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***Wolbachia* heat shock protein 60 induces pro-inflammatory cytokines and apoptosis in monocytes in vitro**

Vijayan Kamalakannan^a, Sreenivas Kirthika^a, Kalyanaraman Haripriya^a, Subash Babu^b, and Rangarajan Badri Narayanan^{a,*}

^a Centre for Biotechnology, Anna University, Chennai 600025, India

^b NIH-TRC-ICER SAIC-Frederick Inc., National Cancer Institute at Frederic, Chetpet, Chennai, India

Abstract

Recombinant *Wolbachia* heat shock protein 60 (rWmhsp60) induces gene expression of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α in human monocytic cell line THP-1. In addition, it inhibits the phagocytic activity and does not alter the nitric oxide production by differentiated THP-1 macrophages, which corroborates with no significant change in inducible nitric oxide synthase gene expression in rWmhsp60 treated THP-1 monocytes. Further, 24 h stimulation of peripheral blood mononuclear cells from normal individuals by rWmhsp60 reveals that monocytes enter the late apoptotic stage, while lymphocytes do not show apoptosis. Thus these findings suggest that rWmhsp60 may contribute to inflammation mediated monocyte dysfunction in filarial pathogenesis.

Keywords

Wolbachia; Filariasis; Cytokines; Monocytes; Heat shock proteins; Apoptosis

1. Introduction

Lymphatic filariasis is a chronic parasitic helminth disease caused by *Wuchereria bancrofti* and *Brugia malayi*. According to a recent report by world health organization, more than 120 million people are currently infected, with about 40 million being disfigured by this disease. These parasitic nematodes are insect borne, responsible for lymphatic or cutaneous filarial infection leading to medical conditions such as elephantiasis or onchocerciasis. Spectrum of clinical manifestations are seen in different clinical groups which comprises asymptomatic patients with circulating microfilaria (MF), individuals with chronic lymphatic obstruction or chronic pathology (CP), endemic normals (EN) who are asymptomatic and amicrofilaraemic and non-endemic normals (NEN) living in non-endemic areas. Pathogenesis of lymphatic filariasis is characterized by acute or chronic inflammation with hydrocele, lymphedema and elephantiasis [1] and in onchocerciasis it is predominated by skin and eye inflammation [2,3].

Parasites responsible for lymphatic filariasis and onchocerciasis harbor intracellular symbiotic bacteria, *Wolbachia* on which they rely on for embryogenesis, growth and survival [4]. The hallmark of the filarial pathogenesis is the host inflammatory responses

provoked by the death of the parasite and subsequent release of *Wolbachia* [5,6]. Studies with *B. malayi* and *Onchocerca volvulus* imply that the inflammation induced by these parasites is dependent on *Wolbachia* [7,8]. Extracts from *B. malayi* and *O. volvulus* infected with *Wolbachia* were found to induce inflammation while that of rodent filariasis devoid of *Wolbachia* failed to induce inflammation, also *Wolbachia* from filarial parasite or insect are found to replicate these inflammatory effects [6]. These reports imply that *Wolbachia* plays a major role in mediating the inflammatory pathogenesis of filariasis. Inflammation which leads to the outbreak of the filarial pathogenesis is found to develop following the death of the parasite and the release of stressed endosymbiont [5,6]. So there is a possibility that inflammatory molecules like heat shock proteins (hsps), lipopolysaccharides (LPS), surface proteins, lipoproteins, CpG motifs in DNA and peptidoglycans from *Wolbachia* may play an important role in filarial pathogenesis [9–11]. Apart from their role in protein biogenesis, studies with bacterial hsps suggests its active involvement in immunostimulatory functions by increasing the production of inflammatory cytokines and thereby providing danger signal to antigen presenting cells which contributes to the inflammation [12–14]. Bacterial hsps are also found to stimulate and regulate innate and acquired immune responses during pathogenesis which leads to severe autoimmunity and chronic inflammation [15,16].

Wolbachia genome project has provided a platform for the production of recombinant proteins for immunological investigations. Sera from individuals harboring microfilaria contained elevated levels of rWmhsp60 [17] and recombinant *Wolbachia* surface protein specific antibodies [18]. Hence we have produced recombinant *Wolbachia* surface protein (rWSP) and recombinant *Wolbachia* heat shock protein 60 (rWmhsp60) in our lab to study their role in filarial pathogenesis. Also, preliminary studies with *Wolbachia* hsp60 shows elevated levels of TNF- α and IL-6 production [19]. Our previous study also shows that *Wolbachia* hsp60 regulates immune activation that mediates an additional level of immune tolerance along with nematode products in filarial patients [20]. Though lot of information is available on lymphocyte responses to *Wolbachia* proteins, but the data available on monocyte and macrophage responses to the *Wolbachia* proteins is scant. In the present study, the role of recombinant *Wolbachia* hsp60 is evaluated by analysis of parameters like inflammatory cytokine and inducible nitric oxide synthase (iNOS) gene expression and phagocytosis in stimulated THP-1 monocytes/macrophages [21] and apoptosis in peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Cloning and expression of rWmhsp60

