The Major Surface Protein of *Wolbachia* Endosymbionts in Filarial Nematodes Elicits Immune Responses through TLR2 and TLR4[1]

Norbert W. Brattig,[2]* Chiara Bazzocchi,[†] Carsten J. Kirschning,[§] Norbert Reiling,[‡] Dietrich W. Büttner,* Fabrizio Ceciliani,[†] Frank Geisinger,* Hubertus Hochrein,[§] Martin Ernst,[‡] Hermann Wagner,[§] Claudio Bandi,[†] and Achim Hoerauf [¶]

More than 150 million humans in tropical countries are infected by filarial nematodes which harbor intracellular bacterial endosymbionts of the genus *Wolbachia* (Rickettsiales). These bacteria have been implicated in adverse effects of drug treatment in filariasis. The present study provides evidence that purified major *Wolbachia* surface protein (rWSP) acts as an inducer of the innate immune system through TLR2 and TLR4: 1) recombinant, stringently purified rWSP elicited the release of TNF-α, IL-12, and IL-8 from cultured blood cells of both *Onchocerca volvulus*-infected and uninfected people; 2) the inflammatory response to rWSP challenge was TLR2- and TLR4-dependent as demonstrated with TLR-transfected fibroblastoid cells, as well as macrophages and dendritic cells from functional TLR-deficient mice; 3) blood cells of onchocerciasis patients exposed to rWSP also generated down-regulating mediators IL-10 and PGE_2 after 6 days of culture; 4) furthermore, rWSP-reactive IgG1 Abs were present in sera of *O. volvulus*-infected people but not in those of uninfected Europeans. The lack of rWSP-reactive IgE and IgG4 in serum indicated a bias toward a Th1-type adaptive immune response. Abs against rWSP stained endobacteria in living and degenerating adult *O. volvulus* filariae, tissue microfilariae and host tissue macrophages that apparently had engulfed microfilariae. Thus, filarial helminths, through products of their endobacteria such as WSP, acquire characteristics of a typical microbial pathogen inducing immune responses via TLR2 and TLR4. ***The Journal of Immunology,*** **2004, 173: 437–445.**

More than 150 million humans in tropical countries are infected by filarial nematodes leading to serious diseases including river blindness (caused by *Onchocerca volvulus*), chronic lymphoedema, and elephantiasis (*Brugia* spp. and *Wuchereria bancrofti*) (1, 2). In temperate countries, heartworm disease in dogs and cats is caused by the filaria *Dirofilaria immitis*.

Immunopathological reactions are thought to play a major role in the development of filarial diseases (3, 4, 61). Thus, clinical symptoms of chronic infection by *O. volvulus* appear to result from the cumulative effects of inflammation evoked mainly by the microfilarial stage of the worms that invade tissues (2). Abundant data from the literature suggests that filarial infection is associated with Th2 cell responses thereby activating potentially toxic effectors such as eosinophils (4–6). Host immune responsiveness is supposed to be counteracted by T regulatory mechanisms (6, 7).

Much interest has focused recently on *Wolbachia* endosymbionts which are essential for worm fertility and contribute to the pathogenesis of filarial disease (8–10, 61). The mutualistic relationship between filariae and their abundant *Wolbachia* endobacteria has led to a novel chemotherapeutic approach with tetracycline that causes long-term worm sterility (11). Adverse reactions following microfilaricidal therapy have been associated with potent innate responses including release of TNF-α and IL-6, as well as with the presence of *Wolbachia* DNA in the blood, apparently liberated from microfilariae after treatment (12–14). In a mouse model, *Wolbachia* constituents have been implicated in inflammatory reactions leading to corneal opacity and stromal thickening (keratitis), and TLR4 signaling appeared to be operative (15). *Wolbachia* and their products are reported to evoke pronounced innate immune responses in vitro mediated by monocytes/macrophages (Mϕ) and neutrophilic granulocytes (16–18). Until now, endotoxin-like molecules, present in the nematode extracts, were thought to be the major inducers of these responses.

Wolbachia surface protein (WSP),[3] an abundantly expressed protein of *Wolbachia*, was first identified in the endobacteria of *Drosophila* spp. (19), and more recently has been characterized for *Wolbachia* residing in *D. immitis* (20, 21). WSP appears to be highly conserved in *Wolbachia* from filarial nematodes and has been used for investigating the endosymbiont phylogeny (20, 22). This study presents, for the first time, evidence for the role of this unique *Wolbachia* molecule in the induction of innate immune responses in humans and in the implication of two TLR protein

*Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; [†]Sezione di Patologia Generale e Parassitologia, Università di Milano, Milan, Italy, [‡]Research Center Borstel, Borstel, Germany; [§]Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany; and [¶]Institute of Medical Parasitology, University of Bonn, Bonn, Germany

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[2] Address correspondence and reprint requests to Dr. Norbert W. Brattig, Tropical Medicine Section, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, D20359 Hamburg, Germany. E-mail address: NBrattig@bni.uni-hamburg.de

[3] Abbreviations used in this paper: WSP, *Wolbachia* surface protein; PPD, purified protein derivative; HEK, human embryonic kidney; DC, dendritic cell; mf, microfilaria; Ov.Extr., *O. volvulus* extract.

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family members, namely TLR2 and TLR4, as its cellular mediators.

Materials and Methods

Patients

The use of blood cells from patients with onchocerciasis was approved by the Ethics Committees of the Hamburg Board of Physicians and of the School of Medical Sciences (Kwame Nkrumah University of Science and Technology, Kumasi, Ghana). Eighteen patients with clinically proven generalized onchocerciasis (5, 11) living in endemic villages in the central region of Ghana were studied. The 12 women and 6 men, aged 17–57 years (median 35 years), were all microfilaria (mf) carriers (15.8 mf/mg skin; range 0.7–91) and presented onchocercomas. No other human filarial infections are endemic in this region. The prevalence of coinfection with *Ascaris lumbricoides* in the endemic area was <7%.

Preparation of rWSP and anti-WSP Abs

Production and purification of rWSP was performed as described (21). One microgram of purified rWSP revealed a single protein band at 24 kDa by silver staining of an SDS gel (21). The identity of the highly pure rWSP was further confirmed by immunoblot analysis because sera of *D. immitis*-infected dogs, recognizing several proteins in crude extracts of *Escherichia coli* clone expressing rWSP, exclusively recognized the respective single band representing purified rWSP. rWSP preparation was used for the production of polyclonal Abs in two rabbits. The specificity of the collected Abs was verified in immunoblot analysis using rWSP as well as extracts of *O. volvulus* worms containing *Wolbachia*.

