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

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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 *Ltg* and *LspA* but not *Lnt*, suggesting *Wolbachia* proteins cannot be triacylated, accounting for recognition by TLR2/6. Lipoprotein databases revealed 3–11 potential lipoproteins from *Wolbachia*. Peptidoglycan-associated lipoprotein (PAL) and Type IV secretion system-VirB6 were consistently predicted, and *B. malayi Wolbachia* PAL (wBmPAL) was selected for functional characterization. Diacylated 20-mer peptides of wBmPAL (Diacyl *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. Dendritic 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)[4] (*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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⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2, Table S1, and additional text.

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[4] The 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; HMVECdly, 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, allophycocyanin; OVA, ovalbumin.

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 Amebocyte 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, PAM_3CSK_4, 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 (CSKRGVNAINKMNFVVKQMK), di-(Diacyl WoLP) or tri-palmitoylated (Triacyl WoLP) at the N-terminal cysteine residue were synthesized by EMC Microcollections (Tubingen, Germany). Diacyl WoLP was labeled with Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Molecular Probes, UK) using the method outlined in Vasselon *et al.* (13), and excess dye was removed using dye removal columns following the manufacturer's instructions (Thermo Scientific, UK). OVA peptide 323-ISQAVHAAHAEINEAGR-339 was provided by Dr. E. Bell (Manchester University, UK) and used at 10 nM.

Cell Lines—HEK293-CD14, HEK293-CD14+TLR2, and HEK293-CD14+TLR4 transfectants were a gift from Prof. M. Yazdanbakhsh, Leiden University, Netherlands. HEK293-TLR2 transfectants and HEK293 parental cell lines were purchased from Invivogen, UK. For transfections, HEK293-TLR2 cells were seeded in 12-well plates at 1×10^5 cells/well. Cells were transfected with plasmids encoding TLR1- or TLR6-specific shRNA under the control of 7SK RNA polymerase III promoter (Autogen BioClear) using FuGENE 6 (Roche Applied Science). For binding assays, HEK293-TLR2 or HEK293 cells were grown to confluence, trypsinized to a single cell suspension, and seeded onto coverslips at 5×10^4 cells/well. Cells were incubated for 24 h, washed 3× in PBS containing 0.5% bovine serum albumin (binding buffer) and incubated with 5 μg/ml Diacyl WoLP:AF488 or equivalent volume PBS:AF488 for 30 min at room temperature before being washed 3× and mounted for confocal microscopy. For flow cytometric quantification, 1×10^5 aliquots of cells were washed 3× in binding buffer before being exposed to 5 μg/ml Diacyl WoLP:AF488 or negative control for 30 min at room temperature. Cells were washed and analyzed by flow cytometry. Human adult, dermal lymphatic microvascular endothelial cells (HMVECdly, Cambrex) were grown to confluence in 24-well plates. 1×10^6 THP-1 cells (human monocytic cell line) were stimulated with 200 μg/ml BMFE or BMFEtet or 10 μg/ml Diacyl WoLP for 24 h. Supernatants were harvested and added to HMVECdly at a 1:3 dilution. TNFα (100 ng/ml) and IL-1β (10 ng/ml, R&D Systems) were used as a positive control.

Animals and in Vivo Procedures—C57BL/6 MyD88$^{-/-}$, TLR1$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ mice were maintained at the University of Liverpool. WT C57BL/6 mice were purchased from Harlan UK. Corneal experiments on TLR1$^{-/-}$, TLR2$^{-/-}$, and TLR6$^{-/-}$ mice were carried out at Case Western Reserve University; C57BL/6 mice were from Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$, TLR1$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ mice were a gift from Prof. S. Akira (Osaka University, Japan). DO11.10 transgenic mice (a gift from Dr. E. Bell, Manchester University, UK) were maintained at Manchester University. Peritoneal macrophages from mice were elicited by 1-ml intraperitoneal injection of sterile 2% thioglycolate solution (BD Biosciences, Oxford, UK). DCs were generated from mouse bone marrow using a modified version of Inabi *et al.* (14), developed by Jiang *et al.* (15). After 6 days of culture and isolation, the resulting yield was typically 90–95% MHCII$^+$/CD11c$^+$. DCs were seeded into 48-well plates at 1×10^6/ml and stimulated for 18 h. For *in vivo* inflammatory responses, mice were inoculated intraperitoneally with 50 μg of Diacyl WoLP and terminated 3–24 h post-inoculation. Terminal bleeds were taken by cardiac puncture into heparin-coated syringes. Plasma was fractionated by centrifugation at 10,000 × *g*. Splenic supernatants were generated by digestion of splenic tissue within RPMI containing 1 mg of collagenase D (Roche Applied Science) followed by mashing through 70-μm gauze and harvesting of supernatant by centrifugation at 10,000 × *g*. For antibody experiments mice were inoculated with 50 μg of BMFE or BMFEtet at day 0 and day 7. Tail veins were bled into heparin-coated tubes at days 0, 7, 14, and 21 days post inoculation, and mice were terminally bled at day 25. Murine $CD4^+$ lymphocytes or $CD11c^+$ DCs were isolated from splenic cell suspensions using positive selection magnetic-associated cell sorting (Miltenyi Bio-tec). For DC-CD4+ co-culture, DCs were washed, γ-irradiated (1500 rads), and cultured with T cells at a ratio of 1:10 in 10% fetal calf serum RPMI. For proliferation assays, cells were seeded in triplicate into 96-well plates at 10^6/ml in 200 μl. Cells were incubated at 37 °C/5% CO_2 for 72 h before being pulsed for 8 h with [^{3}H]thymidine (MBP Biochemicals). Cells were harvested, and ^{3}H incorporation was ascertained by liquid scintillation. For cytokine measurements, cells were seeded into 48-well plates in triplicate at 2×10^6/ml

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 18H5, Acris GmbH, Germany). Data acquisition was performed on a FACS Vantage flow cytometer (BD Biosciences) and analyzed with WinMDI v2.8.

Immunoassay—Murine IL-4, IFNγ (R&D Systems), IL-12/IL-23p40, IL-12p70, IL-23p19/p40 (eBioscience), TNF-α (BioSource), and human IL-8 (BioSource) were determined using matched antibody pair enzyme-linked immunosorbent assay. Levels of BMFE-specific IgG1 and IgG2c were determined using Mouse IgG1 and IgG2c enzyme-linked immunosorbent assay quantification kits (Bethyl) using BMFE (3 μg/ml) as antigen.

Western Blotting—BMFE was diluted with 2× Laemmli buffer, heated to 96 °C for 10 min, and 5 μg was loaded onto a 15% SDS-PAGE gel and run under reducing conditions in a 1× Tris/glycine running buffer at 50 mA. Proteins were transferred to a 0.45-μm pore polyvinylidene difluoride membrane (Millipore), blocked with TBS SuperBlock (Pierce) with 0.05% Tween 20 for 2 h, and incubated overnight with anti-rwBmPAL antibody diluted with 0.1× SuperBlock at 4 °C. Antibody binding was detected by enzyme-linked chemiluminescence.

