**Wolbachia Lipoprotein Stimulates Innate and Adaptive Immunity through Toll-like Receptors 2 and 6 to Induce Disease Manifestations of Filariasis**

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**Abstract**

Wolbachia endosymbiotic bacteria have been implicated in the inflammatory pathogenesis of filariasis. Inflammation induced by *Brugia malayi* female worm extract (BMFE) is dependent on Toll-like receptors 2 and 6 (TLR2/6) with only a partial requirement for TLR1. Removal of Wolbachia, lipids, or proteins eliminates all inflammatory activity. Wolbachia bacteria contain the lipoprotein biosynthesis genes *lts* and *lspA* but not *lnt*, suggesting Wolbachia proteins cannot be triacylated, accounting for recognition by TLR2/6. Lipoprotein databases revealed 3–11 potential lipoproteins that could be triacylated, accounting for recognition by TLR2/6. Peptidoglycan-associated lipoprotein (PAL) and *Wolbachia* lipopeptide (Diacyl WoLP) showed a near identical TLR2/6 and TLR2/1 usage compared with BMFE and bound directly to TLR2. Diacyl WoLP induced systemic tumor necrosis factor-α and neutrophil-mediated keratitis in mice. Diacyl WoLP activated monocytes induce up-regulation of gp38 on human lymphatic endothelial cells and induced dendritic cell maturation and activation. dendritics cells primed with BMFE generated a non-polarized Th1/Th2 CD4+ T cell profile, whereas priming with *Wolbachia* depleted extracts (following tetracycline treatment; BMFEtet) polarized to a Th2 profile that could be reversed by reconstitution with Diacyl WoLP; BMFE generated IgG1 and IgG2c antibody responses, whereas BMFEtet or inoculation of TLR2 or MyD88−/− mice produced defective IgG2c responses. Thus, in addition to innate inflammatory activation, *Wolbachia* lipoproteins drive interferon-γ-dependent CD4+ T cell polarization and antibody switching.

Human filariasis is a major neglected tropical disease. More than 150 million individuals are infected with the filarial worms responsible for lymphatic filariasis (LF) (*Wuchereria bancrofti* and *Brugia malayi*) and onchocerciasis (*Onchocerca volvulus*). Over 40 million suffer from disfiguring and incapacitating disease with an estimated 1.5 billion people at risk of infection, ranking filariasis as one of the major causes of global morbidity (1).

A feature of filarial pathogenesis is a host inflammatory response provoked by the death of larvae and adult stages within parasitized tissues (2). All causative agents of LF and *O. volvulus* harbor an intracellular symbiotic bacterium, *Wolbachia*, and are reliant on this endosymbiont for embryogenesis, growth, and survival (3). Previous studies have determined that the inflammatory potential of *B. malayi* and *O. volvulus* is dependent on the presence of *Wolbachia*. For example, *Wolbachia*-containing filarial extracts induce activation and tolerance in murine macrophages (4, 5), activate human monocytes (6), and activate human and murine neutrophils (7, 8). In addition, *O. volvulus* and *B. malayi* extracts containing *Wolbachia* stimulate neutrophil recruitment to the corneal stroma and development of corneal haze in a murine model of ocular onchocerciasis, in contrast with an aposymbiotic filaria (9). Moreover, isolated *Wolbachia* from filaria or from insect cells can replicate these effects (8, 10). The activation of neutrophils results in further neutrophil recruitment leading to the disruption of normal corneal clarity and development of stromal haze (11).

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**Abbreviations used are: LF, lymphatic filariasis; TLR2/6, Toll-like receptors 2 and 6; PAL, peptidoglycan-associated lipoprotein; psi, plasmids encoding small interfering RNA; TNF, tumor necrosis factor; BMFE, *B. malayi* female worm extract; BMFEtet, *Wolbachia*-depleted extract following tetracycline treatment; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; AF488, Alexa Fluor 488; HMVEC, human adult, dermal lymphatic microvascular endothelial cells; IL, interleukin; DC, dendritic cell; FITC, fluorescein isothiocyanate; IFN, interferon; wBmPAL, *B. malayi* *Wolbachia* PAL; Diacyl WoLP, Diacyl *Wolbachia* lipopeptide; BmDC, bone marrow-derived DC; WSP, *Wolbachia* surface protein; VEGF, vascular endothelial growth factor; MFI, median fluorescent intensity; APC, allopregocyanin; OVA, ovalbumin.

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Activation and subsequent desensitization of macrophages by Wolbachia molecules has been shown to be dependent on TLR2 and the adaptor molecule MyD88 (5, 10). Further studies have established that Wolbachia-induced inflammation is dependent on TLR2 and TLR6 recognition and signaling through the MyD88/Mal pathway and are independent of TRIF and TRAM (12). However, Wolbachia ligands for TLR2/TLR6 have not been characterized. To address this, we used the TLR receptor recognition profile to identify TLR2/6 ligands in the Wolbachia genome. In this study, we demonstrate that Wolbachia-derived diacyl-lipoproteins are candidate stimulatory molecules required for TLR2/6 ligation and production of pro-inflammatory cytokine and chemokine responses. Furthermore, we show that a synthetic Wolbachia lipopeptide (Diacyl WoLP) induces TLR2/6-dependent corneal inflammation, and TLR2-dependent TNFα responses in filarial disease models and up-regulates surface markers of human lymphatic endothelium. Diacyl WoLP also induced activation and maturation of dendritic cells and generated type 1 CD4+ T cell and antibody responses to filarial antigens.

