MAJOR ARTICLE

Schistosomal-Derived Lysophosphatidylcholine Are Involved in Eosinophil Activation and Recruitment through Toll-Like Receptor–2–Dependent Mechanisms

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Parasite-derived lipids may play important roles in host-pathogen interactions and escape mechanisms. Herein, we evaluated the role of schistosomal-derived lipids in Toll-like receptor (TLR)–2 and eosinophil activation in *Schistosoma mansoni* infection. Mice lacking TLR2 exhibited reduced liver eosinophilic granuloma, compared with that of wild-type animals, following *S. mansoni* infection. Decreased eosinophil accumulation and eosinophil lipid body (lipid droplet) formation, at least partially due to reduced production of eotaxin, interleukin (IL)-5, and IL-13 in *S. mansoni*–infected *TLR2*$^{-/-}$ mice, compared with the corresponding production in wild-type mice, was noted. Although no differences were observed in survival rates during the acute schistosomal infection (up to 50 days), increased survival of *TLR2*$^{-/-}$ mice, compared with survival of wild-type mice, was observed during the chronic phase of infection. Schistosomal lipid extract– and schistosomal-derived lysophosphatidylcholine (lyso-PC)–stimulated macrophages in vitro induced TLR2-dependent NF-kB activation and cytokine production. Furthermore, in vivo schistosomal lyso-PC administration induced eosinophil recruitment and cytokine production, in a mechanism largely dependent on TLR2. Taken together, our results suggest that schistosomal-derived lyso-PC may participate in cytokine production and eosinophil activation through a TLR2-dependent pathway in *S. mansoni* infection. Moreover, our results suggest that TLR2-dependent inflammatory reaction, cytokine production, and eosinophil recruitment and activation may contribute to the pathogenesis and lethality in the chronic phase of infection.

Eosinophils are pleiotropic multifunctional leukocytes involved in initiation and propagation of allergic and infectious inflammatory responses and modulators of innate and adaptive immunity [1, 2]. *Schistosoma mansoni* infection exhibits interleukin (IL)–5–dependent increased eosinophilopoiesis and important occurrence of eosinophils in granuloma [3–5]. Accordingly, reduced liver granuloma sizes with decreased numbers of eosinophils were observed in IL-5–deficient mice with *S. mansoni* infection [6]. Although a great number of animal and human studies have been performed over the years, the functions of eosinophils in host protection and in pathogenesis of schistosome infection is still incompletely understood [7, 8]. It has been suggested that, beyond the increase in eosinophils number, there is also an enhancement of the activity of these cells that may participate decisively during *S. mansoni* infection [9]. In fact, eosinophils have been found to kill the *S. mansoni* parasite in vitro [10] and to invade dying and sick schistosomes in vivo [5], suggesting that they might be an important defense mechanism against

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the parasite. However, 2 mouse lineages deficient in eosinophils were recently studied on *S. mansoni* infection and found to have no gross alterations in worm burden, granuloma formation, or liver fibrosis [11]. Although the role of eosinophils as a defense mechanism against *S. mansoni* infection remains to be elucidated, their increased bone marrow differentiation and tissue infiltration during Th2 responses as in schistosomal infection are commonly observed, and eosinophils may play a role in maintaining the Th2 response to infection via cytokine secretion [12–14] and the cytokine-mediated pathogenesis [15–17].

Mechanisms of early recognition in host immune response may participate in driving the course of schistosomiasis pathophysiology. Toll-like receptors (TLRs), capable of identifying pathogen-associated molecular patterns during host innate immune response, have been implicated to schistosomiasis pathology through the recognition of different schistosomal molecules, including soluble egg antigen (SEA), lipids, and double-stranded RNA [18–22]. However, the contribution of TLR recognition and activation to the immunopathology of *S. mansoni*–driven eosinophilic infection is not fully understood. *S. mansoni* has evolved mechanisms to evade host immune effectors. The schistosomes tegument is enriched in lipids. Its tegumental outer-surface structure is crucially involved in complex host-parasite interactions. Studies on the turnover of the phospholipids demonstrated that tegumental lipids have a shorter half-life than those in the worm body, and that lysophospholipids are excreted as degradation products into the environment [23, 24].

Here we investigated the role of TLR2 in cytokine production, eosinophil recruitment, and granuloma formation during *S. mansoni* infection. Because the activation of the innate immune system by *S. mansoni* parasites is likely triggered by its signature molecules, we have focused here on the role of schistosome lipids in interaction with the innate immune system and on the eosinophilic inflammatory response.

MATERIALS AND METHODS

Animals. C57BL/6 mice, C3HeN mice, and C3H/HeJ (nonfunctional TLR4) mice were obtained from Fundação Oswaldo Cruz animal facility. TLR2 and TLR6 genetically deficient mice in a homogeneous C57BL/6 background [25] were kindly donated by Dr Shizuo Akira (Osaka University, Japan). Animals weighing 20–25g from both sexes were used. Protocols were approved by the Fundação Oswaldo Cruz animal welfare committee.