Anti-wBmPAL Antibody Generation—wBmPAL without the signal sequence was PCR amplified from genomic DNA isolated from *B. malayi* using primers WoLP-HindIII 5′-AAG CTT TGC TCA AAA AGA GGA-3′ and WoLP-BamHI 5′-GGA TCC CTA GCT ATA GTT GAA AAA-3′ with incorporated restriction sites. The product was cloned into a pCR 2.1 vector and TOP 10 cells using a TOPO TA Cloning Kit (Invitrogen). Following excision using HindIII and BamHI digestion, the product was ligated into the expression vector pJC40 and the ligation mix was used to transform TOP 10 cells. Following plasmid purification (QIAprep Spin Miniprep Kit, Qiagen) and verification by restriction digestion and sequencing, the expression vector was transformed into competent *Escherichia coli* DE3 cells (Invitrogen). Recombinant (r)wBmPAL was purified using ProBond His-tag resin (Invitrogen) and electro-elution (Bio-Rad). Expression and purification of rwBmPAL was monitored by SDS-PAGE and silver staining. Antibodies to rwBmPAL were produced in a NZW rabbit by subcutaneous injection of 100 μg of antigen in Freund's complete adjuvant (first immunization) or Freund's incomplete adjuvant (subsequent immunizations). Three inoculations were administered 3 weeks apart and serum-harvested 7 days following the final boost. Anti-rwBmPAL antibodies were affinity-purified using HiTrap Protein A and NHS columns (Amersham Biosciences).

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, cytospin preparations of C6/36 cells (an *Aedes albopictus* mosquito cell line) infected with *Wolbachia pipientis* (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 onchocerciasis 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 a >80% knockdown of IL-8 responsiveness to control triacylated PAM_3CSK4 or diacylated FSL-1 lipopeptide, respectively (Fig. 1*A*). 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

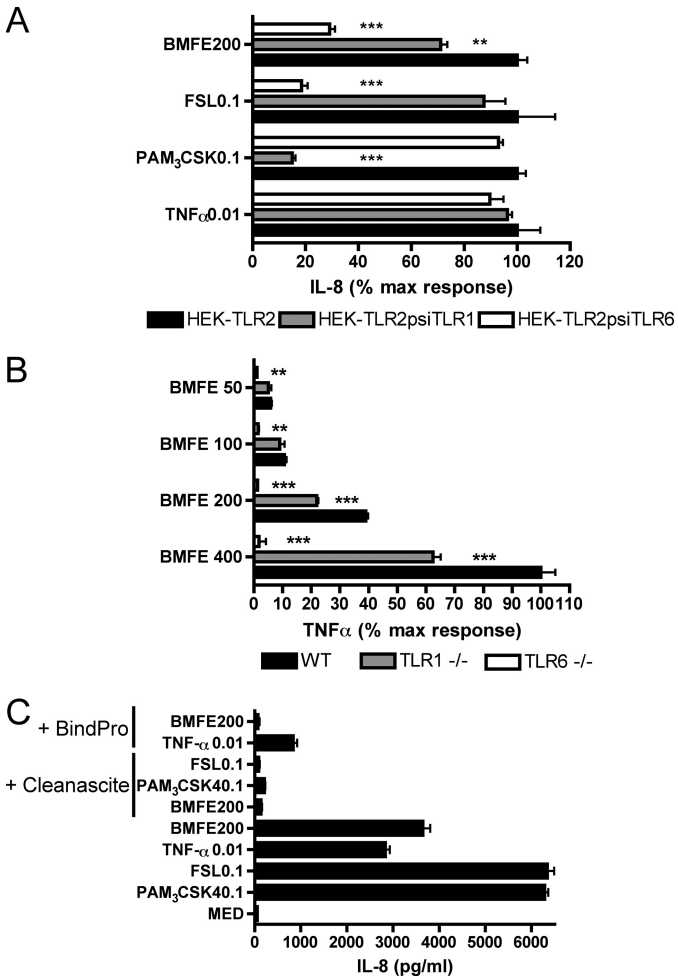


FIGURE 1. **The inflammatory stimuli of BMFE are lipoproteins that primarily signal via TLR2/6.** *A*, HEK-TLR2 cells were transfected with plasmids encoding small interfering (psi) RNA specific for TLR1 or TLR6 before being stimulated with BMFE or control stimuli (doses stated are in micrograms/ml). Accumulations of IL-8 secreted by HEK-psiTLR1 or -psiTLR6 triplicate cultures 20 h post-stimulation are plotted as mean (±1 S.E.) percentages of corresponding HEK-TLR2 responses (mean ± 1 S.E. max IL-8 concentrations as follows: TNFα = 7859 ± 98 pg/ml, PAM_3CSK = 9807 ± 175 pg/ml, FSL-1 = 2001 ± 345 pg/ml, and BMFE = 9495 ± 137 pg/ml). Significant differences compared with HEK-TLR2 responses are indicated: ***, $p < 0.001$; **, $p < 0.01$. *B*, peritoneal macrophages from WT, $TLR1^{-/-}$, $TLR6^{-/-}$ were stimulated with BMFE in triplicate (doses stated are micrograms/ml), and production of TNFα after 20 h is plotted as mean ± 1 S.E. percentages of WT response to 400 μg/ml BMFE (mean ± 1S.E. max TNFα concentration = 188.4 ± 10.36 pg/ml). Significant differences compared with WT are indicated ***, $p < 0.001$; **, $p < 0.01$. *C*, triplicate HEK-TLR2 cultures were stimulated with BMFE or control stimuli (doses stated are micrograms/ml) before or following Cleanascite™ or BindPro™ treatment. Data plotted are mean IL-8 ± 1S.E. All data are representative of three independent experiments.