EXPERIMENTAL PROCEDURES

Parasite Material—B. malayi adults were isolated from Mongolian jirds (TRS Labs, Atlanta, GA). For Wolbachia-depleted B. malayi, jirds were treated with 2.5 mg/ml tetracycline in drinking water for 6 weeks before parasite isolation. B. malayi female worms were processed for soluble extracts (B. malayi female extract (BMFE)) as described previously (5).

Trace endotoxin and mycoplasma contaminants in BMFE were measured by the European Endotoxin Testing Service (colorimetric Limulus Ameboocyte Lysate assay) and MycoAlert assay, respectively (Cambrex). Only extracts with <5 pg of LPS/100 μg of BMFE and negative for mycoplasma were used.

LPS, Lipoprotein, and Lipopeptide Stimuli—Ultra-pure LPS, PAM3CSK4, FSL-1 (Autogen BioClear), and rTNFα (R&D Systems) were used at the doses stated. Synthetic 20-mers of the N-terminal region of wBmPAL (CSKRGVNAINKMFVVKQMK), di-(Diacyl WoLP) or tri-palmitoylated (Triacyl WoLP) at the N-terminal cysteine residue were synthesized by Diacyl WoLP and terminated 3–24 h post-inoculation. Termi-

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in 1 ml, and supernatant was harvested after 96 h with mitogen (1 µg/ml ionomycin and 0.1 µg/ml phorbol myristate acetate) added for the final 24 h. Experimental procedures were reviewed and approved for Liverpool by the Home Office (London, UK), and for the corneal keratitis model by the Case Western Reserve University Institutional Animal Care and Use Committee.

Flow Cytometry—Monoclonal antibodies used for cell surface receptor staining were: rat anti-mouse MHCII-PE (clone M5/114.15.2), CD11c-APC (clone N418), CD40-FITC (clone HM40-3), CD80-PE (clone 16-10A1), and CD86-PE/FITC (clone P03.1/GL1) with appropriate isotype controls (eBioscience). Antibody staining was undertaken as previously described (5). Podoplanin (gp38) surface expression was measured using APC-conjugated mouse-anti-human podoplanin (clone P03.1/GL1) with appropriate isotype controls (eBioScience). Antibody staining was undertaken as previously described (5). Podoplanin (gp38) surface expression was measured using APC-conjugated mouse-anti-human podoplanin (clone P03.1/GL1) with appropriate isotype controls (eBioScience). Data acquisition was performed on a FACS Vantage flow cytometer (BD Biosciences) and analyzed with WinMDI v2.8.

Immunohistochemistry and Immunofluorescence—Human onchocercoma sections and B. malayi adult females were stained using the affinity-purified anti-rwBmPAL antibody. Reactivity was detected using an EnVision G 2 system/Alkaline phosphatase kit with a permanent red chromogenic substrate system (Dako). Sections were counterstained with hematoxylin. For immunofluorescence, cytosin preparations of C6/36 cells (an Aedes albopictus mosquito cell line) infected with Wolbachia pipiensis (5) and Wolbachia-free C6/36 were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then stained with anti-rwBmPAL antibody overnight at 4 °C. Antibody reactivity was detected with a goat anti-rabbit FITC-labeled (Invitrogen) secondary antibody. Cells were counterstained with Evans Blue (Sigma-Aldrich).

Murine Model of Corneal Inflammation—The mouse model of ocular onchoceriasis has been described previously (8). Briefly, mice were anesthetized prior to corneal scarification using a 26-gauge needle. 2 µl of Diacyl WoLP was injected into the corneal stroma using a 30-gauge Hamilton syringe. Neutrophil quantification in the cornea by flow cytometry and in vivo confocal microscopy analysis of corneal stromal haze was evaluated as described previously (11). Mice were treated in accordance with regulations of Association for Research in Vision and Ophthalmology.

Statistical Analysis—In instances where raw data or Log10 transformation of raw data (where indicated) approximated to a normal distribution pattern, differences between two groups were examined by Student’s t test, and differences between three or more groups were examined by one-way analysis of variance with Tukey post-hoc tests. In instances where data were skewed, non-parametric tests (Mann-Whitney tests) were used (non-parametric analysis indicated when used). All data were analyzed using GraphPad Prism v4.0.

RESULTS

Filarial Wolbachia Lipoprotein Activity Mediates Pro-inflammatory Cytokine Expression Principally via Ligation of TLR2/TLR6 and Not TLR2/TLR1 Heterodimers—Several distinct molecular patterns ligate TLR2 and activate the MyD88-dependent pathway (16). Furthermore, TLR2 forms heterodimers with either TLR1 or TLR6, which confers specificity for recognition of microbial lipoproteins, with TLR2/TLR6 recognizing diacylated lipoprotein, and TLR2/TLR1 conferring specificity for triacylated lipoprotein (17, 18). Using knock-out mice, we reported that, in addition to TLR2, macrophage activation by Wolbachia is also dependent on TLR6 (12). However, the involvement of TLR1 was not assessed.