The gene coding for Wmhsp60 was amplified and cloned into the expression vector pRSET-A (Invitrogen, CA, USA). rWmhsp60 expressed in *Escherichia coli* BL21 (DE3) (Invitrogen, USA) was purified by immobilized metal affinity chromatography (IMAC). The purified protein was dialyzed against phosphate buffer saline (PBS) using dialysis membrane-60 (Himedia, India) and lyophilized. The purity of the protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis using rWmhsp60 specific polyclonal antibody and histidine specific monoclonal antibody.

2.2. Study population

Patients were recruited through the Filariasis Control Unit under the Directorate of Public Health (Chennai, India) after obtaining informed oral consent with protocols approved by the Institutional Review Board of Anna University (Chennai). The individuals in the study were informed about the experiment by the DPH medical authorities, and only oral consent was possible as most of them were illiterate. The consent from all the volunteers was

obtained during clinical examination, and it was documented in the form of a spreadsheet. Standardized histories were obtained and physical examinations were done on all the participant residents during epidemiological surveys in and around Chennai, India, an area endemic for *W. bancrofti* infection. The NEN serum samples that were used in the study were a kind gift from Dr. Thomas B. Nutman (NIH, Bethesda, MD, USA). Four asymptomatic amicrofilaremic endemic normals (EN), four asymptomatic microfilareemics (MF) and four symptomatic amicrofilaremic individuals with chronic pathology (CP) were included and their sera were used to assess the immunoreactivity of rWmhsp60. Further, five endemic normals were included for studies on apoptosis. All the individuals were screened for the presence of circulating filarial antigens by Og4C3 mAb ELISA, a marker of *W. bancrofti* infection and adult worm burden (Chanteau et al., 1994; Trop-Bio, Townsville, Queensland, Australia).

2.3. Reactivity of rWmhsp60 with sera from different clinical groups

The specificity of the antiserum against Wmhsp60 and the levels of antibodies in different filarial groups were assessed by western blotting where 25 µg of the protein was resolved using 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Hybond, Amersham Pharmacia) using semi dry blotting transfer apparatus (Amersham Pharmacia). The membrane was blocked using 5% nonfat milk powder in 1% PBS for 3 h at 37 °C and washed thrice in wash buffer, PBST (PBS with 0.05% Tween20) for 10 min followed by incubation with appropriate dilution of primary antibodies overnight at 4 °C. Normal mouse sera (NMS) (1:5000), mouse anti-rWmhsp60 (1:1000) and patient sera in the order of NEN, EN, MF and CP at a dilution of 1:100 were used respectively. The strips were subsequently washed as mentioned above and probed with goat anti-mouse IgG-alkaline phosphate conjugate (1:20,000) (Sigma-Aldrich) in the case of the first two strips and goat antihuman IgG-alkaline phosphate conjugate (1:20,000) (Sigma-Aldrich) in the case of the strips probed with the patient sera and incubated for 1 h. The strips were finally developed with 33 µl BCIP (5-bromo 4-chloro 3-indolyl phosphate) and 66 µl NBT (nitroblue tetrazolium salt), USB Cleveland, Ohio.

2.4. Endotoxin determination

The LPS content of the recombinant Wmhsp60 was determined using Limulus amebocyte lysate (LAL) test. All reagents were purchased from Sigma Chemical Co. (St.Louis, USA), and the assay was carried out according to manufacturer's instructions. Further, to assess the biological activity of traces of LPS that could contribute to the effects of the recombinant protein preparation, PBMCs from healthy individuals were subjected to stimulation with rWmhsp60 and *E. coli* LPS in the presence and absence of polymyxin-B sulfate (10 µg/ml) to examine the difference in the PBMCs proliferation.

2.5. Cell culture

THP-1 cells procured from NCCS (National Centre For Cell Science, Pune) were cultured in RPMI1640 with L-glutamine (Gibco, Invitrogen, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco, Invitrogen), 1.5 g/L sodium bicarbonate (Sigma Chemical Co., St.Louis, USA), 10 mM HEPES (USB, Cleveland, Ohio, USA), 1 mM sodium pyruvate (Gibco, Invitrogen, China), antibiotic and antimycotic solution (Gibco, Invitrogen, USA) and incubated at 37 °C in humidified 5% CO₂. THP-1 monocytes (1million cells/ml) were cultured with 5 µg/ml of rWmhsp60, 10 µg/ml of LPS and 10 µg/ml of mycobacterium purified protein derivative (PPD). After 24 h, cells and culture supernatants were collected for gene and protein level analysis of cytokines.