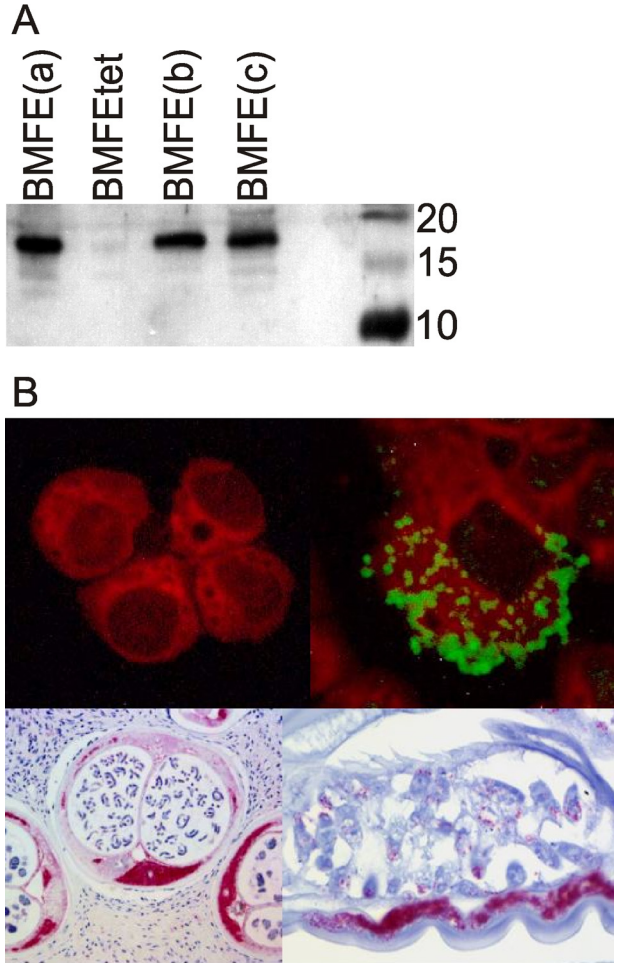


FIGURE 2. *A*, anti-rwBmPAL antibodies bind to a *Wolbachia* product present within BMFE. Anti-rwBmPAL polyclonal antibody immunoblot of three batches of BMFE (*a–c*) and a corresponding preparation of *Wolbachia*-depleted BMFEtet, separated by one-dimensional SDS-PAGE. Molecular weight markers are stated in kilodaltons. *B*, anti-rwBmPAL staining of *Wolbachia*-infected C6/36 cells (an *A. albopictus* mosquito cell line, *top right panel*) and *Wolbachia*-free C6/36 cells (*top left panel*). Antibody reactivity was detected with goat anti-rabbit FITC conjugate and counterstained with Evans Blue. Human onchocercoma sections (*lower left panel*) and *B. malayi* adult females (*lower right panel*) were stained using the affinity-purified anti-rwBmPAL antibody. Antibody reactivity was detected using alkaline phosphatase with a permanent red chromogenic substrate and counterstained with hematoxylin.

reduced by 70% (Fig. 1*A*). 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. 1*B*). 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.

To determine if TLR2/6 ligands of *Wolbachia* are lipoproteins, we treated the filarial extracts with Cleanascite™, which selectively removes lipids and lipoproteins, or with BindPro™, a polymeric protein removal suspension reagent (Biotech Support Group). Both treatments completely ablated (to background levels) HEK-TLR2 cell IL-8 reporter gene activity to BMFE (Fig. 1*C*) thereby showing that the TLR2/6 activity depends on both lipid and protein moieties.

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

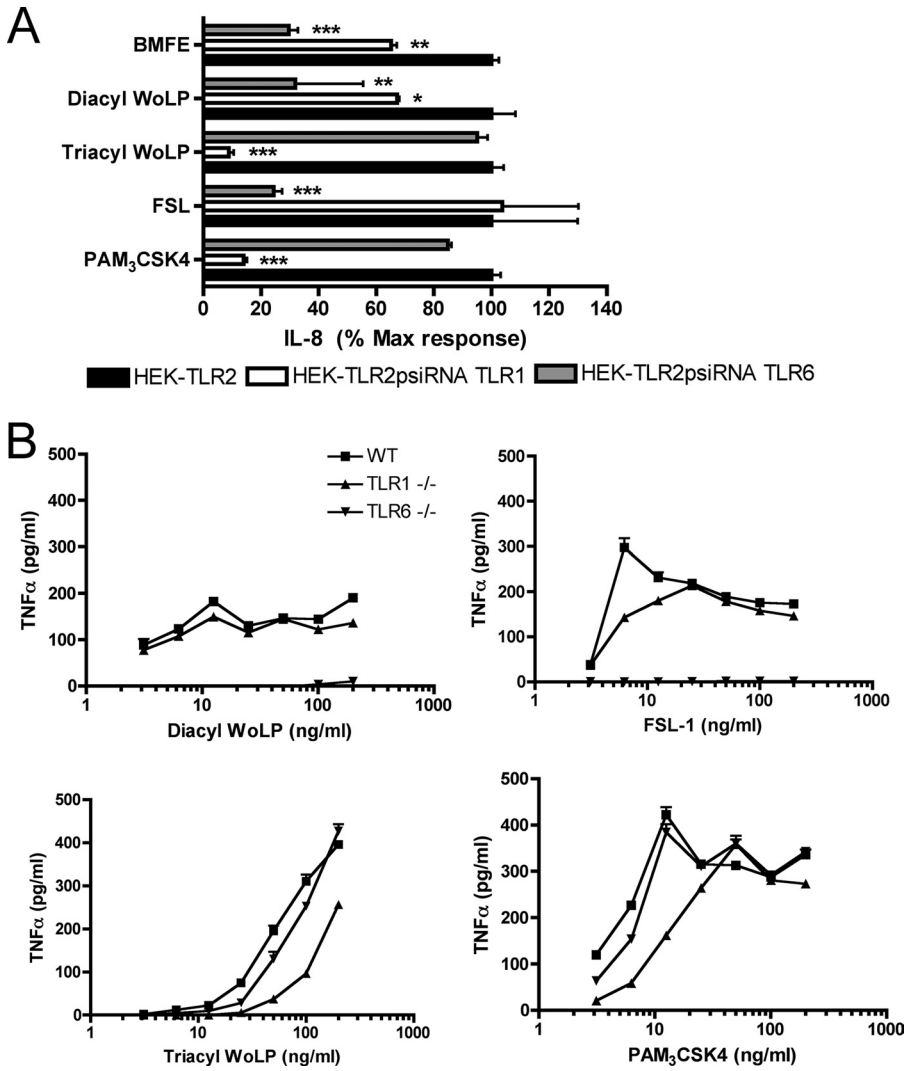


FIGURE 3. **Synthetic diacyl-lipopeptide analogue of wBmPAL (Diacyl WoLP) replicates BMFE-TLR2/6-specific activation of inflammation.** *A*, HEK-TLR2 cells were transfected with plasmids encoding small interfering (psi) RNA specific for TLR1 or TLR6 before being stimulated with Diacyl WoLP or Triacyl 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 ± 1 S.E. percentages of corresponding HEK-TLR2 responses (mean ± 1S.E. max IL-8 concentrations are as follows: PAM_3CSK = 9807 ± 175 pg/ml, FSL-1 = 2001 ± 345 pg/ml, Diacyl WoLP = 8195 ± 199 pg/ml, Triacyl WoLP 571 ± 27 pg/ml, and BMFE = 9495 ± 137 pg/ml). Significant differences compared with HEK-TLR2 responses are indicated: ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$. *B*, peritoneal macrophages from WT, $TLR1^{-/-}$, and $TLR6^{-/-}$ were stimulated with Diacyl WoLP or Triacyl WoLP and control TLR1/6 ligands PAM_3CSK_4 and FSL-1 (doses stated are in nanograms/ml) in triplicate, and production of TNFα after 20 h is plotted as mean ± 1S.E. 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: YP198624, were predicted by all three 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. 2*A*), 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. 2*B*).