Following affinity chromatography and subsequent 4-fold filtering with an anionic exchanger membrane (Sartorius, Göttingen, Germany) that was equilibrated with the solubilization buffer before loading of the recombinant protein, the rWSP was tested for the presence of *E. coli* LPS (LPS or endotoxin) contamination using a quantitative *Limulus* Amebocyte Lysate test (LAL test QCL 1000; <0.4 U/mg protein; BioWhittaker, Walkersville, MD). We tested different pH conditions for dialysis in 100 mM Tris-HCl and 50 mM NaCl. At pH 7.5, ~50% of the total protein remained in solution. After dialysis, the protein was retested for endotoxin presence, which was <0.4 U/mg protein. For control experiments, rWSP and *E. coli* LPS (0127:B8; Sigma-Aldrich, Deisenhofen, Germany) were incubated at 37°C for 1 h with proteinase K on agarose (Sigma-Aldrich) and were separated by centrifugation before testing in cell cultures. The degradation of rWSP was confirmed by the absence of the WSP band after PAGE and after Western blotting using anti-WSP Abs.

Preparation of worm extracts

Adult *O. volvulus* filariae were isolated from nodules of Ghanian onchocerciasis patients using the standard collagenase digestion method under aseptical conditions (18). Isolated worms were frozen in liquid nitrogen, ground, and homogenized in liquid nitrogen. The resulting powder was solubilized in sterile PBS at 4°C, the debris was removed by centrifugation, the supernatant tested for sterility, and aliquots were stored at −28°C until use. An extract of *A. lumbricoides*, obtained from a Ghanian patient, was prepared similarly as a control.

Immunohistology

Paraffin sections from onchocercomas fixed in 80% ethanol, in 4% buffered formaldehyde solution, or in modified Karnovsky fixative were available from several previous studies (11, 18, 23). The localization of the *Wolbachia* endobacteria in sections from these nodules had previously been shown using Abs against *Ov-Wolbachia* heat shock protein 60 and others. The reactivity of these Abs to *Wolbachia* had been confirmed by immunogold electron microscopy (8, 24). For the detection of rWSP by immunohistology the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was applied (Dako Diagnostika, Hamburg, Germany). The polyclonal antiserum raised in rabbits against rWSP from *D. immitis Wolbachia* (see above) was used at a dilution of 1/2000. A secondary polyclonal anti-rabbit antiserum (Dako Diagnostika) was used. Fast Red salt (Sigma-Aldrich) was used as chromogen and hematoxylin (Merck, Darmstadt, Germany) served as counterstain. The assessment of viability was based on worm morphology (23) and detection of filarial proteins expressed only by live worms using antisera against filarial GST1 (OvGST1) (25) kindly donated by Dr. E. Liebau (Bernhard Nocht Institute, Hamburg, Germany).

Ab analysis

Sera from the 18 onchocerciasis patients described above and from 2 healthy European volunteers were analyzed for the presence of Abs specific for WSP and Ags present in the *O. volvulus* extract, respectively, using an ELISA (26). Briefly, multiple dilutions of the sera were used in Ag-coated microtiter plates and human class- and subclass-specific mAbs (for IgG1, clone NL16; IgG4, clone RJ4 and IgE, clone CIA-A-7.12; Calbiochem, La Jolla, CA) were applied. The results were expressed as endpoint titers in arbitrary units (U). Sera from an additional 87 West African patients with onchocerciasis and 20 healthy Europeans were separately studied for WSP-reactive IgG Abs by ELISA using goat anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA). The cut-off values for IgG1 and IgG4 were OD = 0.15, and for IgE, OD = 0 determined by the median plus SD obtained from five normal sera.

Cytokine and PGE_2 secretion of human blood cells

Blood cell responses of 18 patients with onchocerciasis were tested in a whole blood cell culture system as described (5). PBMC and purified monocytes (Mϕ) from six healthy European donors were prepared by density gradient centrifugation and counterflow centrifugation, respectively, as described (5, 16). Blood cells, PBMC, and Mϕ were cultured in triplicate in the absence of stimulus or in the presence of 5–10 μg/ml each of rWSP, mycobacterial tuberculin (purified protein derivative (PPD); Statens Serum Institut, Copenhagen, Denmark), total extract from *O. volvulus* worms (Ov.Extr.), or extract from adult *A. lumbricoides* as a control nematode extract. Cultures were incubated at 37°C either overnight (for potential release of TNF-α, IL-1β, and IL-6) or for 3 days (for IL-4, IL-5, IFN-γ, IL-8, IL-12, IL-10, PGE_2) and the supernatants were conserved in liquid. The limitations of reagent and blood volumes did not allow a determination of varying concentrations of the stimuli, an inclusion of all stimuli for TNF-α responses, and incubations for more than two culture periods.

The concentrations of the cytokines TNF-α, IL-1β, IL-6, IL-4, IL-5, IL-10, and IFN-γ were measured by flow cytometry applying a cytometric bead array kit (BD Biosciences, San Diego, CA) using six bead populations with distinct fluorescence intensities coated with capture Abs specific for the cytokines. The cytokine capture beads were mixed with the PE-conjugated detection Abs and then incubated with recombinant standards (20–5000 pg/ml) or test samples to form sandwich complexes. Following acquisition of sample data using the FL3 channel of a flow cytometer (FACS; BD Biosciences, Mountain View, CA), the results were visualized in graphical and tabular format using BD Biosciences cytometric bead array analysis software. By applying the four-parameter curve fit option, we extrapolated values for sample intensities. The intra-assay variation of cytokine replicates for the kit was 2–6%; the interassay variation was 5–10% for the tested cytokines.

ELISAs were performed to analyze IL-12(p40) (27) (by OptEIA; BD Pharmingen, Heidelberg, Germany), IL-8, and PGE_2 (by kits from R&D Systems, Oxon, U.K.) in the culture supernatants according to the manufacturer's protocols. Cytokine concentrations were calculated from standard curves run in each plate with recombinant cytokine standards using linear regression analysis. The detection limit of the cytokine assays was 7.8 pg/ml for IL-12(p40), 12 U/ml for IL-8, and 36.2 pg/ml for PGE_2.

