Responses to Toll-Like Receptor Ligands in Children Living in Areas Where Schistosome Infections Are Endemic

Desiree van der Kleij,1,2 Anita H. J. van den Biggelaar,1 Yvonne C. M. Kruize,1 Kim Retra,1,2 Yvonne Fillie,1 Marion Schmitz,2 Peter G. Kremsner,3 Aloysius G. M. Tielens,2 and Maria Yazdanbakhsh1

1Department of Parasitology, Leiden University Medical Center, Leiden, and 2Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; 3Department of Human Parasitology, Institute for Tropical Medicine, Tübingen University, Tübingen, Germany

To study the effect of repeated challenge of the innate immune system with pathogen-associated molecular patterns, cytokine responses to schistosomal lipids and bacterial lipopolysaccharide (LPS) were analyzed in schoolchildren living in an area in Gabon where schistosomiasis, a helminth infection that is chronic in nature, is endemic. A schistosomal phosphatidylserine (PS) fraction containing the Toll-like receptor (TLR)–2 ligand lyso-PS stimulated the production of interleukin (IL)–8, IL-10, IL-6, and tumor necrosis factor (TNF)–α in children without Schistosoma haematobium infection. However, in infected children, the responses to this stimulus were lower, in particular for production of IL-8 and TNF-α. Responses to the TLR4 ligand, LPS, followed a similar pattern. In contrast, schistosomal adult worm glycolipids that did not stimulate any of the TLRs tested induced IL-8 and IL-6 responses that were significantly higher in schistosome-infected children than in schistosome-uninfected children. These results indicate that relentless exposure to pathogens can lead to altered responses to TLR ligands.

Innate immune responses are activated through recognition of pathogen-associated molecular patterns (PAMPs) by specific germline-encoded receptors on the host cells. Activation of pattern-recognition receptors leads to an immediate response to infection and can profoundly influence the development of an adaptive immune response. Toll-like receptors (TLRs) are a class of receptors that play an important role in the recognition of PAMPs. Whereas the first TLR ligands identified were all of bacterial origin, it is becoming more and more clear that the recognition spectrum of TLRs is much broader, also including viruses, yeast, and helminths. Although many studies have been performed on the interaction between bacterial products, which often cause acute infections, and the naive immune system, much less is known about how repeated encounters of the innate immune system with PAMPs would affect these responses. Helminth infections, which are chronic in nature, provide a highly suitable model for studying the effect of repeated challenge of the innate immune system. Schistosomes are trematodes that cause schistosomiasis, a chronic blood-vascular disease. The chronic presence of helminths is mirrored by persistent challenge of the immune system with an array of molecules associated with parasite metabolism and reproduction. Studies in humans, as well as experimental models of schistosomiasis, have shown that pro- and anti-inflammatory responses are crucial to the development or containment of immune-mediated tissue damage [1, 2]. Therefore, the activation of cells of the innate immune response by signature molecules of schistosomes may determine the course of the pathologic process and immunity to infection, emphasizing the importance of studying the response to such parasite molecules.
Lipid moieties have been shown to be essential for activation of TLRs in several bacterial products [3–5]. Moreover, it is becoming increasingly clear that lipids and their receptors may play an important role in regulating immune responses. For example, deletion of the lyso-phosphatidylcholine receptor results in adult-onset autoimmune disease similar to human systemic lupus erythematosus [6], and α-galactosylceramide prevents the onset or recurrence of autoimmune diabetes when presented in the context of the surface molecule CD1d [7]. Recently, we identified schistosomal glycolipids (GLs) [8] and schistosomal lyso-phosphatidylserine (lyso-PS) [9] as molecules that are able to activate cells of the innate immune system and found that the latter molecule is a ligand for TLR2 [9]. We therefore have the opportunity to characterize the effect of these PAMPs in naive, as well as in chronically exposed and/or infected, subjects.

In the present study, we have analyzed the immune responses of newly identified schistosomal PAMPs (lyso-PS and GLs) in a population living in a region of Gabon where schistosomiasis is endemic. We found that schistosome-infected children displayed lower levels of cytokine responses to both TLR2 and TLR4 ligands than did schistosome-uninfected children, whereas, for schistosomal adult worm GLs, which do not stimulate any of the TLR ligands tested so far, the uninfected children displayed higher levels of responses. This indicates that continuous exposure to helminths can affect responsiveness of cells to PAMPs derived from these parasites and, thus, modify the innate immune responses in chronically infected subjects.