2.6. RNA preparation and cDNA synthesis

THP-1 monocytes from the stimulated cultures were harvested and the total RNA was extracted according to the manufacturer's protocol (RNeasy minikit; Qiagen). RNA was dissolved in 20 μ l RNase-free water and heated at 70 °C for 5 min after the addition of 100 pM of random hexamer (New England Biolabs, MA, USA) and chilled on ice. Reverse transcription of RNA was performed in a final volume of 40 μ l containing 0.25 mM mix of the four deoxynucleotide triphosphates (dATP, dGTP, dTTP, and dCTP) (New England Biolabs, MA, USA); 1 $\times$ reverse transcriptase buffer (50 mM Tris HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 20U RNase inhibitor (New England Biolabs, MA, USA) and 200U of MMLV-reverse transcriptase (New England Biolabs, MA, USA) followed by incubation of the tubes at 37 °C for 60 min. The reverse transcription reaction was stopped by heating the tubes at 75 °C for 5 min. The final reaction volume was diluted by addition of 10 μ l DEPC (diethylpyrocarbonate) treated distilled water and the cDNA was stored at –20 °C until use.

2.7. Real time RT-PCR

Real time quantitative PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems) using Taq-Man assays-on-demand reagents for IL-1 β , IL-6, IL-10, IL-12, TNF- α , iNOS and an endogenous 18 s ribosomal RNA control (Applied Biosystems). The end point used in real time PCR quantification is CT, threshold cycle during the exponential phase of amplification, according to the manufacturer's protocol. Quantification of gene expression was performed using the comparative CT method (Sequence Detector User Bulletin 2, Applied Biosystems) and reported as the fold change relative to the house keeping gene. To calculate the fold change, the CT of the house keeping gene (18s rRNA) was subtracted from the CT of the target gene to yield the Δ CT. Change in the expression of the normalized target gene as a result of antigenic exposure was expressed as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ of stimulated – ΔCT of unstimulated or antigen negative (Ag^{–ve}) cells.

2.7.1. ELISA—The levels of cytokines (TNF- α , IFN- γ , GM-CSF and IL-10) in the pooled culture supernatants from 3 independent experiments were measured using Bioplex multiplex cytokine ELISA assay system (Biorad, Hercules, CA).

2.8. Phagocytosis and nitric oxide (NO) release assay

The gene coding for green fluorescent protein (GFP) was cloned into pRSET-B vector and expressed in *E. coli* BL21 (DE3) (Invitrogen, CA, USA) (data not shown). THP-1 monocytes were cultured with 4-phorbol- 3-myristate acetate (PMA, Sigma Chemical Co., St.Louis, USA) for three days to facilitate their differentiation into macrophages. Differentiation of THP-1 cells was enhanced by the incubation of cells in fresh medium for 3 days. Cells were washed and stimulated with rWmhsp60 (5 μ g/ml), LPS (10 μ g/ml) and PPD (10 μ g/ml) for 24 h followed by incubation in fresh antibiotic free medium with *E. coli*-GFP for 2 h. The number of *E. coli* ingested by macrophages was then visualized by fluorescent microscopy. The supernatant was collected before and after the addition of *E. coli*-GFP to assess NO release using Griess assay in which SNP (sodium nitro prusside) served as a positive control.

2.9. Isolation of PBMCs and determination of apoptosis by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood collected from healthy donor by ficoll–diatrizoate gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). PBMCs (1 million/ml) were seeded in 24 well culture plates and stimulated with rWmhsp60, PPD and cyclohexamide (CHX, 100 mM) and incubated. CHX treated cells served as positive control. After 24 h, the cells were then pelleted at 4 °C (500 $\times$ g) for 5 min, washed twice with PBS and resuspended in 100 μ l of 1 $\times$ annexin-V

binding buffer (0.1 M Hepes/NaOH at pH 7.4, 1.4 M NaCl, 25 mM CaCl₂) with 2 µg/ml of annexin V FITC (BD Biosciences, USA) and 2 µg/ml of propidium iodide (Merck, Germany). Finally, cells were washed and resuspended in 2× PBS and acquired on a Becton Dickinson FACS Calibur (BD Biosciences, USA) and analyzed using Cell Quest software (Becton Dickinson). For acquisition, forward and side scatter gates were adjusted to acquire monocytes and lymphocytes separately and the data analysis was performed using FlowJo software (Tree star).