Stimulation of TLR-transfected human fibroblasts and reporter gene assay

Analysis of rWSP for use of TLRs in cellular stimulation was performed by complementation of the rWSP unresponsive human embryonic kidney (HEK) 293 cell line which does not express TLRs, but is responsive to TNF-α and IL-1β challenge. NF-κB-dependent reporter gene activation was indicative for cell activation. HEK293 cells (10^4) were plated per well of a cell culture plate and cotransfected with the NF-κB-dependent ELAM-1 promoter luciferase construct (28), an RSV promoter β-galactosidase reporter plasmid, an MD-2 expression plasmid (kindly provided by Dr. K. Miyake; Ref. 29), as well as CMV promoter-regulated plasmids for expression of human TLR1–10. DNA plasmids for expression of TLRs 2–4 were kindly provided by Tularik (South San Francisco, CA). Stimulation was performed with 0.5–10 μg/ml rWSP, or control stimulants such as 100 ng/ml LPS (*Salmonella minnesota* Re595; List Biological Laboratories, Campbell, CA or *E. coli* O111:B4; Sigma-Aldrich). After stimulation for 16 h, the cells were lysed for measurement of luciferase and β-galactosidase activities in a luminescence reader (Berthold Technologies, Weiterstadt, Germany) and normalized by relation of luciferase to β-galactosidase activities (30). In two control experiments, endotoxin-complexing polymyxin B (25 μg/ml; Sigma-Aldrich) was added to the HEK293 cell cultures.

Stimulation of mouse macrophages

Bone marrow-derived Mϕ were generated from TLR2-deficient and TLR2-competent wild-type (C57BL/6) mice as previous described (31, 32). The

Mϕ were stimulated with rWSP (0.5–15 μg/ml) and LPS (1 ng/ml; *Salmonella enterica* serovar friedenau, kindly was provided by H. Brade (Research Center Borstel, Borstel, Germany) as control stimuli. Cell culture supernatants were collected 24 h after stimulation and mouse TNF-α concentrations in the supernatants were measured by ELISA (R&D Systems).

In another series of experiments, thioglycolate-elicited peritoneal Mϕ were obtained from matched wild-type mice and mice lacking TLR2 as well as functional TLR4 ($TLR2^{-/-}TLR4^{d/d}$). Mice were generated as described (31, 33, 34). Mice descending from the homozygous breeding pairs of the genotypes indicated were matched and used for experiments. Peritoneal cells were isolated after 5 days of thioglycolate induction. Cells (1.5×10^5) were incubated for 2 h under regular cell culture conditions and washed for removal of nonadherent cells. Cells were stimulated for 24 h with rWSP, LPS (0.1 μg/ml), the bacterial lipoprotein analog tripalmytoyl-cysteinyl-seryl-(lysyl)3-lysine (Pam3CSK4, 1 μg/ml; EMC, Tübingen, Germany), or the synthetic DNA analog CpG-ODN (2 μM; TIB MOLBIOL, Berlin, Germany) (35). TNF-α and the MCP-1 (MCP-1; CCL2) concentrations in the cell-free culture supernatants were measured by ELISA (R&D Systems).

Stimulation of dendritic cells

Dendritic cells (DCs) were generated with Flt3-L (FL) supplemented bone marrow cultures (FL-DCs) from wild-type, $TLR2^{-/-}TLR4^{+/+}$, $TLR2^{+/+}TLR4^{d/d}$, and double-deficient mice ($TLR2^{-/-}TLR4^{d/d}$) as described (36) and FL-DCs (2.5×10^6/ml) were stimulated with rWSP (6 μg/ml) or CpG-ODN (1 μM; TIB MOLBIOL). After 17 h of stimulation, FL-DCs were labeled with the combination of anti-CD11c-allophycocyanin and anti-CD45RA-PE together with FITC-labeled anti-CD40 and FITC-labeled anti-CD62L (all BD Biosciences, Heidelberg, Germany) and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

Log values of the original data of the 18 patients with onchocerciasis are presented in box and whiskers plots as medians and percentiles (10; 25; 50; 75; 90%), means and SDs are given for the data obtained from the European controls. Statistical differences between test groups in the patients' study were analyzed by use of the Mann-Whitney *U* test; differences between the other groups were analyzed by use of the unpaired Student *t* test. Values of $p < 0.01$ or $p < 0.05$ were given that include Bonferroni correction.

Results

WSP-induced inflammatory responses in O. volvulus*-infected people*

To investigate cellular responses of *O. volvulus*-infected people to the endobacterial protein WSP abundant in the *O. volvulus* filaria, cultures of whole blood cells from 18 onchocerciasis patients were performed using rWSP that had been stringently purified. The production of cytokines in response to rWSP was compared with the responses to an unrelated bacterial Ag (mycobacterial PPD) as well as to WSP-containing extracts of *O. volvulus*, and to a control extract of the parasitic nematode *A. lumbricoides* which does not harbor *Wolbachia* endobacteria (Fig. 1). Exposure of blood cells to rWSP for 12 h resulted in the secretion of high levels of inflammatory cytokine TNF-α with median levels ~10 times higher than those observed in response to *O. volvulus* extract ($p < 0.01$). After 3 days of cell culture, high levels of two other inflammatory cytokines, IL-12 and IL-8, were observed ($p < 0.01$).

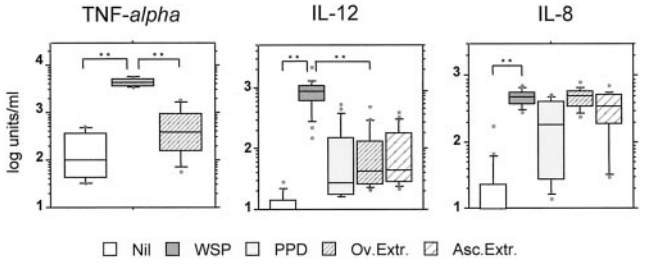

FIGURE 1. Cytokine secretion of peripheral blood cells from 18 *O. volvulus*-infected individuals in the absence (Nil) or in the presence of 10 μg/ml rWSP, PPD tuberculin, *O. volvulus* extract (Ov.Extr.) and *A. lumbricoides* extract (Asc.Extr.), respectively. The values for TNF-α (only two stimuli could be examined) and IL-12 represent log picograms per milliliter, for IL-8 log units per milliliter. Box plots are shown with whiskers and outliers. Significant differences (indicated by **, $p < 0.01$) were found between unstimulated (Nil) and rWSP-stimulated cultures (for all cytokines) as well as between rWSP- and Ov.Extr.-stimulated cultures (for TNF-α and IL-12) (see Figs. 5 and 6).