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

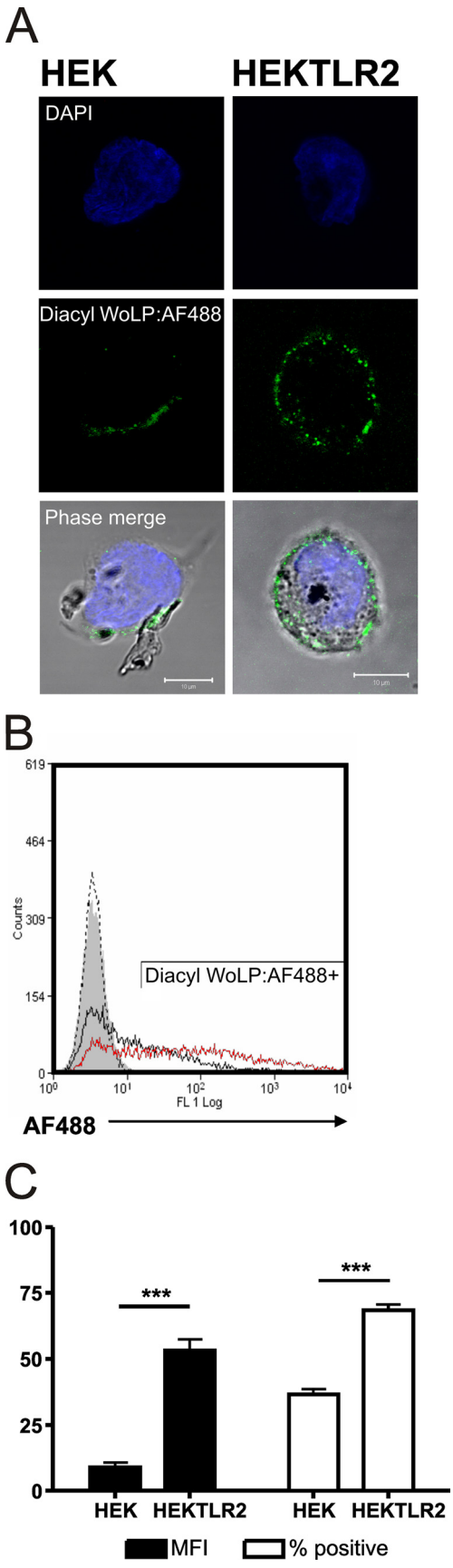
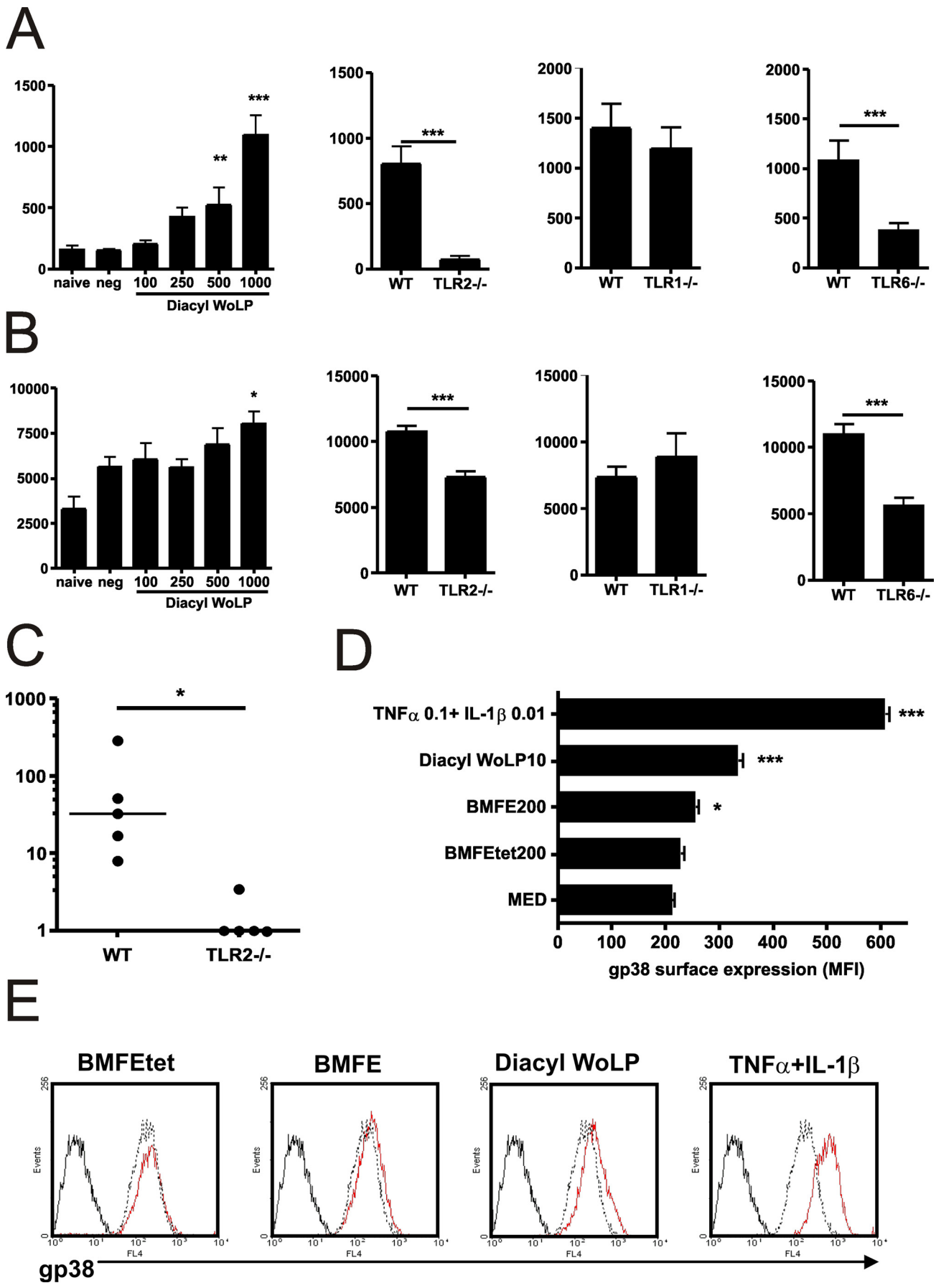


