



Human filarial *Wolbachia* lipopeptide directly activates human neutrophils *in vitro*

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SUMMARY

The host inflammatory response to the *Onchocerca volvulus* endosymbiont, *Wolbachia*, is a major contributing factor in the development of chronic pathology in humans (onchocerciasis/river blindness). Recently, the toll-like pattern recognition receptor motif of the major inflammatory ligands of filarial *Wolbachia*, membrane-associated diacylated lipoproteins, was functionally defined in murine models of pathology, including mediation of neutrophil recruitment to the cornea. However, the extent to which human neutrophils can be activated in response to this *Wolbachia* pattern recognition motif is not known. Therefore, the responses of purified peripheral blood human neutrophils to a synthetic N-terminal diacylated lipopeptide (WoLP) of filarial *Wolbachia* peptidoglycan-associated lipoprotein (PAL) were characterized. WoLP exposure led to a dose-dependent activation of healthy, human neutrophils that included gross morphological alterations and modulation of surface expressed integrins involved in tethering, rolling and extravasation. WoLP exposure induced chemotaxis but not chemokinesis of neutrophils, and secretion of the major neutrophil chemokine, interleukin 8. WoLP also induced and primed the respiratory burst, and enhanced neutrophil survival by delay of apoptosis. These results indicate that the major inflammatory motif of filarial *Wolbachia* lipoproteins directly activates human neutrophils *in vitro* and promotes a molecular pathway by which human neutrophils are recruited to sites of *Onchocerca* parasitism.

Keywords filariasis, human neutrophils activation, *Onchocerca volvulus*, river blindness, *Wolbachia*, *Wolbachia* lipoproteins

INTRODUCTION

Onchocerciasis (river blindness) is a parasitic disease affecting 37 million people worldwide. An estimated 270 000 people have been blinded by infection with an additional 500 000 suffering visual impairment (1, 2). The causative agent, the filarial worm *Onchocerca volvulus*, resides in subcutaneous nodules, also known as onchocercomas. Disease is caused by the migration into and subsequent death of numerous microscopic larval progeny (microfilariae; mf) in dermal and ocular tissues. Inflammatory responses invoked by the release of somatic antigens from dead mf are important in the initiation of disease pathogenesis. In particular, liberated *Wolbachia*, endosymbiotic bacteria found in many filarial species, are major innate inflammatory stimuli (3).

Granulocytes are the principal component of the early inflammatory infiltrate around damaged and dying mf in the cornea. Neutrophils contribute to the *Wolbachia*-mediated pathogenesis of onchocerciasis (4–7) and their infiltration into ocular tissues following mf exposure is dependent on the presence of *Wolbachia*. In mouse models of ocular *Onchocerca* keratitis, neutrophils surround mf in the cornea and are recruited early after injection of isolated *Wolbachia* bacteria and *Wolbachia*-containing filarial extracts but not of extracts from worms *Wolbachia*-depleted or naturally devoid of the symbiont, resulting in corneal haze and opacity (4, 8, 9). After injection of mf into the cornea, *Wolbachia* are found in neutrophil phagosomes fusing with granules (4).

In dermal and subcutaneous tissues, recruitment and maintenance of neutrophils at the site of *Onchocerca* infection and inflammation appears to mirror that occurring in the cornea (5, 10). In both human and bovine

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onchocerciasis, neutrophil recruitment and maintenance within adult worm nodules is sensitive to anti-*Wolbachia* tetracycline-based chemotherapy (11–14). In the latter infection system, neutrophil depletion occurs at a point following sterilisation of the adult *Onchocerca* tissues of endosymbionts but prior to significant decline in adult worm viability (14). Further evidence to support a central role of nematode *Wolbachia* in promoting neutrophilic responses to filariae comes from observations that neutrophils accumulate around *Wolbachia*-containing *Onchocerca spp* but not around *Onchocerca spp.* naturally devoid of the endosymbiont (11, 15).

Systemically, neutrophil activation is also observed during the adverse reactions following microfilaricidal treatment of filariasis patients, which correlate with pretreatment mf loads and the presence of liberated *Wolbachia* DNA or whole bacterial cells in the circulation (16–18). Systemic adverse events and levels of liberated circulating *Wolbachia* also positively correlate with neutrophilia, circulating levels of pro-inflammatory cytokines, including the neutrophil CXC chemokine IL-8 (CXCL8) and neutrophil-derived molecules, such as calprotectin and calgranulin B (16–19).