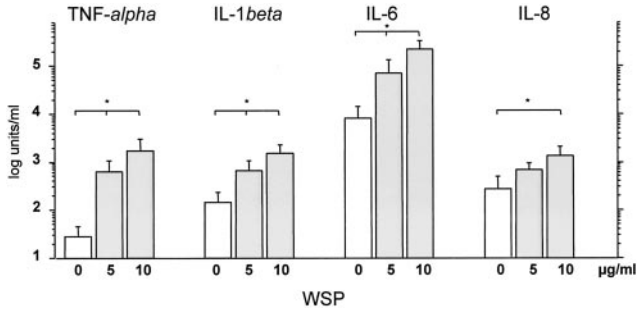

FIGURE 2. Cytokine secretion of peripheral PBMC from three healthy Europeans in the absence (0) or in the presence of 5 or 10 μg/ml rWSP. Dose-dependent production of TNF-α, IL-1β, IL-6, and IL-8 with significantly elevated concentrations ($p < 0.05$). The values for TNF-α, IL-1β, IL-6 represent log picograms per milliliter and for IL-8 log units per milliliter. Means and SDs of mean replicate values of the three healthy controls are shown.

Because these results obtained with cells from people infected with *O. volvulus* strongly indicated that WSP represents a stimulant of the innate immune system, we subsequently investigated cytokine responses of PBMC to rWSP in three healthy Europeans (Fig. 2). rWSP strongly stimulated the release of TNF-α, IL-1β, IL-6, and IL-8 ($p < 0.05$) in contrast to the absence of significant IL-2 and IL-4 amounts (data not shown). The control stimulant LPS elicited higher amounts of IL-1β ($p < 0.05$) in the cell cultures when compared with rWSP and induced a release of similar amounts of IL-6 and IL-8. The absence of endotoxin in the purified rWSP preparation and the dependence of the observed cellular responses on the presence of the *Wolbachia* protein was confirmed by stimulating PBMC from three additional healthy Europeans:

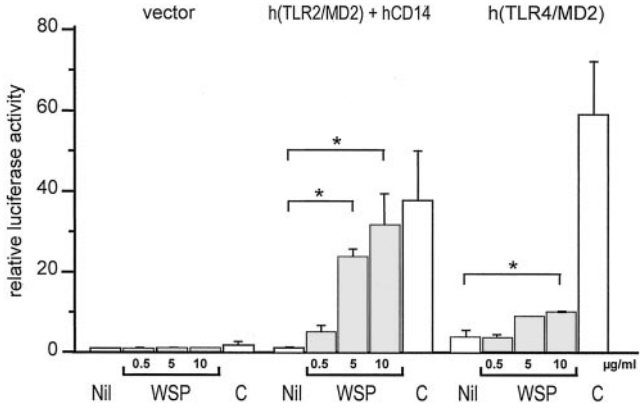

FIGURE 3. NF-κB-dependent reporter gene activation upon challenge with increasing amounts (0.5–10 μg/ml) of rWSP using TLR2 or TLR4 transiently overexpressing human fibroblastoid HEK293 cells. HEK293 cells were cotransfected with an NF-κB-dependent luciferase reporter construct, as well as expression plasmids for TLR2/MD2/CD14 or TLR4/MD2. Control cells were transfected with empty vector and MD2/CD14. The activity of luciferase was quantified 16 h after start of exposure to increasing amounts of rWSP or with LPS (from *S. minnesota*) as positive control (C). The figure shows the means and SD of duplicates. *, Significant increase in comparison to unstimulated cultures ($p < 0.05$).

