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

Received for publication, March 6, 2009. Published, JBC Papers in Press, May 19, 2009, DOI 10.1074/jbc.M901528200

Joseph D. Turner 1,2, R. Stuart Langley 31, Kelly L. Johnston 3, Katrin Gentili 3, Louise Ford 3, Bo Wu 4, Maia Graham 5, Faye Sharpley 6, Barton Slatko 7, Eric Pearlman 8, and Mark J. Taylor 9,10

From the 5Filaria Research Laboratory, Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom, the 6Department of Ophthalmology, Case Western Reserve University, Cleveland, Ohio 44106, and 4New England Biolabs, Ipswich, Massachusetts 01938

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 Ids, or proteins eliminates all inflammatory activity. From Wolbachia. Peptidoglycan-associated lipoprotein (PAL) and Type IV secretion system-VirB6 were consistently predicted, lipoprotein databases revealed 3–11 potential lipoproteins. 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, representing 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).

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, L. 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.

* This work was supported by the Wellcome Trust for Senior Fellowship support (to M. T., J. T., S. L., and K. J.). The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2, Table S1, and additional text.

1 Both authors contributed equally to this work.

2 Current address: Schistosomiasis Research Group, Centre for Immunology and Infection, Dept. of Biology, University of York, York Y010 5YW, United Kingdom.

3 To whom correspondence should be addressed: Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom. Tel.: 44-(0)151-705-3112; Fax: 44-(0)151-705-3371; E-mail: mark.taylor@liverpool.ac.uk.

© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 33, pp. 22364 – 22378, August 14, 2009
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, 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 (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 323-339 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-CD14+TLR2 cells were seeded in 12-well plates and 1 × 105 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 × 104 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 × 106 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 × 106 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−/−, TRIF−/−, 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 × 106/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 105/ml in 200 μl. Cells were incubated at 37°C/5% CO2 for 72 h before being pulsed for 8 h with [3H]thymidine (MBP Biochemicals). Cells were harvested, and [3H] incorporation was ascertained by liquid scintillation. For cytokine measurements, cells were seeded into 48-well plates in triplicate at 2 × 105/ml
Wolbachia Lipoprotein Induces Disease via TLR2/6

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.

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 G2 system/Alkaline phosphatase kit with a permanent red chromogenic substrate system (Dako). Sections were counterstained with hematoxylin. For immunofluorescence, cytopsin preparations of C6/36 cells (an Ae. 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 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.

Anti-rwBmPAL Antibody Generation—rwBmPAL 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).

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, 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

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.
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

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 (± S.E.) percentages of corresponding HEK-TLR2 responses (mean ± S.E. max IL-8 concentrations as follows: TNFα = 7859 ± 98 pg/ml, PAM3CSK4 = 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 ± S.E. percentages of WT response to 400 µg/ml BMFE (mean ± S.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 ± S.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.

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. 1C) thereby showing that the TLR2/6 activity depends on both lipid and protein moieties.
Wolbachia Lipoprotein Induces Disease via TLR2/6

A

|            | BMFE | Diacyl WoLP | Triacyl WoLP | PAM\(_3\)CSK\(_4\) |
|------------|------|-------------|--------------|-------------------|
| IL-8 (%) Max response |        |             |              |                   |
| HEK-TLR2   |      |             |              |                   |
| HEK-TLR2psiRNA |  **   |             |              |                   |
| TLR1       |      |             |              |                   |
| HEK-TLR2psiRNA |      |             |              |                   |
| TLR6       |      |             |              |                   |

B

- **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. max IL-8 concentrations are as follows: PAM\(_3\)CSK\(_4\) 175 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**. 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) in triplicate, and production of TNF\(\alpha\) after 20 h is plotted as mean ± S.E. All data are representative of three independent experiments.

**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 ± S.E. percentages of corresponding HEK-TLR2 responses (mean ± S.E. is as follows: PAM\(_3\)CSK\(_4\) 175 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 *, \(p < 0.05\).