Despite being pivotal effector cells in the pathogenesis of onchocercal disease, few studies have addressed the mechanisms of neutrophil activation and recruitment by filarial *Wolbachia*. In mice, it has been established that production of neutrophil-specific chemokines, neutrophil recruitment and subsequent development of corneal opacity is Toll-like Receptor (TLR)2 and MyD88-dependent, implicating *Wolbachia* TLR ligands in the initiation of neutrophil responses (8, 9). Recently, the diacylated membrane-associated lipoproteins of filarial *Wolbachia* have been promoted as the major pro-inflammatory TLR ligands expressed by filarial endosymbionts and their biosynthetic pathway have been suggested as a potential target for anti-*Wolbachia* filarial therapy (20, 21). Bioinformatic and database searches consistently predict the presence of two lipoproteins in *Wolbachia*: peptidoglycan-associated lipoprotein (PAL), and a type IV secretion system protein (VirB6), which share an N-terminal motif (20). *Wolbachia* PAL of *Brugia malayi* (*wBmPAL*) had a predicted outer membrane location (20) and has been identified as an abundant *Wolbachia* protein in the secretome and proteome of *B. malayi* (22, 23). Depletion of lipid or protein from total *B. malayi* female worm extracts nullifies innate immune activation, supporting a role of native *Wolbachia* lipoproteins as the ligands of TLR2 innate immune reactivity (20). A synthetic, lipolated version of the N-terminus of *Wolbachia* PAL, *WoLP*, has been identified as important in the mediation of filarial inflammation and modulation of antifilarial adaptive immune responses via the ligation of (TLR)2/6 and to a

minor extent of TLR2/1 on myeloid immune cells and stromal cells (20). Most importantly, *WoLP* can replicate the effects of *Wolbachia* bacteria in mediating experimental *Onchocerca* keratitis in a TLR2/6 dependent manner (20).

The responses of purified human neutrophils to *Wolbachia* lipoproteins have not been elucidated, but these likely represent responses to both PAL and VirB6, due to the shared N-terminal motif (20). In this study, we investigated the interaction between *WoLP* and human neutrophils *in vitro* and determined that this *Wolbachia*-specific lipoprotein motif is sufficient to activate a range of activation phenotypes. Our data therefore demonstrates a direct functional role of the major *Wolbachia* inflammatory ligand in promoting human neutrophil responses. We hypothesise that diacylated *Wolbachia* lipoproteins are critical in the induction and maintenance of neutrophil recruitment during human onchocerciasis.

MATERIALS AND METHODS

Human neutrophil isolation

The use of blood neutrophils from adult healthy volunteers was approved by the Research Ethics Committee of the University of Liverpool, UK. Peripheral blood was collected by venepuncture in lithium heparin vacutainers, and neutrophils were isolated using Polymorphprep (Axis Shield Dundee, Scotland) following manufacturers instructions. Contaminating red blood cells were lysed with 9 : 1 ammonium chloride lysis buffer (13.4 mM KHCO₃, 155 mM NH₄Cl, 96.7 μM EDTA) in RPMI 1640 culture media (Gibco, Life Technologies, Carlsbad, CA, USA). Cell viability was assessed by 0.2% trypan blue staining (Sigma Aldrich Gillingham, UK) and was always ≥98%. The purity of isolated neutrophils was assessed by Rapid Romanowsky stain (HD Supplies, TCS Biosciences, Buckingham, UK) of cytopins (Cytospins3, Shandon, Thermo Scientific, Loughborough, UK) followed by differential count of ≥700 cells by optical microscopy. The purity of isolated neutrophils was always ≥97% with ≤0.14% monocyte contamination. Neutrophils were incubated at 37°C in a humidified incubator in RPMI 1640 (with 25 mM HEPES and 2 mM L-glutamine) culture media (Gibco).

Neutrophil stimuli

Stimuli for neutrophil cultures were synthetic 20-mers of the N-terminal region of *wBmPAL* (CSKRGVNAIN KMNFFVVKQMK) di-palmitoylated at the N-terminal cysteine residue (*WoLP*) (20) (EMC Microcollections Tubingen, Germany); recombinant human TNFα (Calbiochem); recombinant, Merk Millipore, Darmstadt, Germany

human GM-CSF (Roche Welwyn Garden City, UK); and N-formyl-methionine-leucine-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA, both from Sigma Aldrich) at the indicated concentrations. TLR2/6 specificity of *W*oLP was confirmed by lack of TNF α production in response to *W*oLP stimulation in TLR2^{-/-} and TLR6^{-/-} mouse macrophage cultures as previously performed (data not shown). Similarly, lack of LPS contamination was confirmed by determining equivalent TNF α release in WT and TLR4^{-/-}-derived macrophages (data not shown). In all assays, vehicle Dimethyl Sulfoxide (DMSO, Sigma Aldrich) was included as control stimulus dissolved in culture media when stimuli dissolved in DMSO were used.