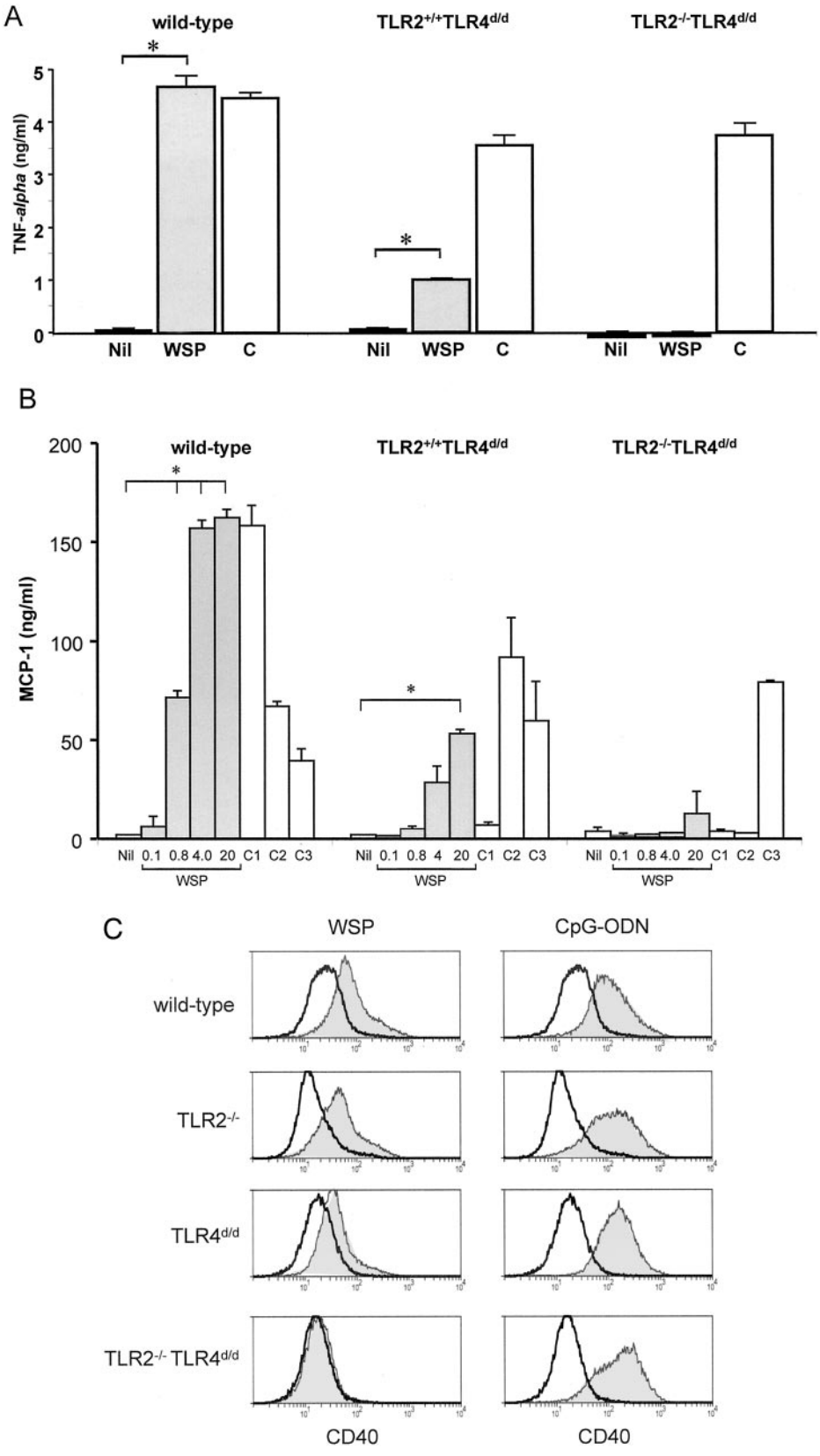

FIGURE 4. Responsiveness of macrophages (Mϕ) and DCs to rWSP is dependent on TLR2 and TLR4. *A*, TNF-α concentrations (nanograms per milliliter) in supernatants of peritoneal Mϕ of wild-type, TLR2$^{+/+}$TLR4$^{d/d}$, or TLR2$^{-/-}$ TLR4$^{d/d}$ mice incubated with 5 μg/ml rWSP or control stimulant (CpG-ODN (C)). Significances are indicated by $*$ ($p < 0.05$). *B*, MCP-1 responses (nanograms per milliliter) of the mouse mutants after stimulation with 0.1–20 μg/ml rWSP or control stimulants (LPS (C1), Pam3CSK4 (C2), or CpG-ODN (C3)). *C*, FACS analysis of CD40 expression in bone marrow-derived DCs from mutant mice without (empty histograms) or after (filled histograms) stimulation with 6 μg/ml rWSP (*left column*) or 1 μM CpG-ODN (*right column*). The up-regulation of CD40 surface expression upon rWSP challenge was only abrogated in the absence of both TLR2 and TLR4.

untreated rWSP caused the release of a concentration of 1368 $\pm$ 450 pg/ml TNF-α (LPS control: 1767 $\pm$ 410 pg/ml), whereas in cells to which proteinase K-treated rWSP had been added the production of TNF-α was abrogated (0 $\pm$ 0 pg/ml; LPS control: 1797 $\pm$ 347 pg/ml). In contrast, treatment of *E. coli* LPS with proteinase K did not alter TNF-α release as compared with untreated LPS control. This result further demonstrates that the biological activity of rWSP does rely on its proteinaceous properties and is not due to possible LPS contamination. Inflammatory cytokine responses were also found when purified Mϕ from three

healthy Europeans were exposed to rWSP (data not shown), underscoring the innate nature of the response.

WSP triggered cellular activation via TLRs 2 and 4

Because WSP is a conserved bacterial surface molecule that triggers inflammatory responses, we asked for its potential TLR dependence. In a first series of experiments, human fibroblastoid cells (HEK293) transiently transfected with TLR2 or TLR4 were exposed to rWSP. Cellular activation was determined as NF-κB-dependent reporter gene activation (30). NF-κB is crucially involved in activation of a broad spectrum of genes encoding proteins relevant in inflammatory processes including TNF-α, IL-8, and IL-12. rWSP-induced reporter gene activation was found to be predominantly TLR2-dependent. Fig. 3 displays rWSP dose-dependent strong activation through TLR2 ($p < 0.05$) and less prominent activation via TLR4 as was observed in separate experiments ($p < 0.05$). No further TLR protein member was implied upon separate transfection of each of TLRs 1–10 and functional analysis in vitro (data not shown). rWSP-induced activation of TLR2- as well as of TLR4-overexpressing cells also occurred in the presence of polymyxin B (25 μg/ml) with only a slight reduction in activation levels while polymyxin B led to an obstruction of the LPS response (data not shown).

Because TLR2 was involved in the recognition of WSP as evidenced upon overexpression, we next used bone marrow-derived mouse Mϕ of the genotype TLR2$^{-/-}$ and compared their response to rWSP with the reactivities of Mϕ from wild-type mice. Stimulation of Mϕ from wild-type mice with rWSP in three separate experiments resulted in the release of high amounts of TNF-α (3365 $\pm$ 586 pg/ml; unstimulated control: 40 $\pm$ 23 pg/ml) while significantly reduced levels (41%) were found in supernatants of Mϕ from TLR2$^{-/-}$ mice (1936 $\pm$ 66 pg/ml; unstimulated control: 39 $\pm$ 20 pg/ml). This result not only further suggests an important role of TLR2 in immune responses toward rWSP, but also implies the involvement of TLR2-independent pathways.

In subsequent experiments, inflammatory responses to rWSP were compared using peritoneal Mϕ from wild-type mice, Mϕ from TLR2$^{+/+}$TLR4$^{d/d}$ mutant mice and from mice lacking the expression of TLR2 and of functional TLR4 (TLR2$^{-/-}$TLR4$^{d/d}$). Experiments revealed significantly reduced inflammatory responses in terms of release of TNF-α and MCP-1 (Fig. 4, *A* and *B*), as well as of IL-6 and NO (data not shown) in TLR2$^{+/+}$TLR4$^{d/d}$ Mϕ as compared with wild-type Mϕ, as well as the absence of responses in TLR2$^{-/-}$TLR4$^{d/d}$ Mϕ. These data confirm an exclusive involvement of TLR2 and TLR4 in WSP-induced cell activation.

Furthermore, rWSP induced the maturation of murine DC in a TLR2- and TLR4-dependent manner. As parameters of DC activation, up-regulation of CD40 (Fig. 4*C*) and down-regulation of L-selectin (CD62L, data not shown) were analyzed by flow cytometry. Whereas DC maturation was clearly detectable in DCs from single-deficient mice (TLR2$^{-/-}$TLR4$^{+/+}$ or TLR2$^{+/+}$TLR4$^{d/d}$), it was completely abrogated in DCs from double-deficient mice (TLR2$^{-/-}$TLR4$^{d/d}$) (Fig. 4*C*).

WSP-induced anti-inflammatory cellular responses in O. volvulus*-infected people*

Inflammatory responses have recently been shown to be limited by subsequently generated anti-inflammatory mediators which also can be induced through TLRs (37). Therefore, we investigated the release of IL-10 and PGE_2 (38). After 3 days of exposure to rWSP, cultured cells from the 18 onchocerciasis patients released significantly increased amounts of IL-10 and PGE_2 ($p < 0.01$; Fig. 5). rWSP induced higher levels of both inhibitory mediators ($p < 0.01$) when compared with *O. volvulus* extract as stimulant even if quantitative comparison is difficult due to the undefined nature of the extract. Low but significantly increased IL-10 release was also observed in rWSP-exposed PBMC cultures of the three uninfected Europeans (110 $\pm$ 33 pg/ml; unstimulated 0 pg; $p < 0.05$).