FIGURE 4. **TLR2 enhances binding of Diacyl WoLP.** *A*, confocal microscope images of a HEK293 cell (*upper panels*) or HEK-TLR2 stable transfectant (*lower panels*) cultured on coverslips with 5 μg/ml Diacyl WoLP:AF488. *Scale bar* = 10 μm. *B*, flow cytometric analysis of HEK293 (*black histogram*) or HEK-TLR2 (*red histogram*) transfectants labeled with 5 μg/ml Diacyl WoLP:AF488. PBS processed for AF488 dye labeling was used as a negative control to determine background levels of fluorescent labeling (*gray*, *filled histogram* = PBS:AF488-treated HEK293; *dashed black histogram* = PBS:AF488-treated HEK-TLR2). *C*, quantification of differences in frequency of cells labeled (% positive) and amount of labeling (median fluorescent intensity (MFI)) of Diacyl WoLP:AF488 in HEK *versus* HEK-TLR2 transfectants determined by flow cytometry. *Bars* are mean MFI or % positive ±1S.E. from triplicate labeling reactions each containing 100,000 cells. Significant differences are indicated ***, $p < 0.001$. All data are representative of two independent experiments.

(PAM_2-CSKRGVNAINKMNFVVKQMK; Diacyl WoLP) or triacylated (PAM_3-CSKRGVNAINKMNFVVKQMK; 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 TNFα 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. 3*A*). 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 μg/ml ($p < 0.002$) and 0.2 μg/ml ($p < 0.001$), Fig. 3*B*). Triacyl WoLP showed minor dependence on TLR6 at concentrations <0.05 μg/ml but was partially dependent on TLR1 at all concentrations, which was similar to PAM_3CSK_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. 4*A*). However, the frequency and quantity of Diacyl WoLP:AF488 bound to HEK-TLR2 was significantly enhanced when studied by flow cytometry (Fig. 4*B*). 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. 4*C*). 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 TNFα—Previous studies using a murine model of ocular onchocerciasis in which *O. volvulus* extracts or isolated *Wolbachia* induced neutrophil and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Diacyl WoLP10
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gp38 surface expression (MFI)
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BMFEtet
BMFE
Diacyl WoLP
TNFα+IL-1β
Events
FL4
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. 5*A*, *left panel*). Similarly, corneal haze increased with the dose of Diacyl WoLP injected (Fig. 5*B*, *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. 5*A*, 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. 5*B*).

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.

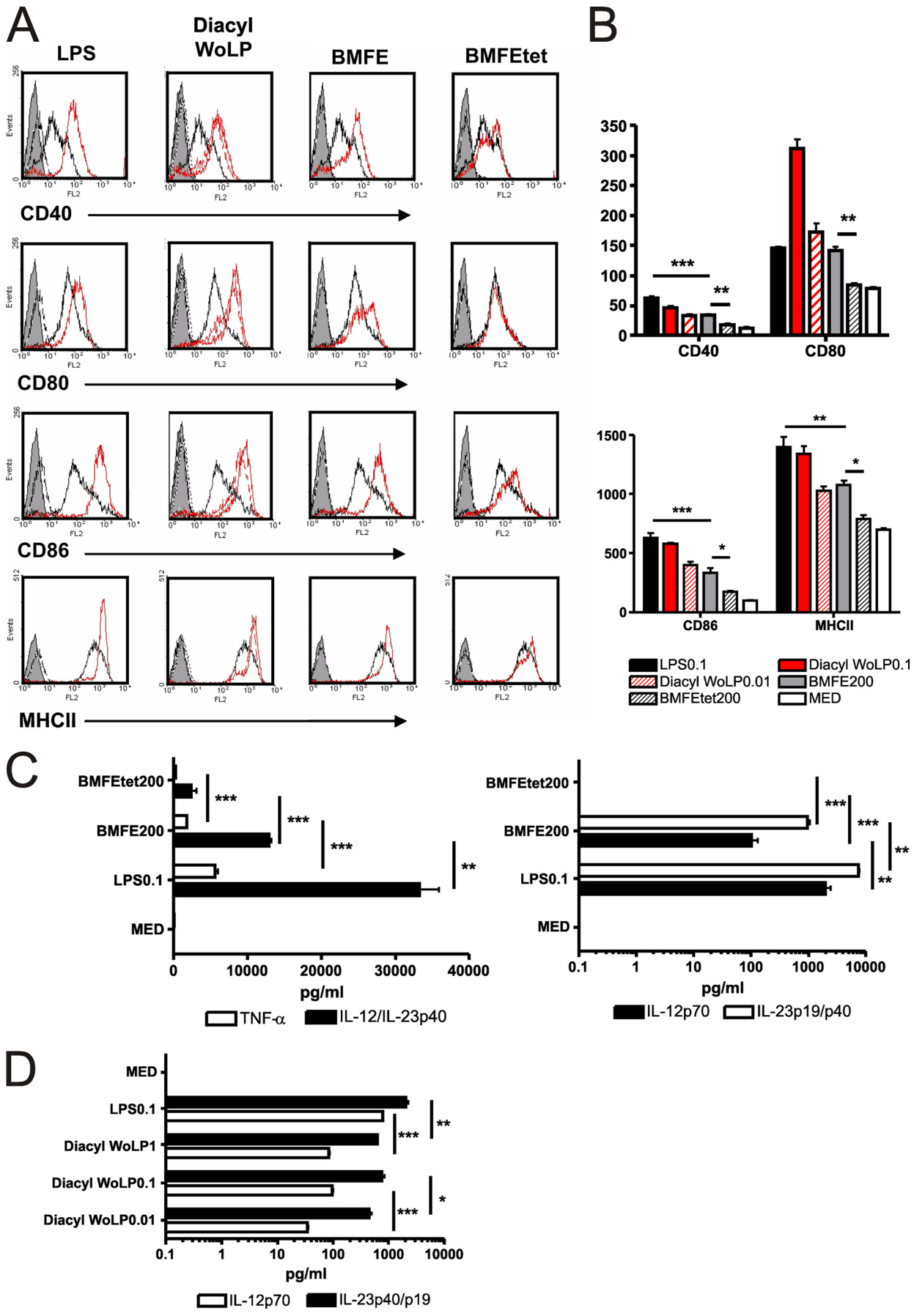
To determine whether exposure to WoLP could induce inflammation beyond the local site of inoculation we used intraperitoneal injection with Diacyl WoLP and observed the induction of systemic TNFα production in the blood 6 h later in WT but not TLR2$^{-/-}$ mice (Fig. 5*C*).