Cell culture

For incubations <8 h, neutrophils were cultured at 5×10^6 cells/mL in 1.5 mL screw-top tubes (Eppendorf Stevenage, UK) with gentle rotation. For incubations ≥ 8 h, neutrophils were cultured at 1×10^6 cells/mL in 24-well culture plates in culture media supplemented with 10% heat-inactivated human AB serum (Sigma Aldrich) in the presence of 5% CO₂.

Morphological assessment of neutrophil activation

After 1.5 h culture with *W*oLP (1 μ g/mL) and control stimuli DMSO and fMLP (0.01 μ M), the morphology of neutrophils was visualized using a Zeiss Axiovert S100TV microscope (Carl Zeiss, Cambridge, UK) supporting a Hamamatsu multiformat CCD camera (Hamamatsu Corporation, Welwyn Garden City, UK) with AQM ADVANCE 6 software (Kinetic Imaging, Liverpool, UK).

Chemotactic and chemokinetic assays

Chemotactic and chemokinetic assays were performed using a transwell system (Millicell 24-wells Cell Culture Hanging Inserts, Millipore, Darmstadt, Germany) in 24-well culture plates precoated with sterile Poly-Hema (Sigma Aldrich), according to the manufacturers instructions. For the chemotaxis assay, *W*oLP (0.5–5 μ g/mL), and control stimuli DMSO and fMLP (0.01 μ M) were added in the culture wells in RPMI 1640 culture media, while neutrophils were added at 5×10^6 cells/mL in RPMI 1640 culture media in the upper hanging insert. To differentiate between chemotaxis (migration towards a chemotactic gradient) and chemokinesis (increased random movements upon exposure to a stimulus in the absence of a gradient), the assay was then carried out with equal concentrations of stimuli *W*oLP (1 μ g/mL) and control DMSO and fMLP

(0.01 μ M) in both the upper and lower chambers of the transwell system. The chambers were incubated for 1.5 h at 37°C and the cells migrating into the lower chamber were counted using a Beckman Coulter cell counter supporting MULTISIZER 3 software (Beckman Coulter, High Wycombe, UK), counting only particles between 8 and 12 μ m diameter.

Assessment of expression of surface adhesion molecules and apoptosis by flow cytometry

After 1 h culture in the presence of *W*oLP (0.1 μ g/mL) and control stimuli DMSO and GM-CSF (5 ng/mL), the surface expression of β_2 -integrins CD11b and CD18, and of L-Selectin by neutrophils was assessed by flow cytometry (FC). Briefly, neutrophils were stained for 30 min on ice in FC buffer (0.2% Bovine Serum Albumin (Sigma Aldrich) in PBS) with FITC-conjugated rat antihuman CD11b IgG_{2b} (Miltenyi Biotec, Bisley, UK), mouse antihuman CD18 IgG₁ (R&D Systems, Minneapolis, MN, USA), mouse antihuman L-Selectin IgG₁ (R&D Systems) and mouse IgG₁ isotype control (Santa Cruz Biotechnology Dallas, TX, USA). After fixation in 2% paraformaldehyde in FC buffer, cells (2×10^5 /mL in FC buffer) were analysed with a Guava EasyCyte Plus (Millipore) flow cytometer and CYTOSOFT 5.3 software.

For the assessment of apoptosis, neutrophils were cultured with *W*oLP (1 ng–5 μ g/mL) and control stimuli DMSO and GM-CSF (5 ng/mL) for 15 and 20 h. Apoptotic cells were labelled for 15 min at room temperature with Annexin-V-FITC (Biosource Life Technologies, Carlsbad, CA, USA) 1 : 100 in HBSS (Gibco). Propidium iodide (PI, from Sigma Aldrich) was added at 1 μ g/mL in HBSS to wells to label late apoptotic and necrotic cells. Unstained cells in HBSS were included as control for background fluorescence. Neutrophils (1×10^5 /mL) were analysed as above.

Respiratory burst assay

Production of total intra- and extra-cellular reactive oxygen species (ROS) was measured using a luminol-enhanced chemiluminescence assay. Neutrophils were primed for 30 min with *W*oLP (1 ng–5 μ g/mL) and control stimuli DMSO and TNF α (10 ng/mL). Cells were then added to white, low-adhesion 96-well plates and stimulated with fMLP (1 μ M), PMA (100 ng/mL) or DMSO (unstimulated cells) in the presence of luminol (10 μ M in HBSS, from Sigma Aldrich). Chemiluminescence was read every 30 s for 30 min in a Wallac Victor™ Light 1420 Luminescence Counter (Perkin Elmer, Waltham, MA, USA) at 37°C. Background chemiluminescence was assessed by inclusion

of one well without cells per each stimulus. Total chemiluminescence was calculated using the area under the curve (AUC) method. Cell viability at the time of peak ROS production was assessed by 0.2% trypan blue staining and was always $\geq 93\%$.