To determine if there is a role for TLR1 in the recognition of Wolbachia, we used small interference RNA knockdown of constitutive TLR1 or TLR6 expression in HEK-TLR2 cells. Suppression of TLR1 or TLR6 expression was confirmed by >80% knockdown of IL-8 responsiveness to control triacylated PAM;CSK4 or diacylated FSL-1 lipopeptide, respectively (Fig. 1A). In TLR1-suppressed HEK-TLR2 cells, responsiveness to BMFE was reduced by 30% of control cells, whereas in TLR6-suppressed HEK-TLR2 cells, responsiveness to BMFE was...
reduced by 70% (Fig. 1A). As a second approach to examine the relative contribution of TLR1 and TLR6, we used peritoneal macrophages from TLR1/−/− and TLR6/−/− mice. BMFE induced a dose response from C57/BL6 wild-type (WT) cells, which was partially dependent on TLR1 only at the higher concentrations (200 μg/ml, p < 0.001, 400 μg/ml, p < 0.001) but completely dependent on TLR6 at all concentrations tested (Fig. 1B). Thus, although TLR6 is essential for Wolbachia recognition, TLR1 has only a minor contribution to the activation of MyD88-dependent pro-inflammatory responses induced by BMFE.

Bioinformatic Analysis and Characterization of Candidate Wolbachia TLR2/6-reactive Lipoproteins—Using the annotated B. malayi Wolbachia (wBm) genome (19), we identified the lipoprotein biosynthesis-encoding genes: Ltg, prolipoprotein diacylglyceryl transferase and LspA, lipoprotein signal peptidase. Importantly, in contrast to most other bacterial genomes, Lnt, apolipoprotein aminoacyl transferase gene, the enzyme responsible for acylation of the N terminus amide...
Synthetic diacyl-lipopeptide analogue of wBmPAL (Diacyl WoLP) replicates BMFE-TLR2/6-specific activation of inflammation. A, HEK-TLR2 cells were transfected with plasmids encoding small interfering (psi) RNA specific for TLR1 or TLR6 before being stimulated with Diacyl WoLP or Triacyl WoLP (doses stated are in micrograms/ml). Accumulations of IL-8 secreted by HEK-psiTLR1 or -psiTLR6 triplicate cultures 20 h post-stimulation are plotted as mean ± S.E. All data are representative of three independent experiments.

B. malayi Wolbachia-labile extracts were stimulated with Diacyl WoLP or Triacyl WoLP and control TLR1/6 ligands PAM₃CSK₄ and FSL-1 (doses stated are in nanograms/ml) in triplicate, and production of TNFα after 20 h is plotted as mean ± 15E. All data are representative of three independent experiments.

group was absent from the wBm genome (supplemental information). This suggests that Wolbachia proteins can be diacylated, but not triacylated, which is consistent with the predominant recognition of Wolbachia by TLR2/6.

Putative candidate Wolbachia lipoproteins (WoLP) from the wBm genome were identified using three bioinformatic sources: the Data base of Lipoproteins, Lipo, and LipoP programs, which select lipoproteins using the predicted features of an N terminus lipobox and lipoprotein signal peptides (supplemental information). Only two proteins, 1) peptidoglycan-associated protein, PAL-like: YP197985, and 2) VirB6 component-like, putative type IV secretion system protein: YP198182, were predicted by all programs and had predicted lipoprotein analogues in Drosophila melanogaster Wolbachia (wMel). A third protein, small protein A (smpA/omlA): YP198099 was predicted by both Data base of Lipoproteins and Lipo but not LipoP. Lipo uniquely predicted two proteins YP198553 and YP198182 without supporting orthologues in wMel. LipoP predicted a further six lipoproteins, four with supporting orthologues in the wMel genome. Further analysis of a fourth protein predicted exclusively by Data base of Lipoproteins, an uncharacterized protein involved in an early stage of isoprenoid biosynthesis YP197882, revealed that this predicted protein has an orthologue in wMel, both of which are most similar to enhancing lycopene biosynthesis protein 2. It lacks both a signal peptide (signalP) and a trans-membrane domain (TMHMM), and it was not predicted as a lipoprotein by either Lipo or LipoP programs. All of this evidence suggests it is not a lipoprotein and is a mis-annotation. Together these bioinformatic databases and predictive programs identified a total of eleven potential lipoproteins in the wBm genome.

Of the two lipoproteins identified by all bioinformatic programs, we selected the peptidoglycan-associated lipoprotein (PAL) for further characterization based on its predicted outer membrane location. In addition, triacylated Escherichia coli PAL is a potent TLR2 ligand capable of inducing septic shock and also displays synergistic inflammatory properties with LPS (20). B. malayi Wolbachia PAL (wBmPAL) consists of 159 amino acid residues with a typical lipobox and lipoprotein signal peptide (supplemental information). We cloned, expressed, purified, and raised rabbit polyclonal antisera against recombinant (r)wBmPAL. IgG was purified and used for Western blot analysis. An 18-kDa band was consistently reactive to anti-rwBmPAL in BMFE containing Wolbachia, but not in Wolbachia-depleted B. malayi-soluble extracts (BMFETet) (Fig. 2A), indicating the presence and Wolbachia specificity of wBmPAL in TLR2/6-reactive BMFE. Anti-rwBmPAL antibodies also identified Wolbachia in the mosquito A. albopictus Wolbachia infected C6/36 cell line and in O. volvulus and B. malayi adult worms (Fig. 2B).

