell line–derived *Wolbachia* to determine LPS activity of the bacteria in the absence of nematode tissue and after antibiotic treatment. Fig. 3, D and E, shows that extracts derived from *Wolbachia*-infected mosquito cells induced TNF-α and NO from C3H/HeN (LPS-responsive), but not C3H/HeJ (LPS-nonresponsive) macrophages. Mosquito cell lines treated with tetracycline, which eliminated *Wolbachia* infection, failed to demonstrate LPS-like activity (Fig. 3, D and E). These results suggest that *Wolbachia* bacteria from both arthropods and nematodes contain LPS in accordance with other related members of the Rickettsiaceae.

PCR analysis with primers for eubacterial 16S rDNA confirmed that *A. viteae* was free of symbiotic bacteria, and that *Wolbachia* was the only bacterial sequence obtained from *B. malayi*. Although this does not rule out the possibility of other symbiotic bacterial species in *B. malayi*, it does suggest that *Wolbachia* are the most abundant. Remarkably high levels of infection can occur in adult parasites and microfilariae, with numerous bacteria almost entirely filling their intracellular environment (24). Previously, we have reported that in a population of *B. malayi* adult worms, although all female worms were infected, only ~25% of male worms appeared to be infected when screened with *Wolbachia*-specific primers (13). We reanalyzed these samples using a nested PCR incorporating eubacterial primers and *Wolbachia*-specific primers and found that all male worms are infected, although many at a lower intensity than that observed in female worms.

We next investigated whether or not LPS was released by living parasites during in vitro culture. Previously, we have reported that 4-d cultures of *B. malayi* microfilariae in the presence of macrophages did not generate any detectable NO (12). Similar experiments on living adult worms and microfilariae cultured in the presence of macrophages, or the addition of spent culture medium incubated on fresh macrophages, failed to induce detectable IL-1β, TNF-α, or NO responses (data not shown). This suggests that living parasites do not release LPS in sufficient amounts to activate macrophages under the conditions tested. Alternatively, living parasites may secrete products that are inhibitory to LPS-induced responses. Furthermore, the vigorous motility of filarial parasites is also not sufficient to induce inflammatory responses from macrophages.

Therefore, the release of LPS by parasites in vivo is likely to occur after death of the parasite through natural attrition, after immunologically mediated clearance or pharmacological intervention and the subsequent release of *Wolbachia* bacteria. Such a situation is consistent with the inflammatory nodules produced after death of filarial parasites, and the systemic and localized responses that accompany the adverse reaction to chemotherapy (1–4). Indeed, a study using d-gal sensitization of rodents, which renders animals susceptible to TNF-α–induced hepatotoxiticity, showed that chemotherapy of *B. malayi* in d-gal–sensitized animals resulted in shock-like lethal side effects (29). These adverse reactions could be prevented by inhibitors of TNF-α and NO and would be consistent with the release of LPS after treatment of filarial parasites. Therefore, we tested whether or not LPS-induced proinflammatory responses occurred after chemotherapy. C3H/HeN and C3H/HeJ mice were infected with *B. malayi* microfilariae and treated with ivermectin and d-gal. Analysis of plasma TNF-α showed that at 6 h after treatment, TNF-α could be detected in eight out of nine LPS-responsive C3H/HeN mice (mean 175 pg/ml, range 0–915 pg/ml), whereas only one out of nine LPS-hyporesponsive C3H/HeJ mice showed elevated TNF-α (mean 9 pg/ml, range 0–81 pg/ml, $P < 0.05$; Fig. 3 F). TNF-α was not detected in plasma samples before treatment, at 3 h after treatment, or in control uninfected animals receiving ivermectin and d-gal. The production of TNF-α in C3H/HeN mice at 6 h after treatment coincided with a 89–100% reduction in parasites from the peripheral circulation. A repeat experiment showed similar results. These data support the idea that proinflammatory responses induced after treatment are predominantly caused by LPS.

The release of proinflammatory cytokines and inflammatory mediators including IL-1, IL-6, IFN-γ, TNF-α, and NO after filarial chemotherapy has been reported (30–33) and shown to correlate with the severity of adverse reaction (32). The severity and presentation of fever associated with acute lymphatic filarial disease also correlate with elevated levels of TNF-α (34). Together with reports of the local production of proinflammatory cytokines in pathologies of animal models (8), these studies suggest a role for these proinflammatory responses in the pathogenesis of filarial disease. Products from endosymbiotic *Wolbachia* bacteria may also contribute to the recruitment and activation of granulomatous inflammatory responses that occur early during *Brugia* infection in animal models (35), in response to *O. volvulus* in vitro (36), and on death of parasites following chemotherapy (3, 4).

The presence of symbiotic *Wolbachia* in all of the major pathogenic filariae of humans (24) and the apparently exclusive induction of proinflammatory responses by bacterial LPS in *B. malayi* extracts suggest that *Wolbachia* LPS may be one of the major mediators of inflammatory pathogenesis in filarial nematode disease. With the current studies in our laboratory, we hope to further clarify the contribution of this fascinating symbiont to human filarial disease.

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