Assessment of cytokine production by ELISA

Levels of IL-1 β , IL12-p70, IL-8, GM-CSF and TNF α in neutrophil culture supernatants were analysed using Duo-Set ELISA Development kits (R&D Systems) as per the manufacturers instruction. Absorbance was read in a FLUOstar Omega plate reader supporting MARS data analysis software 1.20 (BMG Labtech, Ortenberg, Germany). The best-fit curve method was used to calculate the cytokine concentration in each sample.

Statistical analysis

Samples from ≥ 3 donors were used for cell cultures and neutrophil functional assays. Means were compared using independent samples *t*-test. For neutrophil surface adhesion molecule expression, mean percentage changes in mean fluorescence intensity compared with control were analysed using one-sample *t*-test. A $P \leq 0.05$ was considered significant. All analyses were carried out using SPSS STATISTICS 20.0 (IBM Postsmouth, UK).

RESULTS

Neutrophil morphology

Activation of neutrophils resulted in a change in cell morphology, with resting neutrophils having a typical round shape, but an elongated aspect after activation. After 1.5 h exposure to *WoLP*, neutrophils from peripheral blood of human volunteers showed an evident activated cell shape (Figure 1a), similar to that obtained by exposure to fMLP (Figure 1b), while cells exposed to culture media \pm DMSO had a resting morphology (Figure 1c, d).

Chemotaxis and chemokinesis

Migration of neutrophils in response to *WoLP* was analysed in a transwell system using fMLP as a positive control. In the presence of a concentration gradient, *WoLP* and fMLP induced the migration of cells in higher numbers compared with control stimuli at all concentration used ($P \leq 0.004$, Figure 2a). It was then assessed whether this enhanced migration was due to chemotaxis or to increased random movement (chemokinesis). As shown in Figure 2b, the *WoLP*- and fMLP-induced migration of neutrophils into the lower chamber was significantly impaired when the stimulus was present in both chambers, that is, in the absence of a concentration gradient ($P \leq 0.007$),

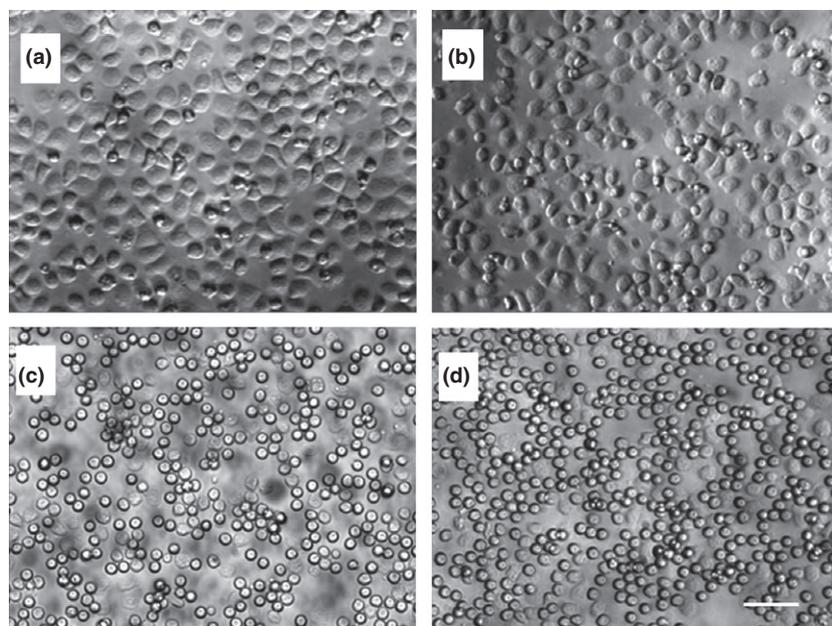


Figure 1 Isolated neutrophils acquire activated cell morphology upon exposure for 1.5 h to *WoLP* (a), similar to that induced by N-formyl-methionine-leucine-phenylalanine (fMLP) (b). Exposure to media alone (c) and Dimethyl Sulfoxide (DMSO) control (d) did not result in any evident change in cell morphology. Original magnification 32 \times . Scale bar, 50 μ m. Images are representative of $n = 3$ independent experiments.