### SUBJECTS, MATERIALS, AND METHODS

**Study population.** The study population consisted of schoolchildren from an area in the Lambarene region of Gabon, where schistosomiasis is endemic and where focal pockets of transmission are found. We identified a school in the area of Lambarene (Agrozile) with high transmission levels of *S. haematobium*. This is a rural area with no paved roads and infrequent access to public transportation, located 15–30 km from the Albert Schweitzer Hospital (ASH) in Lambarene. Uninfected children from a school in the vicinity of the ASH (Lalala) were included and identified as control subjects. PBMCs were isolated from 10 schistosome-infected schoolchildren and from 15 schistosome-uninfected schoolchildren. As shown in table 1, there were no significant differences with regard to sex, age, or intestinal helminth infection between the children from the 2 areas. The present study was approved by the ethics committees of Tübingen University and of the International Foundation of the Albert Schweitzer Hospital in Lambarene, Gabon. Written, informed consent was obtained from parents or guardians of the children participating in the present study.

**Preparation of lipids.** *S. mansoni* adult worms were collected by perfusion of golden hamsters 45–48 days after infection. *S. mansoni* eggs were isolated from livers of infected hamsters after treatment of the liver homogenate with trypsin. GL and PS preparations were made from *S. mansoni* eggs and adult worms, as described elsewhere [10]. In brief, a total lipid extract was made in accordance with the method described by Bligh and Dyer [11]. Lipids were separated into different fractions by use of triethylaminoethyl cellulose column chromatography, as described by Rouser et al. [12], which results in the following 8 fractions containing mainly the specified lipids: (1) cholesterol, glycerides, and other neutral lipids; (2) cerebrosides, glycerol diglycerides, phosphatidyl choline, and sphingomyelin; (3) ceramide-polyhexosides; (4) inorganic substances; (5) phosphatidyl ethanolamine and free fatty acids; (6) phosphatidyl serine; (7) none (washing step); and (8) phosphatic acid, cardiolipin, phosphatidyl glycerol, and phosphatidyl inositol, as well as other acidic lipids. The presence of carbohydrates in the ceramide-polyhexoside-containing fraction was confirmed by orcinol staining of this fraction on high-performance thin layer chromatography plates. Although this fraction (3) also contained other components, it will be named schistosomal GL in this report. Mass spectrometry (as detailed elsewhere [13]) was used to confirm the presence of PS and lyso-PS in the PS fraction (6).

### Table 1. Characteristics of the study population, which comprised schoolchildren from 2 areas in Gabon.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Lalala (n = 15)</th>
<th>Agrozile (n = 10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range), years</td>
<td>10 (9–15)</td>
<td>11 (8–14)</td>
<td>.179</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>10:5</td>
<td>4:6</td>
<td>.530</td>
</tr>
<tr>
<td>Schistosome egg load, geometric mean (range), eggs/10 mL of urine</td>
<td>...</td>
<td>3.7 (1–440)</td>
<td></td>
</tr>
<tr>
<td>Trichuris trichiura</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects infected/no. of subjects examined</td>
<td>5/13</td>
<td>4/8</td>
<td>.472</td>
</tr>
<tr>
<td>Egg load, geometric mean (range), eggs/50 mg of feces</td>
<td>26 (2–326)</td>
<td>22 (6–78)</td>
<td>.948</td>
</tr>
<tr>
<td>Ascaris lumbricoides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects infected/no. of subjects examined</td>
<td>5/13</td>
<td>4/8</td>
<td>.388</td>
</tr>
<tr>
<td>Egg load, geometric mean (range), eggs/50 mg of feces</td>
<td>410 (1–3298)</td>
<td>285 (56–1749)</td>
<td>.343</td>
</tr>
</tbody>
</table>

**NOTE.** Egg loads are represented as geometric means for infected individuals only.
The lipopolysaccharide (LPS) content of the lipid preparations was determined by use of a limulus amebocyte lysate assay (LAL COATEST Endotoxin; Chromogenix). No endotoxin could be detected in any of the lipid preparations. To exclude effects of undetectable amounts of endotoxin on cytokine production, all cell culture experiments (except stimulations with LPS as a ligand) were performed in the presence of 10 μg/mL polymyxin B sulphate (Sigma), which was found to be sufficient to completely abrogate the cytokine response of the peripheral blood mononuclear cells (PBMCs) to 5 ng/mL LPS, a concentration that is easily detectable by use of the limulus amebocyte lysate assay.