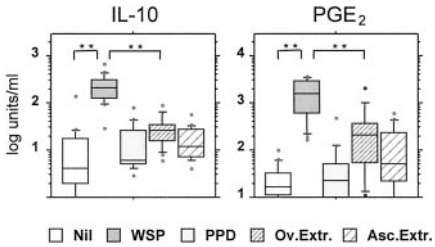

FIGURE 5. IL-10 and PGE_2 release of peripheral blood cells from 18 *O. volvulus*-infected individuals in the absence of stimuli (Nil) or in the presence of 10 μg/ml rWSP, PPD tuberculin, *O. volvulus* extract (Ov.Extr.) and *A. lumbricoides* extract (Asc.Extr.), respectively (see Figs. 1 and 6). **, Significant differences were found between unstimulated (Nil) and rWSP-stimulated cultures for both mediators ($p < 0.01$) and between rWSP- and *O. volvulus* extract-stimulated cultures ($p < 0.01$).

WSP-induced lymphokine release and Ab recognition of WSP

In addition to the parameters representative for the innate immune system, the culture supernatants of blood cells from the 18 onchocerciasis patients were examined for the production of Th1 and Th2 type lymphokines in response to rWSP and *O. volvulus* extract. Although *O. volvulus* extract stimulated the release of higher amounts of IL-4 and IL-5 when compared with rWSP as stimulant ($p < 0.01$), rWSP exposure resulted in higher IFN-γ levels in the cell cultures of the patients ($p < 0.01$; Fig. 6). Similar high IFN-γ release occurred in the cultures of rWSP-exposed PBMC from the uninfected controls (2171 $\pm$ 1190 pg/ml; unstimulated control: 7 $\pm$ 9 pg/ml; $p < 0.05$). The IFN-γ concentrations correlated significantly with the amounts of TNF-α ($r = 0.82$).

To further look for possible adaptive immune responses to WSP, we examined patients' sera for anti-WSP Abs. In contrast to sera from Europeans, we observed IgG1 Abs in the sera of the 18 *O. volvulus*-infected people reactive with rWSP in ELISA. The median titer, however, was significantly lower when compared with the IgG1 response to *O. volvulus*-extracted Ags ($p < 0.01$; Table I). Interestingly, in contrast to the IgG1 reactivities, 16 of the 18 sera showed IgG4 titers below 200 U and none showed a significant rWSP-reactive IgE titer, while both types of Abs, representing hallmarks for filarial infections (4, 6), were prominently found

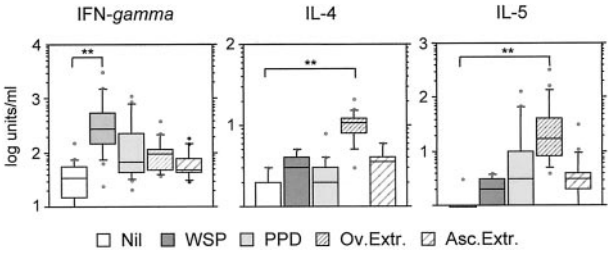

FIGURE 6. Lymphokine release of peripheral blood cells from 18 *O. volvulus*-infected individuals in the absence of stimuli (Nil) or in the presence of 10 μg/ml rWSP, PPD tuberculin, *O. volvulus* extract (Ov.Extr.) and *A. lumbricoides* extract (Asc.Extr.), respectively. The box plots represent log picograms per milliliter. Significant differences ($p < 0.01$; indicated by **) were found between unstimulated and rWSP-stimulated cultures for IFN-γ and between unstimulated and OvExtr-stimulated cultures for IL-4 and IL-5 (see Figs. 1 and 5).

Table I. *Recognition of rWSP by IgG1 and of* O. volvulus *Ags by IgG1, IgG4, and IgE*[a]

Ab Type	Serum Donor	Ag	
		WSP	*O. volvulus* Ag
IgG1	Onchocerciasis patients	320 (183; 1200)[b]	1450 (160; 5550)[b]
	Healthy European controls	50; 50[c]	110; 100[c]
IgG4	Onchocerciasis patients	0 (0; 125)[b]	2500 (700; 6300)[b]
	Healthy European controls	0; 0[c]	75; 75[c]
IgE	Onchocerciasis patients	0 (0; 0)[b]	100 (1; 602)[b]
	Healthy European controls	0; 0[c]	0; 0[c]

[a] Recognition of rWSP by IgG1 and of *O. volvulus* Ags by IgG1, IgG4, and IgE in sera from 18 patients with onchocerciasis and as compared to sera from two healthy Europeans. The endpoint titers (U) obtained in an ELISA are presented as medians and percentiles for the 18 patients and as single values for the two European sera. Significant differences were found between rWSP and *O. volvulus* extract for IgG1, IgG4, and IgE ($p < 0.01$; Mann-Whitney *U* test).
[b] Median titer (10%; 90%).
[c] Single titers.

in the patients' sera ($p < 0.01$). The two sera of Europeans included in the analysis exhibited activities below the cut-off levels. In a separate study, 82 of 87 sera from onchocerciasis patients contained significant IgG activities specific for rWSP while 2 of 20 sera from healthy controls studied together with the 87 patients showed titers weakly higher than the cut-off level (data not shown).

Detection of WSP in onchocercoma of human host

The localization of WSP in onchocercomas was analyzed by immunohistology to explain the possible exposure of WSP to the host immune system. By use of a polyclonal antiserum, WSP was detectable in a large number of sections of microfilariae and adult *O. volvulus* representative for different geographic endemic locations (e.g., Ghana, Liberia, Burkina Faso, Uganda, Yemen, Guatemala). The endobacteria were strongly stained in "live" microfilariae (Fig. 7*A*) and "live" adult worms (Fig. 7, *E* and *F*). WSP-positive endobacteria were also seen in degenerating (Fig. 7, *B* and *C*) and dead microfilariae, as well as in the hypodermis, in oocytes (Fig. 7*G*), as well as embryos of moribund and dead adult worms from untreated people and from patients treated with a macrofilaricide (suramin). WSP-positive endobacteria from oocytes and embryos spread from dying or dead adult worms into the human tissues (Fig. 7*H*). Free WSP-positive endobacteria outside of the filariae were also seen in host's tissues. Mϕ and giant cells were found attached to degenerating (Fig. 7, *B* and *C*) and dead microfilariae, as well as to oocytes or embryos from ruptured adults. In some sections, WSP-positive endobacteria, free or in remnants of filariae, were phagocytized by the Mϕ and giant cells (Fig. 7, *D* and *J*). This indicates the occurrence of close contacts between WSP-carrying bacteria and host immune cells.