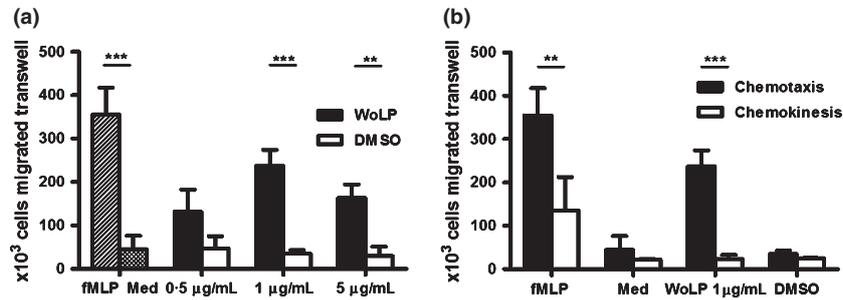


Figure 2 (a) In a transwell chemotaxis assay isolated neutrophils migrate towards a concentration gradient of *WoLP* (0.5–5 µg/mL) and control stimulus N-formyl-methionine-leucine-phenylalanine (fMLP), but not Dimethyl Sulfoxide (DMSO) and media alone. ***P* = 0.004; ****P* = 0.001. (b) Chemokinesis assay: isolated neutrophils migrate only towards (chemotaxis, black bars) but not in the absence of (chemokinesis, white bars) concentration gradient of *WoLP* and fMLP. ***P* = 0.007; ****P* < 0.001. Bar graphs mean ± SD of cells migrated transwell of *n* = 3 donors each tested in duplicate for chemotaxis and of *n* = 6 donors for chemokinesis experiment.

indicating that *WoLP* exerts a chemotactic rather than chemokinetic effect on human neutrophils *in vitro*.

Surface adhesion molecule expression

L-Selectin is the major selectin expressed on neutrophils, contributing to leucocyte rolling on endothelial cells of blood capillaries, and is rapidly shed from the cell surface upon activation. CD11b/CD18 is one of the major neutrophil integrins, involved in strong binding of activated neutrophils to the endothelium, and its surface expression is upregulated upon activation. One hour incubation with *WoLP* led to significant downregulation of surface expression of L-Selectin (*P* < 0.001) and upregulation of CD11b (*P* = 0.001) and CD18 (*P* = 0.025) compared with incubation with DMSO control, mirroring the effects of stimulation with the positive control, GM-CSF (Figure 3).

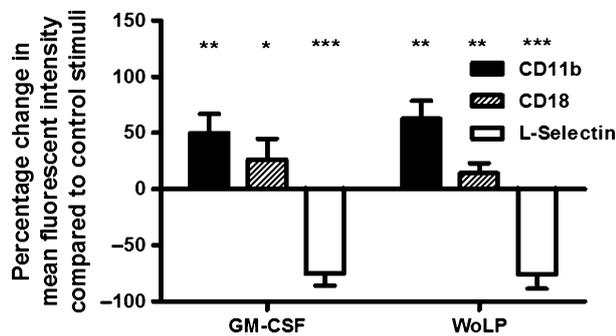


Figure 3 *WoLP* (0.1 µg/mL) induces upregulation of integrins (CD11b and CD18) and shedding of L-Selectin on isolated neutrophils, similar to the effects of GM-CSF. **P* = 0.025 for *WoLP* and *P* = 0.033 for GM-CSF; ***P* = 0.003; ****P* ≤ 0.001. Bar graph represents percentage change (mean ± SD) in mean fluorescence intensity compared with stimulation with control Dimethyl Sulfoxide (DMSO) (for *WoLP*) and media alone (for GM-CSF) of neutrophils from *n* = 5 donors.

Apoptosis

While resting neutrophils are short-lived cells undergoing apoptosis after ~12 h, their activation leads to an increase in their lifespan. A marker of apoptosis is the re-arrangement of molecules present on the inner and outer leaflet of the cell membrane. In particular, phosphatidylserine appears on the outer surface of the cell membrane of apoptotic cells, where it can be labelled with Annexin-V-FITC. Cells in late apoptosis and necrosis show an increased cell membrane permeability which can be detected by PI staining. After 20 h culture in media alone (Figure 4), 56.96% (±8.11%) neutrophils underwent apoptosis. At this time point, *WoLP* induced a decrease in the percentage of neutrophils undergoing apoptosis (Annexin-V⁺ PI⁻) in a concentration-dependent manner (*P* = 0.004 upon stimulation with *WoLP* 0.1 µg/mL and *P* = 0.047 upon stimulation with *WoLP* 1 µg/mL) (Figure 4). Comparable findings were observed after 15-h incubation (data