**Cell lines.** Human embryonic kidney (HEK)-TLR2 and HEK-TLR4 cell lines were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 10 μg/mL ciprofloxacin, and 0.5 mg/mL G418-sulfate. For stimulation of the innate immune system by this lipid fractioncontaining fraction 3 of eggs can activate cells of the innate immune system. The other lipid fractions did not induce cytokine production. We described elsewhere that activation by schistosomal PS is mediated via TLR2 [9]. To determine whether the schistosomal GL preparation also affects cells of the innate immune system via TLRs, we stimulated HEK cell lines that were stably transfected with TLR2 or TLR4 (stimulations were performed in the presence of CD14 and MD-2–transfected cells. Production of interleukin (IL)–8 was measured in supernatants after 20 h, by use of a commercial kit (CLB), in accordance with the manufacturer’s recommendations.

**Parasitologic examination.** Infection with *S. haematobium* was determined by passing 10 mL of urine through a filter with a 10-μm pore size and staining the eggs with a solution of ninhydrin. Infection with *Ascaris lumbricoides* and *Trichuris trichiura* was determined by use of the Kato Katz method [14].

**Stimulation and fluorescence-activated cell sorter (FACS) analysis of PBMCs.** Freshly isolated PBMCs were cultured in 96-well flat-bottom plates (Nunc) at 10^6 cells/well in 200 μL of RPMI 1640 culture medium, as described elsewhere [8]. The cells were stimulated with 10 μg/mL lipids from the various fractions that were dissolved in 0.1% DMSO by water-bath sonication. Cells were incubated at 37°C in a 5% CO2 atmosphere, and supernatants were collected 20 h after stimulation. Cytokine levels were determined in the supernatants by ELISA, by use of commercial kits (CLB), in accordance with the manufacturer’s recommendations. For analysis, cytokine production in unstimulated wells was subtracted from cytokine production in response to the stimulus tested.

For FACS analysis and stimulation in the presence of 10 μg/mL TLR2-blocking antibody (TL2.1; a gift from D.T. Golenbock, University of Massachusetts Medical School, Worcester) or an IgG2a control antibody (CLB), cells were cryopreserved in liquid nitrogen and transported to Leiden. After thawing, the cells were stimulated and cultured, as described above, or were stained with CD14-fluorescein isothiocyanate (FITC) (BD Biosciences) and CD3-phosphatidylethanolamine (PE) (BD Biosciences) or CD14-FITC and CD19-PE (BD Biosciences). Staining was analyzed by use of a FACscan (Becton Dickinson). For FACS analysis, PBMCs from 10 schistosome-infected children and PBMCs from 10 schistosome-uninfected children were used.

**Statistical analysis.** The distribution between the 2 study groups, concerning age and the prevalence of infection with intestinal helminths, was tested by use of Fisher’s exact test. Cytokine levels and infection intensities (calculated in infected children) were not normally distributed, and differences between groups were tested by use of the Mann-Whitney U test. Outcomes of statistical tests were considered to be significant when *P* < .05.

**RESULTS**

**Schistosomal lipids activate the innate immune system.** To determine which classes of lipids can activate the innate immune system, we isolated lipids from *S. mansoni* eggs and adult worms; separated these lipids into different fractions (fractions 1–8); used them to stimulate PBMCs from healthy, unexposed Dutch donors; and measured production of IL-6, tumor necrosis factor (TNF)–α, IL-10, and IL-8 in 20-h supernatants (figure 1). As we reported elsewhere [8, 9], the schistosomal PS–containing fraction 6 of both eggs and worms and the GL-containing fraction 3 of eggs can activate cells of the innate immune system. The other lipid fractions did not induce cytokine production.

We described elsewhere that activation by schistosomal PS is mediated via TLR2 [9]. To determine whether the schistosomal GL preparation also affects cells of the innate immune system via TLRs, we stimulated HEK cell lines that were stably transfected with TLR2 or TLR4 (stimulations were performed in the presence of CD14 and MD2), and we monitored TLR activation by measuring production of IL-8 in supernatants.

In agreement with our previous findings, the fraction containing schistosomal PS of both eggs and adult worms and heat-killed *L. monocytogenes* (HKLM) activated TLR2-transfected cells, whereas LPS activated TLR4-transfected cells (figure 2). Stimulation with PS that was isolated from *S. haematobium* resulted in the same TLR activation pattern (data not shown). In contrast, the schistosomal GL fraction did not activate any of these stable transfectants. HEK cells transiently transfected with TLR1, TLR3, TLR5, TLR6, TLR7, and TLR9 were also tested and did not respond to either schistosomal PS or schistosomal GLs (data not shown). Thus, these schistosomal GLs did not stimulate any of the TLRs tested so far, indicating that activation of the innate immune system by this lipid fraction is probably mediated via other pattern-recognition receptors.