Discussion

The interaction between *Wolbachia* bacteria, its filarial host *Onchocerca*, and the human host of the filaria as three-party interrelationship currently has drawn increased attention (8–10, 61). The presented immunohistological studies revealed that Mϕ phagocytized remnants of degenerating microfilariae or of adult worms with their oocytes and embryos. Because this material contains endobacteria, an intense contact of host immune cells with the endobacteria and its dominant surface protein WSP clearly is indicated. Patients in hyperendemic areas carrying high microfilaria loads have daily turnovers of 20,000–300,000 microfilariae or more (39). The daily uptake of these *Wolbachia*-harboring microfilariae over a period of 10–40 years and of additional endobacteria from adult *O. volvulus* presents an enormous immunogenic stimulus.

The intracellular bacteria in filariae have been implicated in the immune responses and pathogenesis of filarial infections (8–10, 61). In particular, inflammatory responses following treatment of filariae with diethylcarbamazine or ivermectin have been suggested to result in part from the release of high numbers of endobacteria from degenerating tissue microfilariae (12, 13). Thus, increased levels of inflammatory mediators and acute phase reactants including TNF-α, IL-6, and LPS-binding protein in the serum of infected patients have been repeatedly reported after anti-filarial treatment (13, 40) and inflammatory cytokine levels correlated with the presence and amount of *Wolbachia* DNA (13, 14). However, causative *Wolbachia* molecules have not yet been identified, even though in vitro studies have indicated a putative LPS-like activity as a possible candidate (16, 17).

In the present study, however, we have shown for the first time that a *Wolbachia*-derived distinct protein (WSP) can elicit in vitro inflammatory responses consistent with those observed previously in treated filariasis patients in vivo. A possible LPS contamination of rWSP due to the bacterial expression system was excluded by 1) procedures applied for rWSP purification including anionic exchange membrane filtration which leads to undetectable endotoxin levels; 2) total abrogation of Mϕ TNF-α release after degradation of rWSP with proteinase K, that did not affect the Mϕ response to commercial LPS; and 3) by polymyxin B treatment of rWSP before application to TLR4- or TLR2-transfected HEK293 cells which did not inhibit cellular activation.

WSP appears to represent a novel proinflammatory bacterial (*Wolbachia*) molecule that interacts with cells of the innate immune system. This WSP responsiveness could be observed in *O. volvulus*-infected people infected with *Wolbachia*-containing filariae, in healthy Europeans as well as using murine Mϕ. Similar reactivities have been described for surface proteins from other intracellular Rickettsiaceae that bind to Mϕ and stimulate cytokine production. It has been well documented that conserved molecules from pathogens, pathogen-associated molecular patterns, are able to activate innate immune cells, particularly Mϕ (41). TLRs have been shown to function as pattern recognition receptors which mediate the activation of innate and adaptive immune responses (42, 43). To date, 10 TLRs have been identified, along with a variety of microbial agonists that bind to them. Cell activation through TLRs triggers signaling that leads to activation of NF-κB that result in activation of genes encoding inflammatory cytokines including TNF-α, IL-12, and IL-8.

TLR2 and TLR4 have been reported to be involved in the recognition of numerous ligands including lipoproteins and proteins for the first and LPS for the latter (42, 44, 45). In the present study, rWSP stimulated human fibroblasts transfected with either TLR2