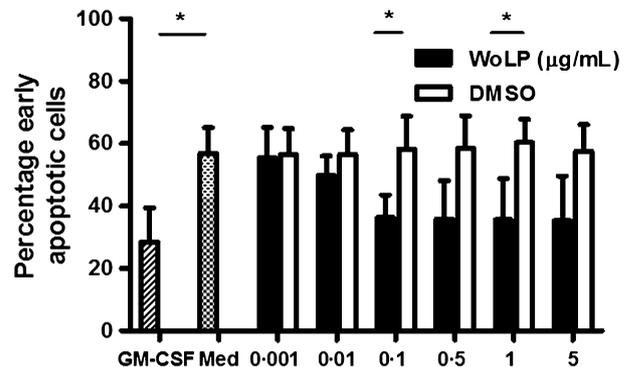


Figure 4 Exposure to *WoLP* (0.001–5 µg/mL) for 20 h delays apoptosis of isolated neutrophils similar to exposure to the positive control stimulus, GM-CSF. **P* = 0.040 *WoLP* 0.1 µg/mL and *P* = 0.047 *WoLP* 1 µg/mL; *P* = 0.023 GM-CSF. Bar graphs represent mean ± SD percentage of early apoptotic neutrophils from *n* = 3 donors each tested in duplicate.

not shown). No differences were found in the percentage of cells in late apoptosis (Annexin-V⁺ PI⁺, range 0.06%–4.98%) or necrosis (Annexin-V⁻ PI⁺, range 0.1–2.84%) (data not shown).

Respiratory burst

The production of reactive oxygen species (ROS) by stimulated neutrophils is typically low unless cells have been pretreated (primed) with other agents, which induce the upregulation of the surface expression of the NADPH oxidase complex components. Priming typically results in a greater ROS production upon stimulation with fMLP and in a more rapid response to PMA stimulation. Levels of ROS produced by neutrophils incubated with *WoLP* were significantly higher compared with cells incubated in media alone, and increased in a *WoLP* concentration-dependent manner, reaching a plateau for concentrations ≥ 0.5 $\mu\text{g/mL}$ *WoLP* (Figure 5a). When cells were stimulated with fMLP after priming with *WoLP*, the production of ROS approximately doubled compared with unprimed cells, showing a similar concentration-dependent pattern (Figure 5b). Also, stimulation of *WoLP*-primed neutrophils with PMA induced a concentration-dependent decrease in the time needed to reach the peak of ROS production compared with unprimed cells (Figure 5c).

IL-8 production

To investigate whether the activation of neutrophils *in vitro* by *WoLP*, resulted in cytokine/chemokine expression, levels of IL-1 β , IL-8, IL-12p70, GM-CSF and TNF α were measured in the supernatant of neutrophils exposed to increasing concentrations of *WoLP* for 30 min, 1 h and 15 h. Only IL-8 was detected by ELISA in neutrophil culture supernatants at ≥ 1 h time points. Levels of IL-8 increased with time and in a *WoLP* concentration-dependent manner (Figure 6).

DISCUSSION

Neutrophils are pivotal effector cells in the pathogenesis of onchocerciasis and their presence in *O. volvulus* infected tissues depends upon the filarial endosymbiont *Wolbachia*. However, precisely how neutrophils are recruited and maintained at *Onchocerca* infection sites by *Wolbachia* has not been defined. This work investigated the role of the TLR2/6 ligand motif, *WoLP*, expressed within a surface filarial *Wolbachia* lipoprotein PAL, a major inflammatory molecule expressed by the endosymbiont, in the activation, chemotaxis and survival of