2.10. Acridine orange/ethidium bromide (AO/EtBr) staining

PBMCs were cultured and stimulated as described previously. After 24 h, cells were washed with PBS and the cell suspension was stained with 1 µl of AO/EtBr solution (100 µg/ml of AO and 100 µg/ml of EtBr prepared in PBS) and examined under fluorescent microscopy. The following criteria was used to identify the difference in the cellular status; intact cells were stained green due to AO which is membrane permeable, while only cells with compromised plasma membranes stained red due to incorporation of membrane impermeable EtBr.

2.11. DNA fragmentation assay

Internucleosomal cleavage of DNA was analyzed after treating PBMCs (2×10^6 cells/ml) with rWmhsp60, PPD and CHX. After 24 h of incubation, cells were collected by gentle centrifugation and were lysed in DNA lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, and 2 g/L Triton X-100, pH 7.4). Cell debris were removed by centrifugation and cell supernatant was treated with RNase (60 µg/ml) (Bangalore Genei, India) at 55 °C for 1 h. The DNA was precipitated with 0.5 M sodium chloride and equal volume of isopropyl alcohol and separated by electrophoresis on 1% agarose and visualized by UV after staining with EtBr.

2.12. Statistical analysis

For comparative analysis, Mann–Whitney *U* test and Wilcoxon signed rank test were used. All statistics were performed using GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Production of recombinant Wmhsp60

The *B. malayi* *Wolbachia* hsp60 gene was amplified from the genomic DNA of *B. malayi*, cloned in pRSET-A (Invitrogen, USA) and the rWmhsp60 was purified by IMAC as reported previously [17]. Sera from patients with microfilaria and chronic pathology reacted with rWmhsp60 in western blot while sera from asymptomatic endemic and non-endemic normal individuals did not exhibit any reactivity (Fig. 1).

3.2. Differential cytokine and iNOS gene expression in rWmhsp60 stimulated THP-1 monocytes

Gene expression analysis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-12) and anti-inflammatory cytokine (IL-10) in THP-1 monocytes were assessed in 24 h monocyte culture followed by incubation with LPS, PPD, and rWmhsp60 using Real Time PCR (Fig. 2A. I–V). THP-1 cells exhibited significant upregulation of IL-1β gene expression upon rWmhsp60 stimulation (10.3 Geometric mean (GM) fold change vs control cells; *p* = 0.032). Cells treated with PPD, LPS and control cells had similar levels of expression. IL-6 mRNA expression was maximal among tested cytokines upon rWmhsp60 stimulation (75.4 GM fold change vs control; *p* = 0.0249). As expected, 24 h LPS stimulated THP-1 monocytes showed elevated expression of IL-6 (12.3 GM fold change vs control; *p* =

0.0009). Gene expression of TNF- α (3 GM fold change vs control; $p = 0.0341$) was increased upon rWmhs60 stimulation. However the LPS and PPD stimulated cultures exhibited no significant change in the expression. Surprisingly, anti-inflammatory cytokine IL-10 upon rWmhs60 stimulation was found to be upregulated (15.9 GM fold change vs control; $p = 0.0249$) when compared with control cells. LPS and PPD stimulation does not show significant change in the expression of IL-10. However, the expression levels of IL-12 was not altered on exposure to PPD, LPS and rWmhs60 stimulations.

iNOS expression in mRNA level remained unaltered following the stimulation with rWmhs60 and LPS while the PPD stimulation showed two fold increase (Fig. 2A. VI).

3.3. rWmhs60 results in augmented release of proinflammatory cytokines

rWmhs60 stimulated pro-inflammatory cytokines (TNF- α , IFN- γ and GM-CSF) production were apparently increased in THP-1 monocytes when compared with the control THP-1 monocytes. Further, the level of anti-inflammatory cytokine IL-10 was decreased in rWmhs60 stimulated culture supernatants when compared with the unstimulated THP-1 monocytes. As expected, LPS and PPD stimulations resulted in perceptible increase in the production of pro-inflammatory cytokines (TNF- α , IFN- γ and GM-CSF) and no change in the anti-inflammatory cytokine IL-10 production when compared with the unstimulated THP-1 monocytes (Fig. 2B. I–IV).

3.4. rWmhs60 induced phagocytic activity in THP-1 cells

THP-1 cells were maintained in the conditioned medium and were confirmed CD14 positive (data not shown). For Phagocytosis experiments, THP-1 cells were differentiated into macrophage by 72 h incubation with PMA and cells were confirmed CD14 positive (data not shown). As shown in the Fig. 3A, LPS stimulated THP-1 macrophages showed increased phagocytic activity, as evident from the GFP expressing *E. coli* uptake compared to control cells (61.91 GM % phagocytosis of control cells vs. 84.13 GM % phagocytosis of LPS stimulated cells; $p < 0.0001$) while the uptake by PPD stimulated THP-1 macrophages were similar to that of control THP-1 macrophages (61 in PPD vs. 61.91 in control cells; $p > 0.0001$). In contrast rWmhs60 stimulation inhibited *E. coli* uptake (9.94 in rWmhs60 vs. 61.91 in control cells; $p < 0.0001$) and thus rWmhs60 stimulation on THP-1 cells resulted in active down regulation of phagocytosis, Fig. 3B.