Synthetic Diacylated WoLP Replicates BMFE, TLR1/2/6-dependent Effects on Innate Cell Activation—Synthetic analogues of the wBmPAL N-terminal 20 amino acids, either diacylated
(PAM$_2$-CSKRGVNAINKMNFFVVKQMK; Diacyl WoLP) or triacylated (PAM$_3$-CSKRGVNAINKMNFFVVKQMK; Triacyl WoLP) at the terminal cysteine residue, were generated in preference to the use of E. coli expression systems (which would result in triacylation) and to avoid the potential for E. coli-derived TLR ligand contaminants, including lipoprotein and LPS. These synthetic analogues were used to determine if wBmPAL could replicate the BMFE/TLR2-dependent effects on macrophage activation. Diacyl WoLP and Triacyl WoLP induced TNF$\gamma$/H9251 production by primary murine macrophages and IL-8 production in HEK-TLR2 but not HEK-TLR4 cells (data not shown), demonstrating that synthetic WoLP induces pro-inflammatory effects via TLR2 ligation, and that there is no LPS/TLR4 activity. Using TLR1 and TLR6 small interference RNA expression knockdown in HEK-TLR2 cells, we compared TLR heterodimer usage between native WoLPs in BMFE and synthetic Diacyl WoLP or Triacyl WoLP. Diacyl WoLP had an identical heterodimer requirement compared with BMFE, with 70% reduction in IL-8 production in TLR6-suppressed HEK-TLR2 cells versus 30% reduction in TLR1-suppressed HEK-TLR2 cells (Fig. 3A). Incubation of peritoneal macrophages from TLR1$^{-/-}$ and TLR6$^{-/-}$ mice with Diacyl WoLP was entirely dependent on TLR6 with only a marginal effect for TLR1 at certain concentrations (0.0125 $\mu$g/ml ($p < 0.002$) and 0.2 $\mu$g/ml ($p < 0.001$), Fig. 3B). Triacyl WoLP showed minor dependence on TLR6 at concentrations <0.05 $\mu$g/ml but was partially dependent on TLR1 at all concentrations, which was similar to PAM$_3$CSK$_4$ and FSL-1 control peptides. These data clearly demonstrate that Diacyl WoLP replicates the TLR2/6 pro-inflammatory responses generated by native WoLPs present in BMFE.

**TLR2 Expression Enhances Diacyl WoLP Binding to the Surface of HEK293 Cells**—To study the physical association between WoLP and the TLR2 receptor, we labeled Diacyl WoLP with Alexa Fluor® 488 and measured the degree of binding to HEK293 cells or HEK cells expressing the human TLR2 receptor. Specific binding of Diacyl WoLP:AF488 localized to the cell surface was observed in both cell lines (Fig. 4A). However, the frequency and quantity of Diacyl WoLP:AF488 bound to HEK-TLR2 was significantly enhanced when studied by flow cytometry (Fig. 4B). This typically equated to a 2-fold increase in the number of cells with Diacyl WoLP:AF488 molecules bound and an approximate 5-fold increase in the degree of binding to WoLP:AF488 molecules (Fig. 4C). Thus, although other receptor interactions may facilitate binding of WoLP, there is a clear role for the human TLR2 receptor in the physical recognition of WoLP.

**Diacyl WoLP Induces Inflammation via TLR2/6 in a Murine Model of River Blindness and Systemic TNF$\gamma$**—Previous studies using a murine model of ocular onchocerciasis in which *O. volvulus* extracts or isolated *Wolbachia* induced neutrophil and...
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A

B

C

D

E

\[ \text{gp38} \]
macrophage recruitment to the corneal stroma and development of corneal haze are TLR2-dependent (11, 12, 21). To determine the effect of WoLPs such as wBmPAL on corneal disease, WT mice were injected with increasing concentrations of Diacyl WoLP. We found that Diacyl WoLP induced neutrophil infiltration with increasing concentration (Fig. 5A, left panel). Similarly, corneal haze increased with the dose of Diacyl WoLP injected (Fig. 5B, left panel). These results indicated that 1 μg/2 μl Diacyl WoLP was an optimal concentration for intrastromal injections and was used in subsequent experiments.

To further examine the role of TLRs in WoLP-induced corneal disease, we injected 1 μg of Diacyl WoLP into the corneal stroma of TLR1−/−, TLR2−/−, and TLR6−/− mice and measured neutrophil infiltration and corneal haze as before. As shown in Fig. 5A, neutrophil infiltration was significantly reduced in TLR2−/− and TLR6−/− mice compared with WT mice, but not in TLR1−/− mice. Similarly, Diacyl WoLP-induced corneal haze was significantly lower in TLR2−/− and TLR6−/− mice, but not in TLR1−/− mice (Fig. 5B).

Taken together, these findings demonstrate that, as with filarial extracts, corneal disease induced by Diacyl WoLP requires TLR2 (11) and, in the cornea, induces neutrophil infiltration to the corneal stroma and the development of corneal haze. Furthermore, these findings demonstrate that Diacyl WoLP requires TLR6 as a co-receptor for neutrophil infiltration and corneal disease.

To determine whether exposure to WoLP could induce inflammation beyond the local site of inoculation we used intraperitoneal injection with Diacyl WoLP and observed the induction of systemic TNFα production in the blood 6 h later in WT but not TLR2−/− mice (Fig. 5C).

Wolbachia- and Diacyl WoLP-dependent Activation of Monocytes Induces gp38 Up-regulation on Human Lymphatic Endothelial Cells—Inflammation is known to promote lymphangiogenesis and changes to lymphatic endothelial cells. To determine if Wolbachia and Diacyl WoLP-mediated inflammation could affect lymphatic endothelium, THP-1 cells (a human monocytic cell line) were stimulated with 200 μg/ml BMFE or BMFET or 10 μg/ml Diacyl WoLP for 24 h. Monocyte supernatants were harvested and added to HMVECdly cells at a 1:3 dilution. TNFα (100 ng/ml) and IL-1β (10 ng/ml) were added to HMVECdly as a positive control. gp38 surface expression was determined by flow cytometry after 16 h. Supernatants from THP-1 cells activated by BMFE and Diacyl WoLP, but not BMFET, induced a significant up-regulation in the expression of gp38 on lymphatic endothelial cells (Fig. 5, D and E).