The 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 **Escherichia coli** 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\textsubscript{2}\textendash CSKRGVAINKNFVVKQMK; Diacyl WoLP) or triacylated (PAM\textsubscript{2}\textendash CSKRGVAINKNFVVKQMK; Triacyl WoLP) at the terminal cysteine residue, were generated in preference to the use of \textit{E. coli} expression systems (which would result in triacylation) and to avoid the potential for \textit{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\textsubscript{a}/H\textsubscript{92}51 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\textsuperscript{+/+} and TLR6\textsuperscript{+/+} 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\textsubscript{3}CSK\textsubscript{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\textsubscript{a}**—Previous studies using a murine model of ocular onchocerciasis in which \textit{O. volvulus} extracts or isolated \textit{Wolbachia} induced neutrophil and
Wolbachia Lipoprotein Induces Disease via TLR2/6

A

B

C

D

E

TNFα 0.1 + IL-1β 0.01
Diacyl WoLP10
BMFE200
BMFEtet200
MED

gp38 surface expression (MFI)

WT

TLR2/--

TNFα+IL-1β

Diacyl WoLP

BMFEtet

BMFE

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

To further examine the role of TLRs in WolLP-induced corneal disease, we injected 1 μg of Diacyl WolLP 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 WolLP-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 WolLP 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 WolLP requires TLR6 as a co-receptor for neutrophil infiltration and corneal disease.

To determine whether exposure to WolLP could induce inflammation beyond the local site of inoculation, we used intraperitoneal injection with Diacyl WolLP 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 WolLP-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 WolLP-mediated inflammation could affect lymphatic endothelium, THP-1 cells (a human monocytic cell line) were stimulated with 200 μg/ml BMFE or BMFEmet or 10 μg/ml Diacyl WolLP for 24 h. Monocyte supernatants were harvested and added to HMVECdl cells at a 1:3 dilution. TNFα (100 ng/ml) and IL-1β (10 ng/ml) were added to HMVECdl 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 WolLP, but not BMFEmet, induced a significant up-regulation in the expression of gp38 on lymphatic endothelial cells (Fig. 5, D and E).

Wolbachia and Diacyl WolLP 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 WolLP 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 WolLP, 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 maturing 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 BMFEmet (Fig. 6, A and B). Analysis of BmDC cytokine secretions determined that BMFE and Diacyl WolLP, but not BMFEmet, stimulated significant release of IL-12/IL-23 p40 monomer/monomers, 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 WolLP were significantly less compared with LPS-stimulated DCs at all doses tested. The activating signal provided by BMFE and Diacyl WolLP 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 WolLP molecules present within BMFE are primarily responsible for driving enhanced DC maturation and induction of DC cytokine secretion.

Diacyl WolLP 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 WolLP exposure on DC, we inoculated WT or TLR2−/− mice with Diacyl WolLP 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 WolLP-inoculated mice...
identified an increase in IL-12/23p40 levels in WT but not TLR2−/− animals (Fig. 7E).

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. 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 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. 8B). Previous reports indicate that priming medium-DC with OVA at the concentration used in our study (10 μg/ml) 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. 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−/− 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. 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 Lnt (apolipoprotein N-transacylase). We established that one Wolbachia lipoprotein, peptidoglycan-associated protein (wBmPAL), was present within BMFE and antibodies to wBmPAL 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...
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 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 ± S.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 150 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 compared with TLR2−/− 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−/− 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; *, p < 0.05. All data are representative of two independent experiments.
Wolbachia Lipoprotein Induces Disease via TLR2/6

Intraperitoneal injection of Diacyl WolLP 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 correlatve 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

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 WolLP; further studies are needed to validate reactivity of native WSP protein rather than the use of potentially contaminated recombinant preparations.

 Fluorescently labeled Diacyl WolLP 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 WolLP 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 WolLP could induce clinical features of the disease. Injection of Diacyl WolLP 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).
Wolbachia Lipoprotein Induces Disease via TLR2/6

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. Bars represent mean levels of antibody. Significant differences compared with WT mice are indicated: **, p < 0.01. B, 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 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.

Acknowledgments—We greatly appreciate the expert technical assistance of Eugenia Diaconu for the cornell experiments and Dr. Adrian Mountford, University of York, for use of laboratory facilities for the lipoprotein binding studies.