To further analyze the role of rWmhs60 in the process of phagocytosis, we transiently estimated the nitric oxide released by the rWmhs60 stimulated rTHP-1 macrophages before and after challenging with *E. coli*-GFP. We observed that there was no significant difference in the release of NO by rWmhs60, LPS, PPD stimulated and control rTHP-1 macrophages (Fig. 3C). As expected, SNP which served as a positive control, when stimulated the THP-1 cells resulted in high NO levels.

3.5. Assessment of rWmhs60 induced apoptosis in human PBMCs

To examine the effect of rWmhs60 in the survival of PBMCs, morphological changes for cell death was observed by AO/EtBr staining. Cells treated with CHX and rWmhs60 showed typical characteristics of cell death and stained orange. An intact morphology of cells stained green was observed in the unstimulated (UNS) and PPD stimulated cells (Fig. 4A). Further, CHX and rWmhs60 stimulated PBMCs showed a typical ladder pattern of DNA fragments, a characteristic feature of apoptosis while UNS and PPD stimulated PBMCs did not exhibit any such pattern (Fig. 4B), thus supporting the morphological observations. Additionally, to quantify the apoptotic cell death in the stimulated PBMCs, annexin V FITC and propidium iodide (PI) were used for Fluorescence Activated Cell Sorting (FACS) analysis in which PBMCs were outlined and gated using FACS dot blot.

Lymphocytes and monocytes were gated separately from PBMCs based on scattering of light (Fig. 5A). Early apoptotic cells were annexin-V FITC⁺/PI⁻, late apoptotic cells were annexin-V FITC⁺/PI⁺ and dead cells were annexin-V FITC⁻/PI⁺. In lymphocyte population, neither early nor late apoptotic cells were observed in the unstimulated or antigen (CHX, PPD and rWmhsp60) stimulated cultures. In contrast, significant increase in the annexin-V FITC⁺/PI⁺ cells (25.2 in rWmhsp60 vs. 1.5 in unstimulated cells; $p < 0.05$) and annexin-V FITC⁻/PI⁺ cells (22.26 in rWmhsp60 vs. 1.5 in unstimulated cells; $p < 0.05$) were observed among monocytes stimulated with rWmhsp60. However CHX stimulation resulted in enhanced early apoptotic, late apoptotic and dead cells among monocytes compared to lymphocytes (Fig. 5B and C). These findings corroborate with morphological observations and DNA fragmentation assay. Thus, these results suggest that rWmhsp60 induces apoptosis in monocytes and not in lymphocytes.

4. Discussion

When the host cells interact with microfilaria or adult parasites, they are exposed to both the filarial and *Wolbachia* antigens. In order to understand the relative contribution of these antigens in the elicitation of the immune response, it is necessary to have these antigens in pure form for immunological investigations. Our lab has produced well characterized filarial and *Wolbachia* recombinant antigens. rWmhsp60 is one such immunodominant protein that has been identified from the infective L3 stage of the parasite. This protein has been recognized by the sera of filarial patients and not the endemic normals [17]. rWmhsp60 contained negligible amounts of LPS as assessed by LAL assay. PBMCs proliferation observed upon stimulation with rWmhsp60 (10 µg/ml) in the presence and absence of Polymyxin-B sulfate indicated that the observed proliferative response of rWmhsp60 was not due to LPS contamination (data not shown).

Our study has showed that stimulation of THP-1 monocytes by rWmhsp60, induces the gene expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Also increased levels of the pro-inflammatory cytokines IFN-γ, TNF-α and GM-CSF were observed in the pooled culture supernatant. Similar increase in the levels of pro-inflammatory cytokines IL-1β, IL-6, IFN-γ and TNF-α in filarial patient following the release of *Wolbachia* into the blood has been reported earlier [22–24]. Also previous studies from our lab with crude filarial antigen (BMA) stimulated human monocytes have shown increased IL-1β production in filarial patients [25]. Similar observations were made with other bacterial hsp such as GroEL [9] in human monocytes, Lp-hsp60 [14] and Mt-hsp65 [26,27] in THP-1 monocytes/macrophages. Interestingly, the gene expression of IL-10 was elevated on rWmhsp60 stimulation but the protein levels in the pooled supernatant were apparently low. The possible explanation that could be offered for this is the levels of the protein are controlled not only by regulating transcripts but also the regulation at other steps like translation and protein stability.