Wolbachia and Diacyl WoLP Induces Maturation and Activation of Dendritic Cells—It has recently been reported that BMFE mediates DC activation in a TLR2-dependent manner (21). We studied the effects of TLR2/6 WoLP agonists on DC function using 6-day granulocyte macrophage-colony stimulating factor differentiated, CD11c+ /MHCII+ bone marrow-derived DC (BmDC). Following 18-h exposure to either BMFE or Diacyl WoLP, DC exhibited significant increased MHCII, CD40, CD80, and CD86 surface molecule expression compared with medium plus granulocyte macrophage-colony stimulating factor-incubated cells (Fig. 6, A and B). In contrast to the maturation effects of BMFE, no up-regulation or only marginal up-regulation of MHCII molecules or co-stimulatory molecules was observed on BmDC exposed to equivalent doses of BMFET (Fig. 6, A and B). Analysis of BmDC cytokine secretions determined that BMFE and Diacyl WoLP, but not BMFET, stimulated significant release of IL-12/IL-23 p40 monomer/monodimers, IL-12p70 or IL-23p40/p19 heterodimers, and TNFα compared with medium-only-exposed BmDC (Fig. 6, C and D). The quantities of pro-inflammatory molecules secreted by BmDC in response to BMFE or Diacyl WoLP were significantly less compared with LPS-stimulated DCs at all doses tested. The activating signal provided by BMFE and Diacyl WoLP to DC was clearly dependent on TLR2 and TLR6 as adjudged by use of DCs derived from TLR2−/−, −4−/−, or −6−/− mice (Fig. 7, A and B). These data indicate that WoLP molecules present within BMFE are primarily responsible for driving enhanced DC maturation and induction of DC cytokine secretion.

Diacyl WoLP Exposure Increases CD80 and CD86 Surface Expression on Splenic MHCII+ CD11c+ Cells and IL-12/23p40 Levels in a TLR2-dependent Manner—To establish in vivo effects of Diacyl WoLP exposure on DC, we inoculated WT or TLR2−/− mice with Diacyl WoLP via the intraperitoneal route. WT CD11c+ and MHCII+ splenocytes showed ~2-fold increases in surface CD86 expression compared with sham inoculated mice 6 h after inoculation (Fig. 7C). Compared with TLR2−/− mice, WT mouse expression levels of CD86 and CD80 on CD11c+ and MHCII+ splenocytes were significantly higher 6 h following inoculation (Fig. 7D). Analysis of cytokine levels in splenic extracts from Diacyl WoLP-inoculated mice
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identified an increase in IL-12/23p40 levels in WT but not TLR2<sup>−/−</sup> animals (Fig. 7E).

Antigen-specific CD4<sup>+</sup> T-cell Proliferation and Polarization by BMFE-exposed DC Is Modulated by Co-exposure to WoLP TLR2<sub>/6</sub> Ligands—To determine whether the presence of TLR2<sub>/6</sub>-reactive WoLP molecules present within BMFE, such as wBmPAL, were capable of modulating the magnitude and type of CD4<sup>+</sup> T cell response elicited by DC exposed to BMFE, we used an in vitro antigen-restricted assay, utilizing DO11.10 TCR ovalbumin (OVA) transgenic mice. Following exposure to BMFE, BMFETet, or Diacyl WoLP, DCs were irradiated, primed with OVA peptide, and co-cultured with CD4<sup>+</sup> T cells derived from the spleens of DO11.10 mice. We studied the effect of co-exposure to native wBmPAL and native B. malayi molecules within BMFE on DC-mediated T-cell activation and skewing by exposing DCs to BMFEtet spiked with low doses of synthetic Diacyl WoLP. The levels of CD4<sup>+</sup> T-cell proliferation were enhanced following BMFE-, BMFETet-, and Diacyl WoLP-DC co-culture compared with medium-DC co-culture (Fig. 8A). However, the degree of proliferation following BMFE-DC co-culture was significantly greater compared with BMFETet-DC. Spiking BMFETet with Diacyl WoLP significantly increased the potential to induce OVA-specific proliferation (Fig. 8A). We then compared Th2<em>versus</em> Th1 bias in the proliferating anti-OVA CD4<sup>+</sup> T cells following co-culture of BMFE-, BMFETet-, or Diacyl WoLP-DC by contrasting the ratio of IL-4 to IFNγ secretions in culture supernatants to the ratio in medium-DC co-cultured CD4<sup>+</sup> T cells (Fig. 8B). Previous reports indicate that priming medium-DC with OVA at the concentration used in our study (10 nM) will result in mixed Th outgrowth (no Th polarization observed in BMFETet-DC and medium-DC). Moreover, DCs exposed simultaneously to BMFEtet and medium-DC/CD4<sup>+</sup> T cells produced preferential Th1 outgrowth with an IL-4:IFNγ ratio significantly lower than medium-DC co-cultures. BMFE priming failed to elicit significant T cell skewing, in terms of preferential IL-4 or IFNγ production. However, the effect of Wolbachia molecule depletion from BMFE prior to DC exposure was the development of a notable Th2 polarization, with significantly higher (2-fold) IL-4:IFNγ ratios compared with both BMFE-DC and medium-DC. Low dose Diacyl WoLP priming of DCs induced a significant increase in Th1 bias compared with non-primed DC. Moreover, DCs exposed simultaneously to BMFETet and low dose Diacyl WoLP prevented the Th2 polarization observed in BMFETet-DC/CD4<sup>+</sup> T cell co-cultures. Instead, Diacyl WoLP-spiked BMFETet-DC induced a mixed outgrowth of IL-4 and IFNγ producing T cells more in line with BMFE or medium-DC/CD4<sup>+</sup> T cell co-cultures. These results demonstrate that WoLP molecules within BMFE prevent an underlying potential of <i>B. malayi</i> molecules to polarize toward Th2 via effects on DCs.