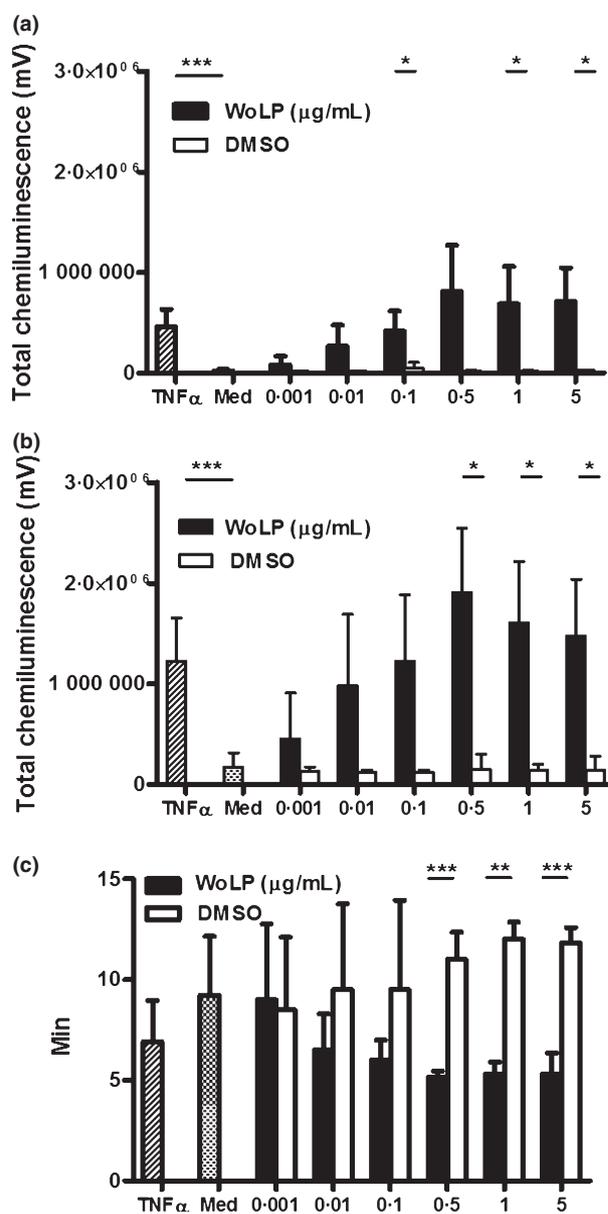


Figure 5 Incubation for 30 min with *WoLP* (0.001–5 $\mu\text{g/mL}$) alone induces reactive oxygen species (ROS) production (a) and primes ROS production upon subsequent stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP) (b) in a concentration-dependent manner, as measured by total chemiluminescence over 30 min. (c) Priming with *WoLP* (0.001–5 $\mu\text{g/mL}$) decreases the time-to-peak ROS production upon stimulation with phorbol 12-myristate 13-acetate (PMA) in a concentration-dependent manner. (a) * $P = 0.036$ *WoLP* 0.1 and 1 $\mu\text{g/mL}$; $P = 0.024$ *WoLP* 5 $\mu\text{g/mL}$; *** $P < 0.001$. (b) * $P = 0.040$ *WoLP* 0.5 $\mu\text{g/mL}$; $P = 0.015$ *WoLP* 1 and 5 $\mu\text{g/mL}$; *** $P < 0.001$. Bar graphs represent mean AUC \pm SD of $n = 3$ donors each tested in duplicate. (c) ** $P = 0.002$; *** $P = 0.001$. Bar graph represents mean \pm SD time-to-peak ROS production expressed in minutes of $n = 3$ donors each tested in duplicate.

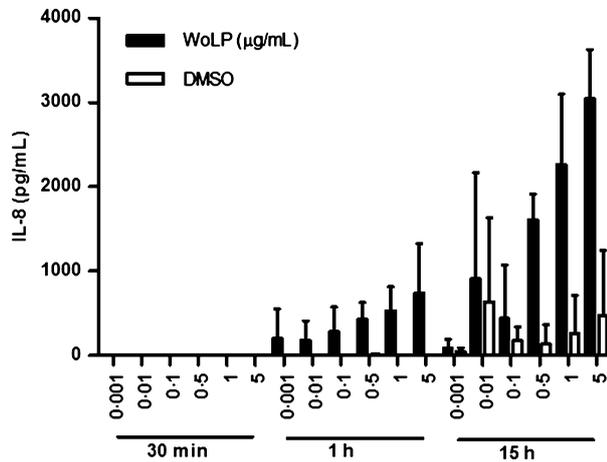


Figure 6 Isolated neutrophils secrete IL-8 upon stimulation with *WoLP* (0.001–5 µg/mL) in a concentration and time dependent manner. Bar graphs represent mean \pm SD levels of IL-8 in culture supernatants of $n = 3$ donors each tested in duplicate.

isolated human neutrophils *in vitro*. We used a synthetically generated *WoLP* 20mer lipopeptide matching the N-terminus of *wBmPAL* in our study. The use of synthetic *WoLP* offers the advantage of scrutinising human neutrophil responses to diacylated *Wolbachia* molecules (matching the motif contained within native *Wolbachia* lipoproteins) rather than triacylated recombinant *Wolbachia* lipoproteins that are generated via expression vectors with fully intact lipolating biosynthetic pathways. This approach also nullifies the risk of inclusion of possible costimulatory effects of trace TLR ligands derived from expression systems that can contaminate recombinant preparations, as evaluated previously (20). The results obtained in this study indicate that the synthetic, diacylated analogue of the N-terminal polypeptide of *Wolbachia* PAL, abundantly expressed on the endosymbiont surface (20, 22), exerts an activating effect on human neutrophils *in vitro*, as shown by changes in their cell shape and IL-8 production upon exposure to this stimulus. The analysis of cell surface adhesion molecules expression confirmed this activating effect: L-Selectin expression was downregulated while CD11b/CD18 integrins were upregulated. These results are in accordance with previous observations upon stimulation of neutrophils with ligands of TLR2/6 (MALP-2) but also TLR2/1 (Pam₃CSK₄) and TLR4 (LPS) (24–29). These functions appear directly mediated by TLR2 and TLR4 ligands (26, 29), but are amplified in the presence of monocytes, at least where TLR4 stimulation is concerned (30). Of note, the TLR2/6 ligand MALP-1 had a 10-fold higher potency than TLR2/1 ligand Pam₃CSK₄ in activating neutrophils *in vitro* (29), comparable to that observed for activation of TLR2 dimers by *WoLP* (20). Shedding of