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 BMFEtet 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 BMFEtet, 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 maturating 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 BMFEtet (Fig. 6, *A* and *B*). Analysis of BmDC cytokine secretions determined that BMFE and Diacyl WoLP, but not BMFEtet, 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. 7*C*). 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. 7*D*). Analysis of cytokine levels in splenic extracts from Diacyl WoLP-inoculated mice

FIGURE 5. **Diacyl WoLP induces filarial disease manifestations.** *A*, Diacyl WoLP induces neutrophil infiltration following intra-corneal injection into WT mice in a dose-dependent manner (*left-hand panel*). *Bars* are mean ± 1S.E. neutrophil numbers from groups of three animals. Doses given are in nanograms/ml. Neutrophil accumulation is impaired in TLR2$^{-/-}$ or TLR6$^{-/-}$ but not TLR1$^{-/-}$ mice following a 1000 ng/ml intra-corneal injection (*right-hand panels*). *Bars* are mean ± 1S.E. neutrophil numbers from groups of three animals. *B*, corneal haze is induced by Diacyl WoLP in a dose-dependent fashion in WT mice (*right-hand panels*). *Bars* are mean ± 1S.E. corneal haze from groups of three animals. Doses given are in nanograms/ml. Corneal haze is significantly reduced in TLR2$^{-/-}$ and TLR6$^{-/-}$ mice but not TLR1$^{-/-}$ mice following a 1000 ng/ml dose. Significance differences between Diacyl WoLP inoculation and negative control or between WT and specific KO are indicated: ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$. *C*, Diacyl WoLP induces a systemic TNFα response in plasma 6 h following a 50-μg intraperitoneal injection in WT but not TLR2$^{-/-}$ mice. *Bars* indicate median TNFα levels from groups of five animals. Significant difference is indicated: **, $p < 0.01$ (Mann-Whitney non-parametric test). *D*, *Wolbachia*- and Diacyl WoLP-dependent gp38 up-regulation on lymphatic endothelial cells. THP-1 cells (human monocytic cell line) were stimulated with 200 μg/ml BMFE or BMFEtet or 10 μg/ml WoLP for 24 h. Monocyte supernatants were harvested and added to HMVECdly at a 1:3 dilution. Recombinant human TNFα (100 ng/ml) and IL-1β (10 ng/ml) were added to HMVECdly as a positive control. Surface expression of gp38 was determined by flow cytometry after 16 h. Differences in MFI of anti-gp38 labeling in HMVECdly exposed to TNFα and IL-1β or supernatants from treated THP-1 cells (treatments given are in micrograms/ml) were compared with unstimulated THP-1 supernatant exposed HMVECdly. *Bars* are mean ± 1S.E. MFI from triplicate labeling reactions. Significant differences between treatment and medium control are indicated: ***, $p < 0.001$; *, $p < 0.05$. All data are representative of two independent experiments. *E*, histograms represent fluorescent intensity of anti-gp38-stained HMVECdly exposed to unstimulated THP-1 supernatant (*dashed line*) or stimulated THP-1 supernatants/positive control (*red lines*). The isotype control is plotted as a *black solid line*.


A
LPS
Diacyl WoLP
BMFE
BMFEtet
CD40
CD80
CD86
MHCII
Events
FL2
B
CD40
CD80
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MHCII
LPS0.1
Diacyl WoLP0.1
Diacyl WoLP0.01
BMFE200
BMFEtet200
MED
C
BMFEtet200
BMFE200
LPS0.1
MED
pg/ml
TNF-α
IL-12/IL-23p40
IL-12p70
IL-23p19/p40
D
MED
LPS0.1
Diacyl WoLP1
Diacyl WoLP0.1
Diacyl WoLP0.01
pg/ml
IL-12p70
IL-23p40/p19

identified an increase in IL-12/23p40 levels in WT but not TLR2$^{-/-}$ animals (Fig. 7*E*).

Antigen-specific CD4$^+$ T-cell Proliferation and Polarization by BMFE-exposed DC Is Modulated by Co-exposure to WoLP TLR2/6 Ligands—To determine whether the presence of TLR2/6-reactive WoLP molecules present within BMFE, such as wBmPAL, were capable of modulating the magnitude and type of CD4$^+$ 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$^+$ T cells derived from the spleens of DO11.10 mice. We studied the effect of co-exposure to native wBmPAL and *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$^+$ T-cell proliferation were enhanced following BMFE-, BMFEtet-, and Diacyl WoLP-DC co-culture compared with medium-DC co-culture (Fig. 8*A*). 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. 8*A*). We then compared Th2 *versus* Th1 bias in the proliferating anti-OVA CD4$^+$ 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$^+$ T cells (Fig. 8*B*). 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 bias), whereas priming of LPS-DC will result in a Th1 bias in this system (22, 23). In agreement with these studies, we observed that LPS-DC co-cultured CD4$^+$ DO11.10 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$^+$ 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$^+$ T cell co-cultures. These results demonstrate that WoLP molecules within BMFE prevent an underlying potential of *B. malayi* 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$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ 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. 9*A* shows BMFE inoculations induced specific IgG1 sero-conversion at 21 days in all groups (defined as significant elevation in anti-BMFE IgG1 compared with sham inoculated control groups). However, anti-BMFE IgG2c sero-conversion was only observed in WT and TLR4$^{-/-}$ mice. Comparing between groups at day 25, BMFE-specific IgG1 levels did not significantly differ between WT and MyD88$^{-/-}$, TLR2$^{-/-}$, or TLR4$^{-/-}$ mice inoculated with BMFE. IgG2c levels were absent or significantly reduced in MyD88$^{-/-}$ and TLR2$^{-/-}$ mice inoculated with BMFE compared with WT controls, whereas TLR4$^{-/-}$-inoculated mice showed comparable IgG2c production (Fig. 9*B*). 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. 9*C*), 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 *Lnt* (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 *O. volvulus* and *B. malayi* worms. In Gram-negative bacteria, PAL is ubiquitous and highly conserved. *E. coli* PAL is anchored

FIGURE 6. **Diacyl WoLP provides necessary signals for DC maturation and activation.** *A*, increases in DC CD40, CD80, CD86, and MHCII following 20-h exposure to 0.1 μg/ml LPS, 0.1 μg/ml Diacyl WoLP, 200 μg/ml BMFE, or 200 μg/ml BMFEtet (all *red line histograms*), 0.01 μg/ml Diacyl WoLP (*red dashed-line histograms*), compared with untreated DC (*black histograms*). *Gray shaded histograms* = untreated DC isotype control, *dashed-line histograms* = exposed DC isotype control. *B*, CD40, CD80, CD86, and MHCII surface expression levels in unexposed or DC exposed to LPS, Diacyl WoLP, BMFE, or BMFEtet. *Bars* are mean ± 1S.E. median fluorescent intensities of triplicate labeling reactions. Doses stated are in micrograms/ml. *C*, production of DC TNFα, IL-12/IL-23p40, IL-12p70, and IL-23p40/p19 following 20-h exposure to LPS, BMFE, or BMFEtet. Doses stated are in picograms/ml. *Bars* are mean ± 1S.E. cytokine production from triplicate cultures. *D*, production of DC IL-12p70 and IL-23p40/p19 following 20-h exposure to various doses (stated in micrograms/ml) of Diacyl WoLP or LPS. *Bars* are mean ± 1S.E. cytokine production from triplicate cultures. Significant differences are indicated: ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$. All data are representative of three independent experiments.