Optimal Anti-BMFE IgG2c Antibody Production Requires Wolbachia, MyD88, and TLR2—To investigate the consequence of Wolbachia-TLR2 engagement on filarial-specific adaptive immune responses in vivo, we inoculated WT, MyD88<sup>−/−</sup>, TLR2<sup>−/−</sup>, and TLR4<sup>−/−</sup> mice with BMFE or WT mice with BMFETet at days 0 and 7 and tracked anti-BMFE serum IgG1 and IgG2c (markers of Th2 and Th1 responses, respectively, in C57BL/6 mice) over a time course of 25 days. Fig. 9A shows BMFE inoculations induced specific IgG1 seroconversion at 21 days in all groups (defined as significant elevation in anti-BMFE IgG1 compared with sham inoculated control groups). However, anti-BMFE IgG2c seroconversion was only observed in WT and TLR4<sup>−/−</sup> mice. Comparing between groups at day 25, BMFE-specific IgG1 levels did not significantly differ between WT and MyD88<sup>−/−</sup>, TLR2<sup>−/−</sup>, or TLR4<sup>−/−</sup> mice inoculated with BMFE. IgG2c levels were absent or significantly reduced in MyD88<sup>−/−</sup> and TLR2<sup>−/−</sup> mice inoculated with BMFE compared with WT controls, whereas TLR4<sup>−/−</sup>-inoculated mice showed comparable IgG2c production (Fig. 9B). IgM levels were also found to be comparable between groups at 25 days (data not shown). Wolbachia-depleted BMFETet-inoculated mice also showed a diminished anti-BMFE IgG2c response in the face of a comparable IgG1 response at day 25 (Fig. 9C), supporting a role for Wolbachia in the mediation of TLR2/MyD88-dependent BMFE IgG2c antibody production. These data suggest that IgG2c subclass production to filarial antigen is dependent on TLR2 reactive molecules such as wBmPAL. Moreover, in the absence of this Wolbachia pattern recognition pathway (or in the absence of native Wolbachia lipoproteins), BMFE exposure leads to an IgG1-polarized rather than IgG1/IgG2c-mixed antibody response.

**DISCUSSION**

Here we provide evidence that filarial Wolbachia lipoprotein induces inflammatory responses through activation of TLR2/6 receptors and to a minor extent TLR2/1 heterodimers. Because TLR2/6 preferentially ligates diacylated rather than triacylated protein (17), we deduced that Wolbachia lacks the ability to add further acyl groups to diacylated protein, due to an absence of Llt (apolipoprotein N-transacylase). We established that one Wolbachia lipoprotein, peptidoglycan-associated protein (wBmPAL), was present within BMFE and antibodies to rwBmPAL detected Wolbachia in infected insect cells and in adult <i>O. volvulus</i> and <i>B. malayi</i> worms. In Gram-negative bacteria, PAL is ubiquitous and highly conserved. <i>E. coli</i> PAL is anchored...
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in the outer membrane by the N-terminal lipid group and has an important role in the structural integrity of the membrane by binding to peptidoglycan meso-diaminopimelate residues and Tol membrane proteins (24, 25). It is interesting to note that Wolbachia is only able to synthesize a single amino acid, meso-diaminopimelate, which is predicted to be a component of an unmodified peptidoglycan in the degenerate wBm Wolbachia cell wall (19), suggesting that Wolbachia PAL may also play a role in membrane structural integrity.

E. coli PAL is a potent TLR2 ligand, which activates inflammation via MyD88 and induces inflammatory mediated cardiac dysfunction and fatality in sepsis (26, 27).