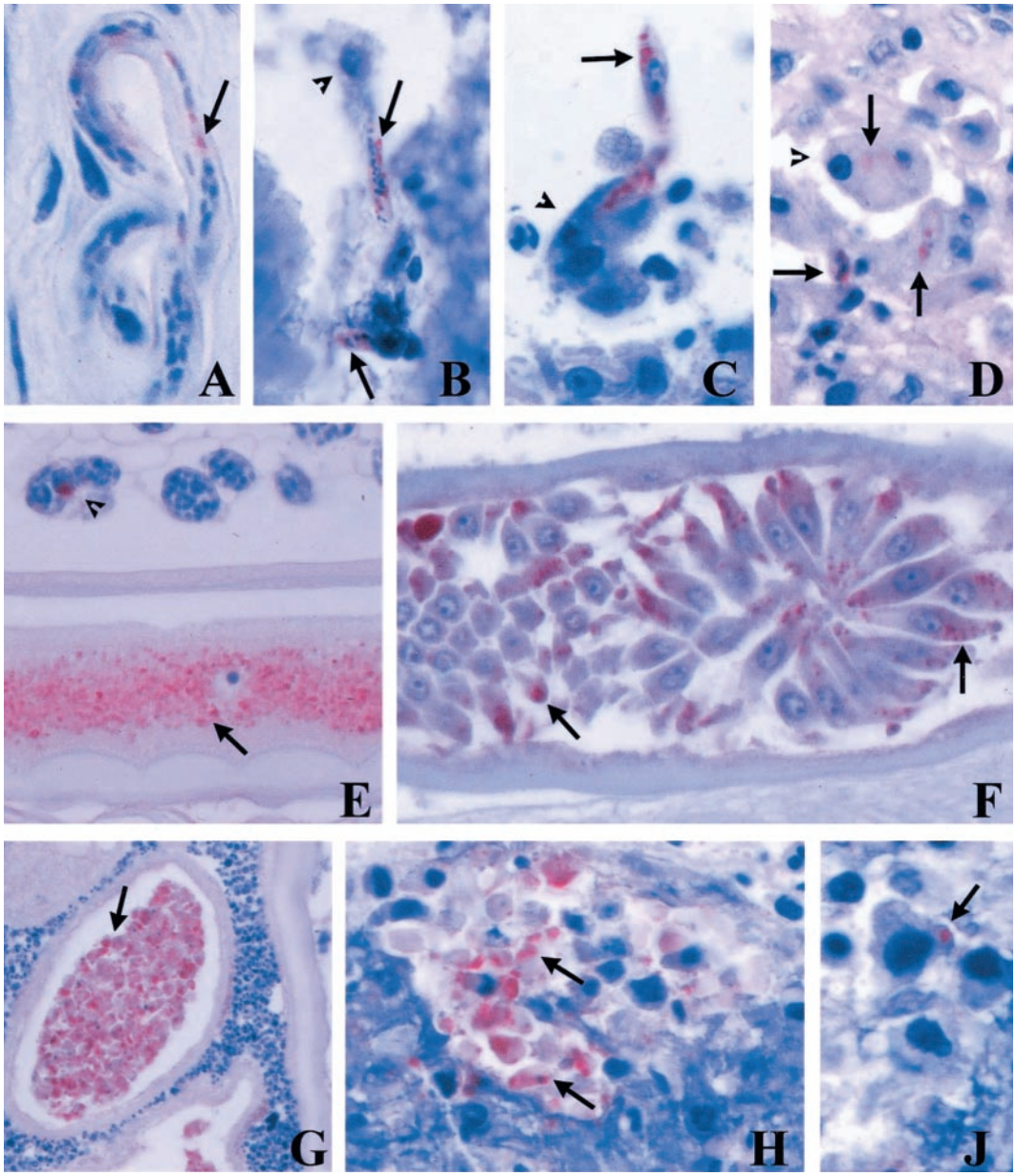

FIGURE 7. Strong red APAAP staining of WSP in immunohistological sections from onchocercomas showing *O. volvulus Wolbachia*. *A–C*, *Wolbachia* in microfilariae. Bacteria (arrow in *A*) in hypodermal cells of a live microfilaria in human nodule tissue and in degenerating microfilariae (arrows in *B* and *C*, same nodule) surrounded by Mϕ (arrowhead). *Wolbachia* (arrows in *D*) in remnants of dead microfilariae are taken up by Mϕ and a small giant cell (arrowhead in *D*). *E–J*, *Wolbachia* in adult filariae. Endobacteria in the hypodermis (arrow in *E*) and in a morula (arrowhead in *E*) of a live female filaria (longitudinal section). Bacteria in oocytes (arrows in *F*) in the ovary of a live worm, in degenerating oocytes in the uterus of a dead worm (arrow in *G*), and in degenerating oocytes (arrows in *H*) from a ruptured filaria lying freely in nodule tissue. A Mϕ phagocytizes a degenerated oocyte with a *Wolbachia* (arrow in *J*, same nodule as *H*). Magnification: ×1050 (*A*), ×570 (*B*), ×800 (*C*), ×520 (*D*), ×600 (*E*), ×730 (*F*), ×380 (*G*), ×650 (*H*), ×2750 (*J*).

or TLR4. Furthermore, Mϕ and DCs from either functional TLR2- or TLR4-deficient mice responded to rWSP with cytokine production and maturation whereas responsiveness was completely abrogated in the combined absence of TLR2 and TLR4. Taken together, our data strongly support the view that both receptors are involved in transducing WSP stimulation. The unresponsiveness of Mϕ and DCs from TLR2$^{-/-}$TLR4$^{d/d}$-deficient mice to rWSP excluded the involvement of additional TLRs or other pattern recognition receptors for the recognition of rWSP as confirmed by an experiment using fibroblasts transfected with TLR1–10. Recent reports showed that intracellular signaling upon challenge with various pathogen-associated molecules can involve TLR4 or TLR2 (30, 32, 46, 47). Thus, activation of Mϕ by the Gram-negative endobacterium *Chlamydia* mainly depends on TLR2 and partially on TLR4 (30). Of note, in addition to Mϕ, also mast cells, characteristic for filarial infections, and neutrophils express TLR2 and TLR4 (48, 49) possibly indicating a direct mast cell activation by TLR ligands like WSP.

Recently, TLR stimulation has also been found to induce, subsequent to proinflammatory stimuli, down-regulators of inflammation (50–52). These responses appear to represent a feedback loop of the innate immune system and may play a critical role in the maintenance of homeostasis of the host organism (51, 53). The observed production of IL-10 and PGE_2 in response to rWSP may relate to similar mechanisms (38, 53). Helminths are now supposed to alternatively activate Mϕ (54–56) which are suggested to sustain an immune

quiescent state in chronic inflammation. The trematode *Schistosoma mansoni* has recently been reported to stimulate DCs through TLR2 thereby leading to IL-10 (57). The role of TLR signaling in helminth infection, therefore, needs further analysis.

Furthermore, of interest was our finding that patients with onchocerciasis, who are chronically exposed to *Wolbachia*-carrying WSP, show marked IgG1 but not IgE and IgG4 responses to WSP, the latter being characteristic for filarial infections (2, 4, 6). Although the pronounced IFN-γ response that we observed in *O. volvulus*-infected as well as noninfected people, may originate from the innate immune system (e.g., NK cells) stimulated by IL-12 from Mϕ, the pronounced WSP-recognizing IgG1 Ab levels appear to represent an acquired immune response to WSP. This indicates a Th1-type bias, typical of exposure to bacteria. The lack of IL-4 and IL-5 production by WSP-exposed immune cells appears to confirm such a bias. Thus, Th1-type responses and not Th2 responses may depend to a considerable degree on the presence of *Wolbachia*. IgG responses against WSP were recently reported in cats infected with *D. immitis* (21), in humans with pulmonary dirofilariasis (58), as well as in humans with lymphatic filariasis (59). Cross-reactions between Ags from *Wolbachia* residing in filariae and those in insects have been shown (60). Transmembrane regions of WSP, which are conserved between arthropod and nematode *Wolbachia*, might be responsible for this cross-reactivity (59). However, in our study, 82 of 87 patients affected by onchocerciasis had high IgG1 titers against WSP, compared with only 2 of 20 healthy Europeans showing marginal titers over the cut-off value. In addition, in cats residing in the same geographic area those infected with *D. immitis* showed significantly higher Ab titers compared with uninfected ones in the same area (21). Similar observations have been reported for humans with pulmonary dirofilariasis (58). Although there is an intense daily turnover of *Wolbachia*-containing microfilariae, an induction of a significant anti-WSP Ab response by insect *Wolbachia* can be neglected because the insects do not transfer *Wolbachia* to the human through the biting act and *Tunga penetrans* is rare in the endemic area.

The present study strongly suggests that *Wolbachia*-associated molecules participate in the interaction between the filarial parasite and the human host. WSP, using TLR2 and TLR4 for immune cell stimulation, might activate the innate and the adaptive immune system of the host. Thus, filarial helminths, through their endobacteria, acquire characteristics of bacteria and signal immune responses via TLR. Th1-type inflammatory responses in filariasis may therefore depend on the endobacteria dormant in the filarial "Trojan horse."

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