surface L-Selectin was also reported in mouse neutrophils stimulated with isolated *Wolbachia* (8). *WoLP* was found to both prime and directly induce the production of ROS and to exert a chemotactic rather than a chemokinetic effect on isolated neutrophils. Moreover, *WoLP* was shown to induce a delay in neutrophil apoptosis. Contrasting results have been reported about the effect of TLR2 ligands on neutrophil apoptosis delay (26, 29, 31, 32). Sabroe and coworkers suggested that apoptosis delay upon TLR2 stimulation was due to the induced production of cytokines with pro-survival activity by monocyte-contaminating neutrophil cultures (26, 33). In our work, a role for *WoLP*-induced monocyte-derived pro-survival stimuli in the observed neutrophil apoptosis delay could not be completely excluded. However, the only measured cytokine that reached detectable levels in *WoLP*-stimulated neutrophil culture supernatants was IL-8, in concentrations compatible with a neutrophil, as opposed to a monocyte origin (26). IL-8 is a potent activator and chemoattractant of neutrophils, and therefore it is possible that this cytokine contributes to the *WoLP*-induced activation of neutrophils in a paracrine manner. Although the mechanism of chemotaxis of neutrophils towards *Wolbachia* is yet to be defined, the observed chemotactic response to *WoLP* *in vitro* may be reflective of a paracrine IL-8 gradient established following initial TLR2/6 ligation on the surface of neutrophils.

In conclusion, our data support an important role of the intrinsic filarial *Wolbachia* TLR2/6 motif expressed at the N-terminus of *Wolbachia* PAL, in mediating neutrophil recruitment from the blood to the skin and corneal sites of parasite infection. These are likely to be representative of neutrophil responses to both *Wolbachia* PAL and VirB6, due to the shared N-terminal motif (20). The interplay between parasite, host and *Wolbachia* in shaping neutrophil functions is intriguing. Clearly, neutrophils are recruited and activated around adult worms in a *Wolbachia*-dependent manner but they do not appear to be harmful to the parasite. It is possible that levels of whole *Wolbachia* or shed/excreted/secreted *Wolbachia* lipoproteins, such as PAL, slowly released by adult worms and their mf in nodular tissues would be sufficient to maintain chronic recruitment but not to fully activate macrofilaricidal responses. In comparison, the bolus release of *Wolbachia* and their lipoproteins after microfilaricidal drug therapy may more potently activate neutrophils, thus mediating onchocercal immunopathology. Nevertheless, defensins and calgranulins have been found on the surface of adult worms, suggesting neutrophil activation and degranulation occurs in living onchocercomas (34–36). This may indicate that living worms have a mechanism of deactivating the harmful effects of neutrophil granule-release products or

are else inherently resistant to the toxic effects of neutrophil products. Indeed, several parasite molecules that neutralize host reactants, such as antioxidants and protease inhibitors, have been characterized (12). Far from being damaging to adult *Onchocerca*, a recent hypothesis has been proposed that the large infiltrate of neutrophils proximal to the nematode cuticle in onchocercomas may actually be beneficial to parasite survival. As such, neutrophil recruitment and release of neutrophil products adjacent to the nematode surface may form a bio-physical barrier blocking infiltration of more macrofilaricidal immune effector cells, such as eosinophils (13, 14). Interestingly, it has been reported that binding of eosinophil peroxidase to human neutrophils *in vitro* leads to reversible inhibition of its peroxidase activity (37), and, in this regard, the recently discovered process of neutrophil death via extrusion of cellular DNA, called NETosis, deserves investigation (38, 39). Finally, recent advances in the understanding of neutrophil functions illustrate that these cells are able to shape the adaptive immune response (40, 41). Thus, *Wolbachia* and its

surface lipoproteins may also influence the development of adaptive immune responses to different *Onchocerca* life cycle stages in humans via the activation and recruitment of neutrophils, a proposal that deserves further investigation.

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AUTHOR'S CONTRIBUTION

FT and HLW performed the experiments; FT, HLW, SWE, JDT and MJT designed the study; JDT and KJJ provided essential reagents; FT and HLW analysed the data; FT wrote the paper; all Authors revised the critically the data and the manuscript and approved the submitted final version of the manuscript.

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