By using synthetic peptides of the N terminus of wBmPAL, which were either diacylated or triacylated at the N-terminal cysteine residue, we clearly demonstrated that the diacylated peptide had a near identical TLR2/6 and TLR2/1 receptor usage compared with native Wolbachia-containing BMFE. We observed some reduction (30%) in pro-inflammatory cytokine production following native or synthetic Diacyl WoLP stimulation when TLR1 expression was selectively knocked down. This suggests that TLR2/1 heterodimers are capable of a degree of Wolbachia diacyl lipoprotein recognition, but they are relatively less reactive with Diacyl WoLP than TLR2/6. TLR6-independent recognition of certain synthetic diacyl lipopeptides has been previously reported and appears to depend on peptide composition and length (28). Thus TLR1 may function as an accessory molecule in optimal responsiveness to native Wolbachia lipoprotein. Similarly, CD36, CD14, and LPS-binding protein have been reported to act as amplifying molecules in bacterial lipoprotein pattern recognition (29–31) indicating that multiple receptors (a receptosome) may coordinate TLR2-facilitated lipoprotein recognition. Fur-
FIGURE 8. Diacyl WoLP modulates antigen-specific CD4+ T-cell activation and differentiation. A, DO11.10 OVA transgenic CD4+ T-cell proliferation following 72-h co-culture of OVA peptide-loaded DC primed with BMFE, BMFEtet, BMFEtet + Diacyl WoLP, Diacyl WoLP, LPS, or untreated (medium only). Doses of stimuli stated are micrograms/ml. Bars are mean ± 1 S.E. tritiated thymidine incorporation (counts per minute). Significant differences to untreated DC are indicated: **, p < 0.001; ***, p < 0.01; and *, p < 0.05. B, ratios of IL-4:IFNγ from DO11.10 OVA transgenic CD4+ T cells following 72-h co-culture of OVA peptide-loaded DC primed with BMFE, BMFEtet, BMFEtet + Diacyl WoLP, Diacyl WoLP, LPS, or untreated (medium only). Doses of stimuli stated are in micrograms/ml. Bars are the mean ratio ± 1 S.E. of IL-4:IFNγ secreted in supernatant from triplicate co-cultures 24 h after polyclonal (phorbol myristate acetate/ionomycin) stimulation. Significant different ratios compared with untreated DC are indicated: **, p < 0.001; *, p < 0.05. All data are representative of two independent experiments.

other characterization of additional Wolbachia lipoproteins in BMFE is ongoing. In this regard, a previous study reported that a recombinant Dirofilaria immitis Wolbachia surface protein (WSP) preparation activated macrophages and DCs in a TLR2- and TLR4-dependent manner (32). We have been unable to reproduce these findings using recombinant B. malayi Wolbachia WSP protein or overlapping 20-mer synthetic peptides of WSP (data not shown). WSP is not predicted to be a lipoprotein based on predictions of three independent bioinformatic databases, and therefore not a likely candidate ligand of TLR2/1 or TLR2/6. Furthermore, we have established that no intrinsic TLR4 activity is present in BMFE and Diacyl WoLP; further studies are needed to validate reactivity of native WSP protein rather than the use of potentially contaminated recombinant preparations.

Fluorescently labeled Diacyl WoLP molecules bound to the surface of human-transfected TLR2-expressing HEK cells with greater frequency and more abundance than non-TLR2-expressing parental cells, demonstrating a degree of direct recognition of the wBmPAl. N terminus by TLR2. The binding observed in the parental line suggests that other surface receptors are involved in Diacyl WoLP recognition, such as TLR1, TLR6, CD36, or other facets of a diacyl lipoprotein receptosome.

Because TLR2 responses are essential for Wolbachia-induced innate immune responses in a murine model of onchocerciasis, we examined if Diacyl WoLP could induce clinical features of the disease. Injection of Diacyl WoLP into the corneal stroma induced neutrophil infiltration and corneal haze previously observed with microfilariae, isolated Wolbachia or soluble filarial extracts containing Wolbachia (8, 10, 11), suggesting Wolbachia lipopeptides mimic the innate inflammatory activation associated with systemic inflammation and onchocercal eye disease. The sequence of events in corneal disease likely begins with death and degeneration of microfilariae and exposure of Wolbachia lipoproteins to resident fibroblasts and bone marrow-derived macrophages and DCs through TLR2/6. Activation of MyD88/Mal-dependent signaling events induces pro-inflammatory cytokine and chemokine production, which mediate the recruitment and activation of neutrophils in the corneal stroma. The activation of neutrophils at this site results in disruption of normal corneal clarity and stromal haze (8). This sequence of events appears to be dominant in the cornea even in the presence of an adaptive immune response, as in immunized animals, parasite specific T-cell cytokine and antibody production is diminished in the absence of TLR2 (21).

Intraperitoneal injection of Diacyl WoLP induced TLR2-dependent elevated systemic TNFα responses in mice. Systemic inflammatory reactions are also a feature of adverse reactions following anti-filarial drug treatment, which are associated with the release of Wolbachia in the blood and tissues and severity of adverse reactions. PCR and immunoelectron microscopy analysis of plasma samples following the treatment of B. malayi with diethylcarbamazine show the persistent presence of Wolbachia in patients with severe systemic inflammation (33). Wolbachia DNA can also be detected in the sera from onchocerciasis patients who have received diethylcarbamazine or ivermectin or bancroftian filariasis patients receiving ivermectin and albendazole (34, 35). In both these studies, the severity of adverse reaction and levels of pro-inflammatory mediators or released neutrophil products correlate with the amount of Wolbachia DNA measured in sera. Recent field trials have determined that prior doxycycline treatment ameliorates adverse reactions and systemic pro-inflammatory cytokines in bancroftian or brugian filariasis (35, 36). However, in doxycycline-treated individuals, significant reductions in microfilaraemia, as well as ablation of Wolbachia from nematode tissues, were evident at the point of standard anti-filarial treatment. Thus, although there is substantial correlational evidence that Wolbachia release from filarial tissues taints with the incidence and magnitude of systemic inflammation and adverse reaction, further experimental and field studies are required to delineate the contribution of Wolbachia and nematode in the provocation of post-treatment reactions.