REFERENCES

1. Molyneux, D. H., Bradley, M., Hoerauf, A., Kyelem, D., and Taylor, M. J. (2003) Trends Parasitol. 19, 516–522
2. Dreyer, G., Noroés, J., Figueredo-Silva, I., and Piessens, W. F. (2000) Parasitol. Today 16, 544–548
3. Taylor, M. J., Bandi, C., and Hoerauf, A. (2005) Adv. Parasitol. 60, 245–284
4. Taylor, M. J., Cross, H. F., and Bilo, K. (2000) J. Exp. Med. 191, 1429–1436
5. Turner, J. D., Langley, R. S., Johnston, K. L., Egerton, G., Wanji, S., and Taylor, M. J. (2006) J. Immunol. 177, 1240–1249
6. Brattig, N. W., Rathjens, U., Ernst, M., Geisinger, F., Renz, A., and Tischendorf, F. W. (2000) Microbes Infect. 2, 1147–1157
7. Brattig, N. W., Büttner, D. W., and Hoerauf, A. (2001) Microbes Infect. 3, 439–446
8. Gillette-Ferguson, I., Hise, A. G., McGarry, H. F., Turner, J., Esposito, A., Sun, Y., Diaconu, E., Taylor, M. J., and Pearlman, E. (2004) Infect. Immun. 72, 5687–5692
9. Saint André, A., Blackwell, N. M., Hall, L. R., Hoerauf, A., Brattig, N. W., Volkmann, L., Taylor, M. J., Ford, L., Hise, A. G., Lass, J. H., Diaconu, E., and Pearlman, E. (2002) Science 295, 1892–1895
10. Gillette-Ferguson, I., Hise, A. G., Sun, Y., Diaconu, E., McGarry, H. F., Taylor, M. J., and Pearlman, E. (2006) Infect. Immun. 74, 2442–2445
11. Gillette-Ferguson, I., Daehnel, K., Hise, A. G., Sun, Y., Carlson, E., Diaconu, E., McGarry, H. F., Taylor, M. J., and Pearlman, E. (2007) Infect. Immun. 75, 5908–5915
12. Hise, A. G., Daehnel, K., Gillette-Ferguson, I., Cho, E., McGarry, H. F., Taylor, M. J., Golenbock, D. T., Fitzgerald, K. A., Kazura, J. W., and Pearlman, E. (2007) J. Immunol. 178, 1068–1076
13. Vasselton, T., Detmers, P. A., Charron, D., and Haziot, A. (2004) J. Immunol. 173, 7401–7405
14. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Mura-matsu, S., and Steinman, R. M. (1992) J. Exp. Med. 176, 1693–1702
15. Jiang, H. R., Gilham, D. E., Mulryan, K., Kirillova, N., Hawkins, R. W., and Stern, P. L. (2006) J. Immunol. 177, 4288–4298
16. Kirschning, C. I., and Schumann, R. R. (2002) Curr. Top. Microbiol. Immunol. 270, 121–144
17. Takeuchi, O., Kawai, T., Mühlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., Takeda, K., and Akira, S. (2001) Int. Immunol. 13, 933–940
18. Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L., and Akira, S. (2002) J. Immunol. 169, 10–14
19. Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kaparat, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moranz, L., Lapidas, A., Omelchenko, M., Kyprides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tuskerman, K., Mazur, M., Comb, D., Koonin, E., and Slatko, B. (2005) PLoS. Biol. 3, e121
20. Liang, M. D., Bagchi, A., Warren, H. S., Tehan, M. M., Trigilio, J. A., Beasley-Toplelfe, L. K., Tesini, B. L., Lazzaroni, J. C., Fenton, M. J., and Hellman, I. (2005) J. Infect. Dis. 191, 939–948
21. Daehnel, K., Gillette-Ferguson, I., Hise, A. G., Diaconu, E., Harling, M. J., Heinzel, F. P., and Pearlman, E. (2007) Parasite Immunol. 29, 455–465
22. Whelan, M., Harnett, M. M., Houston, K. M., Patel, V., Harnett, W., and Rigley, K. P. (2000) J. Immunol. 164, 6453–6460
23. Jenkins, S. J., and Mountford, A. P. (2005) Infect. Immun. 73, 395–402
24. Cascales, E., Bernadac, A., Gavioli, M., Lazzaroni, J. C., and Lloberes, R. (2002) J. Bacteriol. 184, 754–759
25. Parsons, L. M., Lin, F., and Orban, J. (2006) Biochemistry 45, 2122–2128
26. Hellman, I., Roberts, J. D., Jr., Tehan, M. M., Allaire, J. E., and Warren, H. S. (2002) J. Biol. Chem. 277, 14274–14280
27. Zhu, X., Bagchi, A., Zhao, H., Kirschning, C. J., Hajjar, R. J., Chao, W., Hellman, I., and Schmidt, U. (2007) Crit. Care Med. 35, 886–892
28. Buvvitt-Beckmann, U., Heine, H., Wiesmüller, K. H., Jung, G., Brock, R., Akira, S., and Ulmer, A. J. (2005) Eur. J. Immunol. 35, 282–289
Wolbachia Lipoprotein Induces Disease via TLR2/6

29. Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zähringer, U., and Beutler, B. (2005) *Nature* **433**, 523–527

30. Triantafilou, M., Gamper, F. G., Haston, R. M., Mouratis, M. A., Morath, S., Hartung, T., and Triantafilou, K. (2006) *J. Biol. Chem.* **281**, 31002–31011

31. Schroder, N. W., Heine, H., Alexander, C., Manukyan, M., Eckert, J., Hammann, L., Goebel, U. B., and Schumann, R. R. (2004) *J. Immunol.* **173**, 2683–2691

32. Brattig, N. W., Bazzocchi, C., Kirschning, C. J., Reiling, N., Buttner, D. W., Ceciliani, F., Geisinger, F., Hochrein, H., Ernst, M., Wagner, H., Bandi, C., and Hoerauf, A. (2004) *J. Immunol.* **173**, 437–445

33. Cross, H. F., Haarbrink, M., Egerton, G., Yazdanbakhsh, M., and Taylor, M. J. (2001) *Lancet* **358**, 1873–1875

34. Keiser, P. B., Reynolds, S. M., Awadzi, K., Ottesen, E. A., Taylor, M. J., and Nutman, T. B. (2002) *J. Infect. Dis.* **185**, 805–811

35. Turner, J. D., Mand, S., Debrah, A. Y., Muehlfeld, J., Pfarr, K., McGarry, H. F., Adjei, O., Taylor, M. J., and Hoerauf, A. (2006) *Clin. Infect. Dis.* **42**, 1081–1089

36. Supali, T., Djuardi, Y., Pfarr, K. M., Wibowo, H., Taylor, M. J., Hoerauf, A., Houwing-Duistermaat, J. J., Yazdanbakhsh, M., and Sartono, E. (2008) *Clin. Infect. Dis.* **46**, 1385–1393

37. Esterre, P., Plichart, C., Huin-Blondey, M. O., and Nguyen, L. N. (2005) *Parasite Immunol.* **27**, 9–16

38. Debrah, A. Y., Mand, S., Specht, S., Marfo-Debrekyei, Y., Batsa, L., Pfarr, K., Larbi, J., Lawson, B., Taylor, M., Adjei, O., and Hoerauf, A. (2006) *PLoS Pathog.* **2**, e92

39. Debrah, A. Y., Mand, S., Toliat, M. R., Marfo-Debrekyei, Y., Batsa, L., Nurnberg, P., Lawson, B., Adjei, O., Hoerauf, A., and Pfarr, K. (2007) *Am. J. Trop. Med. Hyg.* **77**, 601–608

40. Kaneko, M., Kato, Y., Kunita, A., Fujita, N., Tsuruo, T., and Osawa, M. (2004) *J. Biol. Chem.* **279**, 38838–38843

41. Gröger, M., Loewe, R., Holthnower, W., Embacher, R., Pillinger, M., Horron, G. S., Wolff, K., and Petzelbauer, P. (2004) *J. Immunol.* **173**, 7161–7169

42. Al-Rawi, M. A., Watkins, G., Mansel, R. E., and Jiang, W. G. (2005) *Int. J. Oncol.* **27**, 721–730

43. Maruyama, K., Ii, M., Cursiefen, C., Jackson, D. G., Keino, H., Tomita, M., Van Rooijen, N., Takenaka, H., D’Amore, P. A., Stein-Streilein, J., Losordo, D. W., and Streilein, J. W. (2005) *J. Clin. Invest.* **115**, 2363–2372

44. Bagchi, A., Herrup, E. A., Warren, H. S., Trigilio, J., Shin, H. S., Valentine, C., and Hellman, J. (2007) *J. Immunol.* **178**, 1164–1171

45. Iwasaki, A., and Medzhitov, R. (2004) *Nat. Immunol.* **5**, 987–995

46. Korten, S., Badusche, M., Buttner, D. W., Hoerauf, A., Brattig, N., and Fleischer, B. (2008) *Microbes Infect.* **10**, 313–324

47. Babu, S., Blauvelt, C. P., Kumaraswami, V., and Nutman, T. B. (2005) *J. Immunol.* **175**, 1170–1176

48. Korsmeyer, S. J., and Korsmeyer, S. J. (2005) *Cell* **120**, 275–282