Further, no significant difference in iNOS gene expression by rWmhsp60 treated THP-1 macrophages/monocytes was noticed. Similarly GroEL from *Wolbachia* of *Dirofilaria immitis* administered in BALB/c mice failed to induce iNOS and NO production [28]. iNOS production was low with LPS stimulation and is supported by other investigators [29]. As expected, increased levels of IL-1β [30], IL-6 and TNF-α [25,9] and no significant change in IL-10 and IL-12 was detected from LPS stimulated THP-1 monocytes. Our findings with PPD stimulated cytokine production on THP-1 monocytes supports the previous findings with human monocytes incubated with PPD [31]. These results suggest that, rWmhsp60 stimulates THP-1 monocytes to secrete proinflammatory cytokines.

rWmhsp60 stimulation of THP-1 macrophages resulted in inhibition of phagocytosis with no significant change in the NO release. One possible reason for the inhibition of phagocytosis could be the binding of rWmhsp60 to *E. coli* specific macrophage surface receptors thereby preventing the binding of *E. coli* or could be the induction of apoptosis in monocytes. The latter is possible as rWmhsp60 stimulation of THP-1 monocytes results in the enhanced production of pro-inflammatory cytokines TNF- α and IFN- γ and GM-CSF that are known to initiate apoptotic activity. This synergy of pro-apoptotic cytokines may lead to inflammation and apoptosis leading to monocyte dysfunction as observed in filarial patients [32]. We found that rWmhsp60 induced apoptosis on monocytes and not lymphocytes. It would be appropriate to use THP-1 monocytes/macrophages, for the study on apoptosis but PBMCs were used as these cells are involved in interaction with the parasite during natural infection. However, the molecular mechanism involved requires further investigation. In conclusion, rWmhsp60 induces pro-inflammatory cytokine production, diminished phagocytic function of monocyte/macrophage and promotes apoptosis of human monocytes.

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References

- Ottesen EA. Infection and disease in lymphatic filariasis: an immuno-logic perspective. *Parasitology*. 1992; 104:S71–S79. [PubMed: 1589302]
- Ottesen EA. Immune responsiveness and the pathogenesis of human onchocerciasis. *J. Infect. Dis.* 1995; 171:659–671. [PubMed: 7876613]
- Hall LR, Pearlman E. Pathogenesis of *Onchocerca keratitis* (River blindness). *Clin. Microbiol. Rev.* 1999; 12:445–453. [PubMed: 10398675]
- Taylor MJ, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J. Immunol.* 2005; 174:4924–4933. [PubMed: 15814720]
- Taylor MJ, Cross HF, Bilo K. Inflammatory responses induced by the filarial nematode *Brugia malayi* are mediated by lipopolysaccharide-like activity from endosymbiotic *Wolbachia* bacteria. *J. Exp. Med.* 2000; 191:1429–1436. [PubMed: 10770808]
- Dreyer G, Noroes J, Figuerdo-Silva J. New insights into the natural history and pathology of bancroftian filariasis: implications for clinical management and filariasis control programmes. *Trans. R. Soc. Trop. Med. Hyg.* 2000; 94:594–596. [PubMed: 11198637]
- Turner JD, Langley RS, Johnston KL, Egerton G, Wanji S, Taylor MJ. *Wolbachia* endosymbiotic bacteria of *Brugia malayi* mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner. *J. Immunol.* 2006; 177:1240–1249. [PubMed: 16818783]
- Brattig NW, Rathjens U, Ernst M. Lipopolysaccharide-like molecules derived from *Wolbachia* endobacteria of the filaria *Onchocerca volvulus* are candidate mediators in the sequence of inflammatory and anti-inflammatory responses of human monocytes. *Microbes Infect.* 2000; 2:1147–1157. [PubMed: 11008105]
- de Galdiero M, l'Ero GC, Marcatili A. Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. *Infect. Immun.* 1997; 65:699–707. [PubMed: 9009333]
- Heumann D, Glauser MP, Calandra T. Molecular basis of host pathogen interaction in septic shock. *Curr. Opin. Microbiol.* 1998; 1:49–55. [PubMed: 10066457]
- Krieg AM. The role of CpG motifs in innate immunity. *Curr. Opin. Immunol.* 2000; 12:35–43. [PubMed: 10679406]
- Kaufmann SHE. Heat shock proteins and the immune response. *Immunol. Today.* 1990; 11:129–136. [PubMed: 2187470]