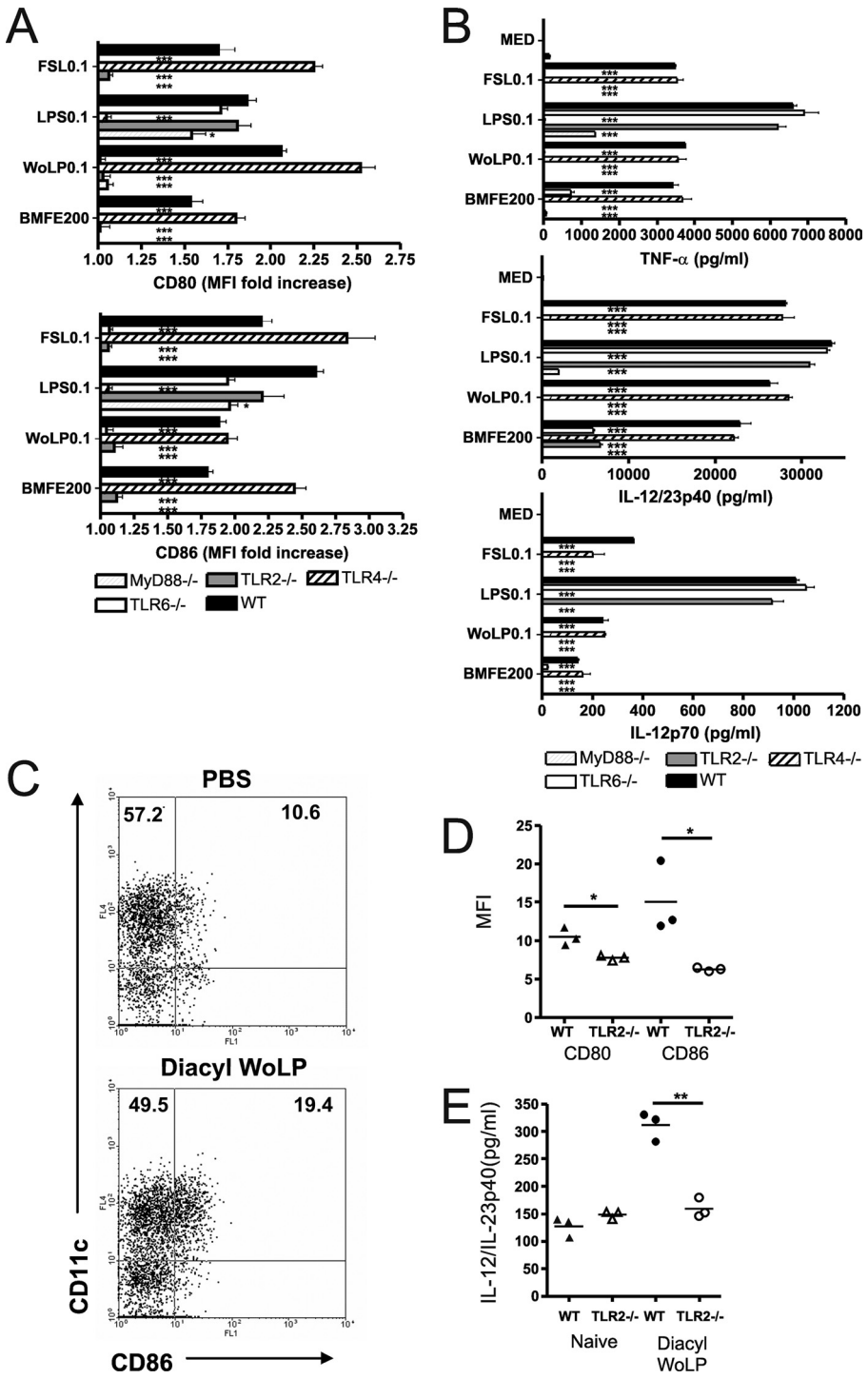

FIGURE 7. **DC maturation and activation by *Wolbachia* and Diacyl WoLP requires MyD88, TLR2, and TLR6 but not TLR4.** *A*, increase in CD80 or CD86 surface molecules following 20-h exposure to LPS, FSL-1, Diacyl WoLP, or BMFE in DC derived from WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$,or TLR6$^{-/-}$ mice. Doses stated are in micrograms/ml. *Bars* represent mean fold increase in MFI ± S.E. compared with unstimulated cells of triplicate labeling reactions. *B*, stimulation of TNFα, IL-12/IL-23p40, and IL-12p70 by LPS, FSL-1, Diacyl WoLP, or BMFE (doses stated are in micrograms/ml) from DC derived from WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, or TLR6$^{-/-}$ mice. *Bars* are mean ± 1S.E. cytokine production from triplicate cultures. *C*, Diacyl WoLP mediates an expansion of mature CD11c^{+} DC *in vivo*. Increases in CD86 surface expression on CD11c^{+} splenocytes 6 h following intraperitoneal inoculation with 50 μg of Diacyl WoLP were compared with sham inoculated WT mice. *Numbers* are percentages of splenocytes in the *upper left* and *right quadrants*. *D*, significant differences in CD80 and CD86 MFI on CD11c^{+} splenocytes derived from WT mice were compared with TLR2$^{-/-}$-deficient mice 6 h following inoculation with 50 μg of Diacyl WoLP intraperitoneally. *Bars* are mean MFI from groups of three mice. *E*, significant differences in levels of IL-12/IL-23 p40 measured in spleen extracts from WT mice compared with TLR2$^{-/-}$-deficient mice 6 h following inoculation with 50 μg of Diacyl WoLP intraperitoneally. *Bars* are mean cytokine levels from groups of three mice. Significant reductions compared with WT are indicated: ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$. All data are representative of two independent experiments.