Purification and analysis of schistosomal lipids. Lipid extracts from both *S. mansoni* eggs and adult worms were obtained as described elsewhere [26]. Briefly, lipids were extracted for 2 hours with a chloroform-methanol-water solution (2:1:0.8, v/v). After centrifugation, the supernatant was collected and the pellet was subjected to a second lipid extraction for 1 hour. Extracted lipids were subjected to 2-dimensional thin-layer chromatography for phospholipid fractionation and analysis, and lysophospholipid extraction was performed essentially as described by Golodne et al [27]. Lipid fractions were tested by limulus amoebocyte lysate (LAL; Bio Whittaker) for the presence of endotoxin.

Schistosomal lysophosphatidylcholine (lyso-PC) was analyzed by mass spectrometry. Spectra were obtained in a ThermoFinnigan LCQ DECA XPplus mass spectrometer (Finnigan; ThermoQuest). Samples were introduced into the electrospray source through a silica capillary at a flow rate of 5 μL/min. Spectra were acquired at 3 s/scan over a mass range of m/z 130–1500. Collision-induced (electrospray ionization tandem mass spectrometry) fragmentation of parent ions was carried at relative collisional energy from 25–35 V. The same analysis was done for the standard phospholipids and lysophospholipids (Sigma Chemical).

For digestion with phospholipase A_2 and C, 100 μg of the schistosomal adult worm lyso-PC fraction was incubated in 2 mL of diethyl ether and 1 mL of Tris-HCl buffer (pH, 7.2) supplemented with 5 mM CaCl2 and 1.3 units/mL phospholipase A_2 and C (Sigma Chemical) during 3 h while shaking. As a control, schistosomal lyso-PC were incubated in the same buffer in the absence of phospholipases.

S. mansoni *infection in vivo.* Wild-type and *TLR2*$^{-/-}$ mice were inoculated intraperitoneally with either 70 cercaria from *S. mansoni* or saline. The intraperitoneal model of *S. mansoni* infection was chosen to ensure that the actual number of cercaria used to infect mice was the same in both experimental mouse strains while maintaining infectivity similar to that of the percutaneous method [28]. Infected and noninfected mice were euthanized at different days of infection. Wild-type and *TLR2*$^{-/-}$ mice were also inoculated intraperitoneally with schistosomal lyso-PC (10 μg/cavity), and mice were euthanized after 24 h. Liver samples were collected with 95 days of infection, fixed in Formalin-Millonig, and embedded in paraffin for histopathological analysis. Sections (5 μm) were stained with hematoxilin and eosin. Granulomata were measured, and the eosinophil number present in granulomata was evaluated.

Stimulation of peritoneal macrophages in vitro. Peritoneal macrophages from naive C57BL/6, *TLR2*$^{-/-}$, C3H/HeJ, C3HeN, and *TLR6*$^{-/-}$ mice were harvested by lavage with sterile Roswell Park Memorial Institute (RPMI) 1640 medium. Macrophages (1×10^6 cells/mL) adhered overnight with RPMI containing 2% fetal calf serum. Nonadherent cells were removed after phosphate-buffered saline (PBS) wash. Macrophages were stimulated by schistosomal lipids extract (0.01–10 μg/mL), lipopolysaccharide (LPS) (500 ng/mL), or lipoarabinomannan (LAM; 300 ng/mL) for 24 h at 37°C in CO_2 atmosphere. In inhibitory studies, macrophages were treated with pertussis toxin (100 ng/mL) for 30 min and then stimulated by schistosomal

lipids (0.01–10 μg/mL) for 24 h. Viability assessed by trypan blue exclusion at the end of each experiment was always ⩾95%.

Cytokine measurements. Cell-free peritoneal fluid or supernatants from in vitro stimulated macrophages were collected after 24 h and stored at −20°C until the day of analysis. IL-5, IL-13, IL-4, and eotaxin were measured in the peritoneal fluid, and tumor necrosis factor (TNF) was measured in supernatants from in vitro stimulated macrophages by enzyme-linked immunosorbent assay, in accordance with manufacturer instructions (R & D Systems).

Leukocyte and lipid body counts. Total leukocytes (diluted with Turk fluid) were counted using a Neubauer chamber. Differential counts were performed in cytopins stained by the May-Grunwald-Giemsa method. Lipid bodies were enumerated by microscopy in 50 consecutively scanned cells, in cytospin slides fixed in 3.7% formaldehyde and stained and contrasted with osmium.