13. Kaufmann SHE, Schoel B, Van Embden JD. Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infections. *Immunol. Rev.* 1991; 12:67–90. [PubMed: 1937535]
14. Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW. Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. *Infect. Immun.* 1994; 62:5689–5693. [PubMed: 7960155]
15. Stewart GR, Young DB. Heat-shock proteins and the host—pathogen interaction during bacterial infection. *Curr. Opin. Immunol.* 2004; 16:506–510. [PubMed: 15245747]
16. Van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat. Rev. Immunol.* 2005; 5:318–330. [PubMed: 15803151]
17. Suba N, Shiny C, Taylor MJ, Narayanan RB. *Brugia malayi* *Wolbachia* hsp60 IgG antibody and isotype reactivity in different clinical groups infected or exposed to human bancroftian lymphatic filariasis. *Exp. Parasitol.* 2007; 116:291–295. [PubMed: 17306254]
18. Shiny C, Krushna NSA, Archana B, Farzana B, Narayanan RB. Serum antibody responses to *Wolbachia* surface protein in patients with human lymphatic filariasis. *Microbiol. Immunol.* 2009; 53:685–693. [PubMed: 19954456]
19. Taylor MJ, Cross HF, Ford L, Makunde WH, Prasad GB. *Wolbachia* bacteria in filarial immunity and disease. *Parasite Immunol.* 2001; 7:401–409. [PubMed: 11472559]
20. Shiny C, Krushna NSA, Babu S, Elango S, Manokaran G, Narayanan RB. Recombinant *Wolbachia* Heat Shock Protein 60 (HSP60) mediated immune responses in patients with lymphatic filariasis. *Microbes Infect.* 2011; 13:1221–1231. [PubMed: 21827871]
21. Tsuchiya S, Yamihi M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer.* 1980; 26:1373–1378.
22. Turner PF, Rockett KA, Ottesen EA. Interleukin-6 and tumor necrosis factor in the pathogenesis of adverse reactions after treatment of lymphatic filariasis and onchocerciasis. *J. Infect. Dis.* 1994; 169:1071–1075. [PubMed: 8169393]
23. Haarbrink M, Terhell AJ, Abadi GK, Mitsui Y, Yazdanbakhsh M. Inflammatory cytokines following diethylcarbamazine (DEC) treatment of different clinical groups in lymphatic filariasis. *Trans. R. Soc. Trop. Med. Hyg.* 1999; 6:665–672. [PubMed: 10717762]
24. Cross H, Haarbrink M, Egerton G, Yazdanbakhsh M, Taylor MJ. Severe reactions to filarial chemotherapy are associated with the release of *Wolbachia* endosymbionts into the blood. *Lancet.* 2001; 358:1873–1875. [PubMed: 11741630]
25. Sasisekhar B, Aparna M, Augustin DJ, Kaliraj P, Kar SK, Nutman TB, Narayanan RB. Diminished monocyte function in microfilaremic patients with lymphatic filariasis and its relationship to altered lymphoproliferative responses. *Infect. Immun.* 2005; 73:3385–3393. [PubMed: 15908365]
26. Friedland JS, Shattock R, Remick DG, Griffin GE. Mycobacterial 65 kDa heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clin. Exp. Immunol.* 1993; 91:58–62. [PubMed: 8419086]
27. Zhang Y, Doerfler M, Lee TC, Guillemin B, Rom WN. Mechanisms of stimulations of interleukin-1 beta and tumor necrosis factor-alpha by *Mycobacterium tuberculosis* components. *J. Clin. Invest.* 1993; 91:2076–2083. [PubMed: 7683696]
28. Morchon R, Bazzocchi C, Belmonte JL, Pacho JRM, Kramer LH, Grandi G, Simon F. iNOS expression is stimulated by the major surface protein (WSP) from *Wolbachia* bacterial endosymbiont of *Diro filaria immitis* following subcutaneous injection in mice. *Parasitol. Int.* 2007; 56:71–75. [PubMed: 17126589]
29. Pham P, Bransteitter R, Petruska J, Goodman MF. Processive AID-catalyzed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature.* 2003; 424:103–107. [PubMed: 12819663]
30. Grahames CB, Michel AD, Chessell IP, Humphrey PP. Pharmacological characterization of ATP and LPS-induced IL-1beta release in human monocytes. *Br. J. Pharmacol.* 1999; 127:1915–1921. [PubMed: 10482924]
31. Babu S, Nutman TB. Proinflammatory cytokines dominate the early immune responses to filarial patients. *J. Immunol.* 1999; 171:6723–6732. [PubMed: 14662876]

32. Semnani RT, Keiser PB, Coulibaly YI. Filaria-induced monocyte dysfunction and its reversal following treatment. *Infect. Immun.* 2006; 74:4409–4417. [PubMed: 16861626]

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Fig. 1.