**Cytokine responses to schistosomal PS and schistosomal GL in Gabonese schoolchildren.** To study the effect of continuous systemic exposure to pathogens, we compared cytokine responses in schistosome-infected children with those in schis-

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Figure 1. Cytokine production induced by schistosomal lipids. Lipids isolated from schistosomal eggs (A) and adult worms (B) were fractionated into 8 lipid fractions, as described in Subjects, Materials, and Methods, and these fractions were used to stimulate peripheral blood mononuclear cells from 6 healthy, unexposed individuals. Cytokines were measured in 20-h supernatants.
Figure 2. Toll-like receptor (TLR)–2 activated by schistosomal phosphatidylserine (PS). Human embryonic kidney cells transfected with human TLR2 or human TLR4 were stimulated with bacterial lipopolysaccharide (LPS) (1 ng/mL), heat-killed Listeria monocytogenes (HKLM) (10⁶ cells/mL), schistosomal egg PS fraction (10 μg/mL), schistosomal adult worm PS fraction (10 μg/mL), schistosomal egg glycolipid (GL) fraction (10 μg/mL), and schistosomal adult worm GL fraction (10 μg/mL). Production of interleukin (IL)-8 was measured in 20-h supernatants. Results are mean ± SD of 3 independent experiments.

tosome-uninfected children. PBMCs were isolated, and the PBMCs were stimulated with schistosomal PS of eggs and worms, schistosomal GLs of eggs and worms, and Escherichia coli LPS. Although data for PS of worms is shown in figure 3, we found similar responses when PS of eggs was analyzed (results not shown). Production of IL-10, IL-6, TNF-α, and IL-8 was measured in 20-h culture supernatants by use of ELISA. We found that responses to TLR ligands differed between infected and uninfected children. The IL-8 response to schistosomal PS and LPS was significantly lower in schistosome-infected children than in schistosome-uninfected children (figure 3). For IL-10 and TNF-α, the same trend was observed, but it only reached statistical significance for TNF-α in the case of schistosomal PS and for IL-10 in the case of LPS stimulation (figure 3). For IL-6, no differences were found (figure 3).

Responses to GLs showed different profiles. Although PBMCs isolated from unexposed Dutch donors did not respond to worm GLs, very low levels of responses were seen in uninfected Gabonese children, which is in agreement with data shown in figure 1. However, in infected children, worm GLs stimulated significant production of IL-8 and IL-6 (figure 3). Egg GLs induced responses in PBMCs from both infected and uninfected children (data not shown), again confirming results seen in figure 1, in which egg GLs stimulated PBMCs from unexposed Dutch donors. The levels of cytokines produced in response to egg GLs were comparable to those produced in response to worm GLs. When we corrected for differences in age and sex by regression analysis, all the differences mentioned above were still significant (data not shown).

Percentages of monocytes, B cells, and T cells in PBMCs. To determine whether differences in cytokine responses could be attributed to differences in numbers of monocytes present, the percentages of cells positive for CD14, CD19, and CD3 were monitored in PBMCs. No differences in the percentage of monocytes (CD14⁺) or B cells (CD19⁺) were found between PBMCs from schistosome-infected children and those from schistosome-uninfected children (P = .3510 and P = .4698, respectively). Only the percentage of T cells (CD3⁺) tended to be higher in uninfected children (P = .0712), but this percentage did not correlate with cytokine production induced by any of the stimuli. Thus, the differences in cytokine responses between schistosome-infected children and schistosome-uninfected children cannot be explained by differences in the percentages of monocytes, B cells, or T cells in PBMCs.

Cytokine production in response to PS, in PBMCs from schistosome-infected children, can be blocked with a TLR2 antibody. To confirm that, after stimulation with the schistosomal PS fraction, cytokine production in PBMCs from the Gabonese children was indeed induced by lyso-PS [9] via activation of TLR2, we stimulated PBMCs from 3 children with schistosomal PS or LPS in the presence or absence of a TLR2-blocking antibody. In presence of the TLR2-blocking antibody, the cytokine responses induced by schistosomal PS were decreased significantly (P = .012 for IL-10, P = .005 for IL-8,
and $P = .033$ for IL-6), whereas responses to LPS were not affected (figure 4).