Vascular endothelial growth factors (VEGF) A and C and VEGF receptor 3 (VEGFR3) have been recently characterized as critical factors in the induction of lymphangiogenesis and are elevated in clinical cases of LF lymphoedema, hydrocoele, and chyluria (37–39). Doxycycline treatment has therapeutic benefits in reducing lymphoedema and supratotal lymphatic dilation in addition to its macrofilaricidal effects (38). Doxycycline-treated patients also exhibit significant decreases in serum levels of VEGFα and sVEGFR3, providing an association between reductions in pro-lymphangiogenic
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**FIGURE 9.** Anti-BMFE IgG2c production is dependent on MyD88 and TLR2 but not TLR4. A, time course of BMFE-specific IgG1 or IgG2c antibody production following inoculation with 50 μg of BMFE intraperitoneally at days 0 and 7 in MyD88−/−, TLR2−/−, TLR4−/−, or WT mice. Data plotted are mean antibody levels from groups of four mice. B, day 25 anti-IgG1 or -IgG2c levels. Bars represent mean levels of antibody. Significant differences compared with WT mice are indicated: **, p < 0.01. C, day 25 anti-IgG1 or -IgG2c levels from WT mice inoculated with 50 μg of BMFE or BMFEtet intraperitoneally at days 0 and 7. Horizontal bars represent mean levels of antibody. Significant differences are indicated: ***, p < 0.001. Data are representative of two independent experiments.

The consequence of DC exposure to native Wolbachia lipoproteins in BMFE on subsequent CD4+ T cell development in vitro was an enhancement of antigen-specific proliferation. This is most likely to be mediated via elevations in one or more of three activating signals provided to T cells by DC following exposure to TLR ligands: increased antigen bound within MHCII molecules, increased adhesion and co-stimulatory receptor/ligand interactions, and increased paracrine effects of cytokines secreted by DCs. IL-12 and IL-23, cytokines produced following exposure to BMFE and Diacyl WoLP, have positive yet divergent effects on facets of T cell development, with IL-12 supplying a positive signal for Th1 development and IL-23 supporting the expansion of IL-17-secreting Th17 cells (48). Given the emerging role for Th17 responses as mediators of immunopathogenesis (48), the identification that Wolbachia lipoprotein can drive a pro-Th17 DC response may indicate that Th17 responses have a role to play in filarial pathogenesis.

The effects of DC exposure to native Wolbachia lipoproteins within filarial extracts on antigen-specific CD4+ T cell subset differentiation were perhaps more surprising, given that synthetic Diacyl WoLP clearly activates DCs, even at low doses.
There was no significant effect of DC pre-exposure to BMFE on Th1 polarization compared with LPS-exposed DCs. However, removal of Wolbachia resulted in notable Th2 polarization. Our data illustrate an intrinsic potential for B. malayi molecules to prime DC for Th2 differentiation, which is modulated by the co-occurrence of native lipoproteins, a theory strengthened by the observation that low dose Diacyl WoLP primes DCs for Th1 differentiation and can effectively nullify Th2 priming by B. malayi molecules within BMFE. These findings are compatible with a "default" hypothesis of Th2 development where antigen processing and presentation following limited/reduced DC activation drives Th2 polarization, whereas increased CD40 expression and IL-12 production preferentially induce Th1 differentiation (49).

The effect of MyD88 or TLR2 deficiency following BMFE exposure in vivo was an almost complete ablation of BMFE-specific IgG2c antibody production in the face of comparable IgG1 production, suggesting that Wolbachia lipoproteins are crucial for IgG2c isotype class switching and so act as naturally occurring B cell adjuvants. Given the requirement for Th1 cell help for this switch, our observations are compatible with an expansion of Th1 CD4^+ clones via effects of Wolbachia lipoprotein-TLR2/6 ligation on APC in vivo. Indeed, it has been identified that IFNγ recall responses of splenocytes following B. malayi microfilariae inoculation are dependent on TLR2 (21), suggesting optimal anti-filarial Th1 expansion requires Wolbachia lipoprotein recognition in vivo. Because TLR2 ligation can activate Th1 cells in the absence of TCR signaling (50) and optimal production of antibody has been shown to be dependent on TLR ligation of B cells (51), we cannot rule out that Wolbachia lipoproteins influence IgG2c subclass production via direct effects on Th1 or B cells.

In conclusion, our data indicate that Wolbachia lipoproteins mediate innate immune activation and Th1-adaptive immune responses. The consequence of co-exposure to Wolbachia in the adaptive immune response to filarial infection is yet to be fully elucidated, although it is known that anti-Wolbachia antibody responses are evident in exposed individuals and increased in symptomatic patients (52, 53). Both endemic normal (putative immune) individuals and elephantiasis patients demonstrate more pronounced anti-filarial Th1 and Th2 responses compared with asymptomatic infected patients (52). This is largely attributed to active suppression of Th effector responses during asymptomatic infection (54, 55). TLR2-specific responses are also notably diminished in asymptomatic infection, indicating that TLR signaling in myeloid cells is regulated in these patients (56, 57). However, when adult worms and larvae die and degenerate, Wolbachia products, including lipoproteins, are released and activate TLR2 on APC. We hypothesize that loss of or defective regulation of TLR2-driven inflammation at this point will lead to heightened Th1-adaptive responses associated with disease pathology. Together our results suggest Wolbachia lipoproteins are the prime candidate ligands for the activation of TLR2/6-dependent innate and adaptive inflammation associated with filarial disease pathogenesis.
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Wolbachia Lipoprotein Stimulates Innate and Adaptive Immunity through Toll-like Receptors 2 and 6 to Induce Disease Manifestations of Filariasis
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