Reactivity of rWmhsp60 with sera from different clinical groups. Western blot analysis shows the reactivity of the rWmhsp60 with sera from different filarial clinical groups. rWmhsp60 resolved on 12% SDS-PAGE and transferred on to a nitrocellulose membrane and probed with appropriate primary and secondary antibodies. Lane 1- protein molecular weight marker; Lane 2- NMS (1:5000) as a negative control showing an absence of reactivity; Lane 3- shows the reactivity with mouse anti-rWmhsp60 (1:1000) as a positive control. Lanes 4–7 show the reactivity with sera (1:100) dilution from different filarial clinical groups (Lane 4: NEN, Lane 5: EN, Lane 6: MF and Lane 7: CP).

Fig. 2. rWmhsp60 resulted in increased expression of pro-inflammatory cytokines. THP-1 monocytes were stimulated with LPS (100 ng/ml), PPD (10 μ g/ml) and rWmhsp60 (5 μ g/ml). (A) Cells were harvested and the gene expression of cytokines (I) IL-1 β , (II) TNF- α , (III) IL-12, (IV) IL-6, (V) IL-10 and (VI) iNOS were determined by Real Time PCR, 24 h post stimulation. Antigen-induced fold change was plotted for the values on the Y-axis upon Ag^{-ve}, LPS, PPD and rWmhsp60 stimulation. Each bar represents expression levels as fold change. Fold change (δ CT) = CT of the house keeping gene (18s rRNA) – CT of the target gene. Change in the expression of the normalized target gene as a result of antigenic exposure ($2^{-\delta\delta$ CT), where $\delta\delta$ CT = δ CT of Stimulated – δ CT of Unstimulated. Values are represented as Mean $\pm$ S.D of 3 independent experiments. (B) rWmhsp60 results in augmented release of pro-inflammatory cytokines. Pooled culture supernatants from 3 independent cytokine gene expression experiments were assayed for levels of cytokines (I)

TNF- α , (II) IFN- γ , (III) GM-CSF and (IV) IL-10 using Bioplex multiplex cytokine ELISA assay system.

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Fig. 3.

rWmhsp60 induced phagocytic activity in THP-1 cells. (A) Photomicrograph of one of the representative experiment of five independent experiments of THP-1 monocytic cells differentiated into macrophages by PMA and incubated with stimulants for 24 h. Subsequently GFP expressing *E. coli* (represented by green dots) was added and incubated for 2 h to examine phagocytosis. (I) Control, (II) LPS (100 ng/ml), (III) PPD (10 $\mu\text{g/ml}$) and (IV) rWmhsp60, (5 $\mu\text{g/ml}$). (B) Percent phagocytosis determined from Fig. 3A. Values are represented as Mean $\pm$ S.D of 3 independent experiments. (C) Assessment of nitric oxide (NO) production: Nitric oxide release was measured in PMA rested THP-1 cells (control) and PMA rested THP-1 cells stimulated with LPS (100 ng/ml), PPD (10 $\mu\text{g/ml}$), rWmhsp60 (5 $\mu\text{g/ml}$) and SNP, 300 $\mu\text{M/ml}$ as positive control and incubated for 24 h. After 24 h stimulation, cells were treated with *E. coli*-GFP for 2 h. The culture supernatants before and after the addition of *E. coli*-GFP were collected and NO was measured by Griess Assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Assessment of rWmhsp60 induced apoptosis in human PBMCs. rWmhsp60 induces apoptosis in PBMCs. (A) Morphological changes were observed by AO/EtBr staining. (I) UNS, (II) CHX (100 μ M/ml), (III) PPD (10 μ g/ml) and (IV) rWmhsp60 (5 μ g/ml). Intact cells were stained green due to AO which is membrane permeable, while apoptotic cells stained red due to incorporation of membrane impermeable EtBr. (B) Induction of DNA fragmentation visualized by agarose gel electrophoresis. Apoptotic cells containing fragmented DNA were visible in rWmhsp60 and CHX stimulated cells, whereas UNS and PPD stimulated cells do not show any DNA fragmentation. Lane 1: UNS, Lane 2: CHX (100 μ M/ml), Lane 3: PPD (10 μ g/ml) and Lane 4: rWmhsp60 (5 μ g/ml). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Quantitative analysis of apoptosis in human PBMCs. (A) Representative flow cytometric dot plot showing gating strategies for analyzing monocytes and lymphocytes subsets from PBMCs based on forward- and side-light scattering. (B) Representative distributions of the fluorescence intensity of annexin V FITC and PI binding of human lymphocytes (Fig. 5B. I) and monocytes (Fig. 5B. II) after 24 h stimulation of PBMCs, with CHX, PPD and rWmhsp60. (C) The percentage of apoptotic and dead cells in lymphocyte (Fig. 5C. I) and monocyte populations (Fig. 5C. II) upon stimulation with CHX, PPD and rWmhsp60 were evaluated using GraphPad Prism version 5. Values are represented as Mean $\pm$ S.D of 5 independent experiments.