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 be dependent 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-

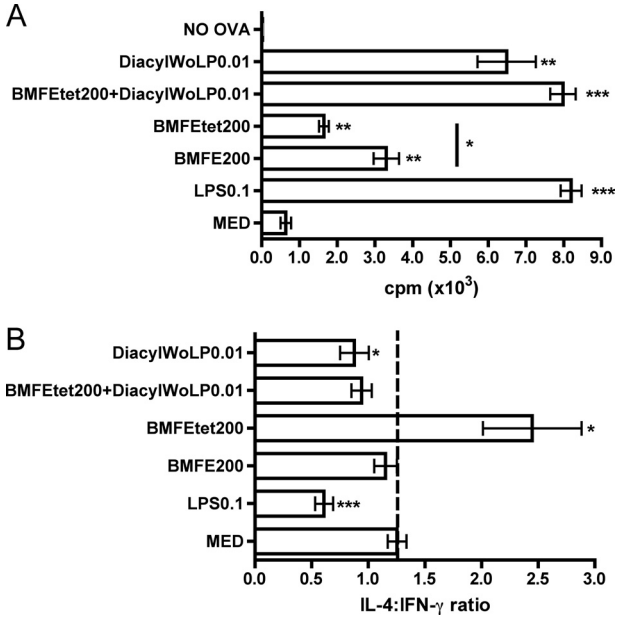

FIGURE 8. **Diacyl WoLP modulates antigen-specific CD4$^+$ T-cell activation and differentiation.** *A*, DO11.10 OVA transgenic CD4$^+$ T-cell proliferation following 72-h co-culture of OVA peptide-loaded DC primed with BMFE, BMFEtet, BMFEtet+Diacyl WoLP, Diacyl WoLP, LPS, or untreated (medium only). Doses of stimuli stated are micrograms/ml. *Bars* are mean ± 1S.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 ±1S.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.

ther 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 correlative evidence that *Wolbachia* release from filarial tissues tallies 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 supratesticular lymphatic dilation in addition to its macrofilaricidal effects (38). Doxycycline-treated patients also exhibit significant decreases in serum levels of VEGFc and sVEGFR3, providing an association between reductions in pro-lymphangiogenic

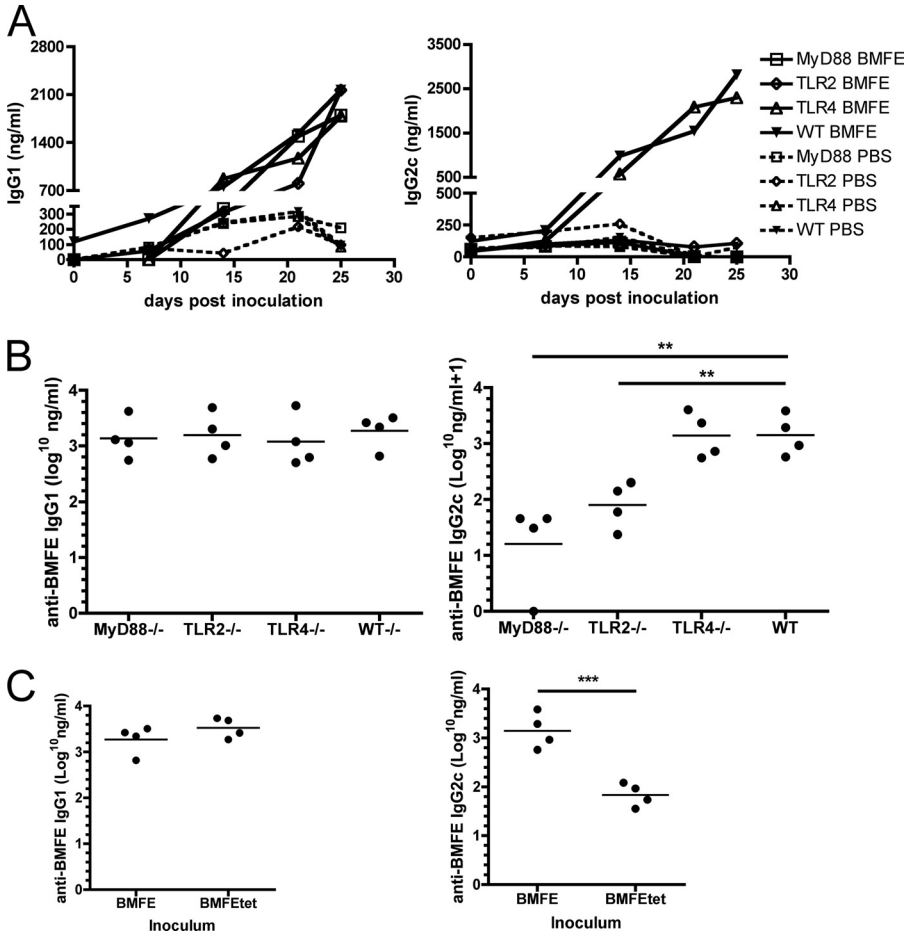

FIGURE 9. **Anti-BMFE IgG2c production is dependent on MyD88 and TLR2 but not TLR4.** *A*, time course of BMFE-specific IgG1 or IgG2c antibody production following inoculation with 50 μg of BMFE intraperitoneally at days 0 and 7 in MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, or WT mice. Data plotted are 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.

factors and amelioration in LF disease pathology (38). We determined that *Wolbachia* and Diacyl WoLP activated human monocyte supernatants, but not those derived from tetracycline treated parasites, induced the up-regulation of gp38, a lymphatic endothelial cell marker. Up-regulation of gp38 is induced in inflammatory conditions and by hematopoietic growth factors IL-3 and IL-7, and its overexpression promotes cell adhesion, migration, and tube formation (40–43). Thus *Wolbachia* lipoprotein-induced inflammation may contribute to lymphatic endothelial activation and lymphangiogenesis through up-regulation of lymphatic endothelial cell gp38 expression in addition to the inflammatory mediated production of VEGFs associated with LF chronic disease pathogenesis.

TLR2 signaling pathways are known to synergize with distinct TLR4 pathways in inflammatory gene expression (44, 45). Because Limulus amoebocyte lysate-negative BMFE preparations do not signal via TLR4 (supplemental information), we speculate that the observed TLR4 dependence previously reported (4, 9) may be due to a synergistic activation of innate inflammation between *Wolbachia* TLR2/6 lipoproteins, such as wBmPAL and trace levels of environmental endotoxin. In support of this, we observed that addition of BMFE to sub-nanogram levels of LPS, but not PAM_3CSK_4, produced a 3- to 4-fold synergy in macrophage activation (supplemental information).

Because TLR ligands can promote antigen presentation via activation of TLR-expressing APC (46), we hypothesized that native *Wolbachia* lipoproteins would regulate adaptive immune responses via TLR ligation of DCs. We have demonstrated that soluble *Wolbachia* products mediate enhanced maturation and activation of DCs *in vitro* in a TLR2/6- and MyD88-dependent manner, which is consistent with our previous studies (21). In the current study, we found that Diacyl WoLP emulates these effects on DCs *in vitro* and also demonstrates the potential to utilize TLR2 receptors *in vivo* to provoke an increase in levels of CD80/CD86 co-expressing CD11c$^+$ cells in the spleen following inoculation. Our data are consistent with previous observations that TLR2-mediated DC maturation and activation are completely dependent on the adaptor molecule MyD88. This is in contrast to TLR4 ligation, which can result in DC maturation, but not cytokine release, independently of MyD88 by utilizing the TRIF-dependent pathway (47).

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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