**DISCUSSION**

We have shown here that, in children with chronic schistosomiasis, responses to schistosome-derived lyso-PS, a TLR2 ligand, and *E. coli* LPS, a TLR4 ligand, are lower than those in children without *S. haematobium* infection. For bacterial PAMPs, it has been shown that, on repeated stimulation, the cells of the innate immune system can become unresponsive to the same ligand, a phenomenon termed “tolerance.” Tolerance has been described for several TLR ligands. Most studies of tolerance involve LPS, but tolerance has also been described for the TLR2 ligands lipoarabinomannan [15], lipoteichoic acid (LTA) [16], and mycoplasmal macrophage–activating lipopeptide (MALP-2) [17]. Certain ligands can also induce tolerance to a heterologous ligand. This cross-tolerance has been described for LPS and LTA [16], as well as for LPS and MALP-2 [17], in which tolerance is induced both ways. The phenomenon of tolerance might also explain our present observations. The lower responsiveness of cells from infected children could be due to the fact that their innate immune system is continuously challenged by a pathogen that releases foreign antigens systemically (*S. haematobium* worms live in the plexus veins around the bladder), resulting in a state of tolerance to certain molecules derived from these pathogens, such as those that stimulate TLR2. This tolerance could also induce cross-tolerance to a bacterial ligand, LPS. It is also possible that children infected with schistosomes have higher exposure to bacterial pathogens, explaining the observed tolerance to LPS. Interestingly, positive associations between *Schistosoma* and *Salmonella* species have been described before, with the possibility of the existence of a symbiotic relationship between them [18].

However, it is also possible that exposure to other microbes that stimulate TLRs is higher in the areas where schistosome-
Figure 4. Cytokine production in Gabonese schoolchildren induced by the schistosomal phosphatidylserine (PS) fraction via Toll-like receptor (TLR)-2. Peripheral blood mononuclear cells of 3 Gabonese schoolchildren were stimulated with bacterial lipopolysaccharide (LPS) (100 ng/mL) or schistosomal adult worm PS fraction (10 μg/mL), in the presence of a TLR2-blocking antibody or an isotype control antibody. Cytokine production was measured in 20-h supernatants. Cytokine production in the presence of the isotype control antibody was set at 100%. Results are mean ± SD.

infected children were identified. The exposure to either Salmonella species or other microbes could indeed explain the lower responses to LPS in schistosome-infected children. A recent immunoepidemiological study of individuals exposed to varying levels of endotoxin indicated that, with increasing exposure, innate immune responses to LPS were down-regulated profoundly [19].

The observation that cytokine production to the schistosomal adult worm GL fraction was induced in schistosome-infected children but not in schistosome-uninfected children, either from Gabon or from The Netherlands, indicates that responses to schistosomal GLs expand only in individuals who are infected. The enhanced responses may be explained by either the up-regulated expression of the receptors that mediate recognition of schistosomal adult worm GLs in infected children or the expansion of the number of cells that respond to schistosomal worm GLs after infection. The latter can be envisaged if worm GLs are recognized by T cells in a CD1-restricted manner [20]. Although we have not yet identified the receptor for schistosomal GLs, experiments described here show that schistosomal GLs do not activate TLRs. Several C-type lectins have been described to recognize and internalize pathogen-derived carbohydrate-containing molecules [21–23]. Whether this class of pattern-recognition receptors or the well-described CD1 molecules are involved in recognition of schistosomal GLs remains to be determined.

The mechanism for unresponsiveness to PAMPs is not yet entirely clear. Although tolerance to TLR-activating molecules has been suggested to be due to down-regulation of TLR surface expression [24], the overexpression of TLRs does not prevent induction of tolerance to TLR ligands [15]. Indeed, there are reports suggesting that continuous exposure to PAMPs leads to up-regulation of receptors that recognize such molecules [25]. Irrespective of the levels of receptors, there is accumulating evidence for modulation of downstream signalling events that render a cell unresponsive to a subsequent stimulation with the same or a cross-tolerizing ligand [26–29]. Future studies should resolve the mechanisms that are responsible for the tolerance in the innate immune system that has been observed in the schistosome-infected schoolchildren described in the present study. Considering that both groups of children (i.e., schistosome-infected and uninfected) had the same levels of intestinal helminth infections, the clear differences seen in stimulation of TLRs indicates that blood-dwelling helminths (S. haematobium) may have a more profound effect on modulating the innate immune responses in the peripheral blood than do intestinal helminths.

In summary, the present study has shown that chronic infection with S. haematobium, which is present in the blood and continuously stimulates the immune system, leads to down-regulated responses to TLR ligands, while leaving responses to other schistosomal PAMPs that do not stimulate the immune system via TLRs unaffected. These results indicate that not only T cell responses [30], but also the innate immune system, can be modulated during chronic helminth infections.

Acknowledgments

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References

1. Wynn TA, Cheever AW, Williams ME, et al. IL-10 regulates liver pathology in acute murine Schistosomiasis mansoni but is not required