NF-κB immunolocalization. The slides containing peritoneal macrophages were fixed in 3.0% formaldehyde at room temperature for 10 min, permeabilized with 0.2% Triton, and then blocked with 1% normal donkey serum. After washing, slides were incubated overnight at 4°C with anti–NF-κBp65 polyclonal antibodies diluted in 1% normal donkey serum/PBS solution. Nonimmune rabbit serum was used as control. After 3 washes of 5 min, slides were incubated for 1 h at room temperature with the secondary Alexa 546-labeled goat anti-rabbit immunoglobulin G antibody. DAPI (Sigma; 5 min) was used for nuclei counterstaining. Immunostained samples were analyzed with a confocal microscope (LSM 510; Zeiss).

Statistical analysis. Survival curves were generated with Prism computer software (GraphPad), and comparisons between curves were made using the Mantel-Cox log-rank test. All other data were expressed as mean (± standard error of the means) and were analyzed statistically with analysis of variance (ANOVA) followed by the Neuman-Keuls-Student test, with the level of significance set at $P < .05$.

RESULTS

S. mansoni–*induced eosinophil recruitment and activation occur in a TLR2-dependent manner.* To characterize the role of TLR2 in the response to *S. mansoni* infection, we infected wild-type and *TLR2*$^{-/-}$ mice with *S. mansoni* cercaria. The histopathological analysis of liver revealed a decrease of granuloma size in *TLR2*$^{-/-}$ mice, compared with granuloma size in wild-type mice, after 95 days of infection (Figure 1*A, B*). The granuloma size reduction was mostly due to a significant reduction of the number of eosinophils infiltrating the granulomata of *TLR2*$^{-/-}$ mice (Figure 1*A, C*). In fact, a significant decrease of eosinophil recruitment was observed in infected *TLR2*$^{-/-}$ mice, compared with that of wild-type mice (Figure 2*A*). To evaluate the roles of TLR2 in eosinophil activation, lipid bodies were quantified. Lipid body formation in leukocytes is a highly regulated event that depends on the interaction of cellular receptors with their ligands, and lipid bodies are considered markers of leukocyte activation [29]. Eosinophils were activated in a TLR2-dependent manner during *S. mansoni* infection, as observed by an increase of lipid bodies in eosinophils from wild-type but not from *TLR2*$^{-/-}$ mice (Figure 2*B, C*).

In vivo *S. mansoni* infection caused a dramatic increase of cytokines and chemokines involved in eosinophil recruitment and activation, including IL-5, IL-13, and eotaxin. Significantly decreased eotaxin production was observed in *TLR2*$^{-/-}$ infected mice, compared with the corresponding production in wild-type mice, as early as 5 days after infection (Figure 3*A*). Significantly decreased production of IL-5 and IL-13 in *TLR2*$^{-/-}$ infected mice, compared with that of wild-type mice, was noted only after 70 days of infection (Figure 3*B, C*). Although a similar pattern of decreased IL-4 production was observed in *TLR2*$^{-/-}$ infected mice, compared with IL-4 production in wild-type mice, it did not reached statistical significance, and future studies will be necessary to better characterize the role of TLR2 in IL-4 production during *S. mansoni* infection (Figure 3*D*).

No differences were observed in survival rates of infected *TLR2*$^{-/-}$ mice, compared with survival rates of wild-type mice, during the acute period of schistosomal infection, whereas increased survival of infected *TLR2*$^{-/-}$ mice, compared with survival of wild-type mice, was observed during the chronic phase of infection (Figure 4). Together, these results indicate that TLR2 regulates cytokine production and eosinophil recruitment and activation, contributing to the pathogenesis of *S. mansoni* infection.

Schistosomal lipid extract–induced TLR2-dependent cytokine production and NF-κB activation. To evaluate whether schistosomal lipids are recognized by TLRs, wild-type, *TLR2*$^{-/-}$, C3h/HeJ, and *TLR6*$^{-/-}$ peritoneal macrophages were stimulated with schistosomal lipid extract from eggs and adult worms from *S. mansoni*. Lipids from eggs and adult worms were capable of inducing TLR2-dependent TNF production in vitro, as demonstrated by the impairment of TNF production in *TLR2*$^{-/-}$ mice (Figure 5*A*). In contrast, macrophages from C3H/HeJ (TLR4-deficient) and *TLR6*$^{-/-}$ mice stimulated by schistosomal lipid extract had TNF production comparable with that of wild-type controls (Figure 5*B, C*). Schistosomal lipid extract also induced TLR2-dependent translocation of NF-κB toward the nucleus after 18 h (Figure 5*D*). As expected, *TLR2*$^{-/-}$ mice retained the capacity to induce TNF production in response to LPS. TLR2-dependent LAM-induced TNF production was used as a positive control. These results demonstrate a requisite role for TLR2 in schistosomal lipid extract recognition and signaling to induce TNF production.

Functional and structural characterization of schistosomal lyso-PC. Schistosomal lyso-PC–purified fractions induced a

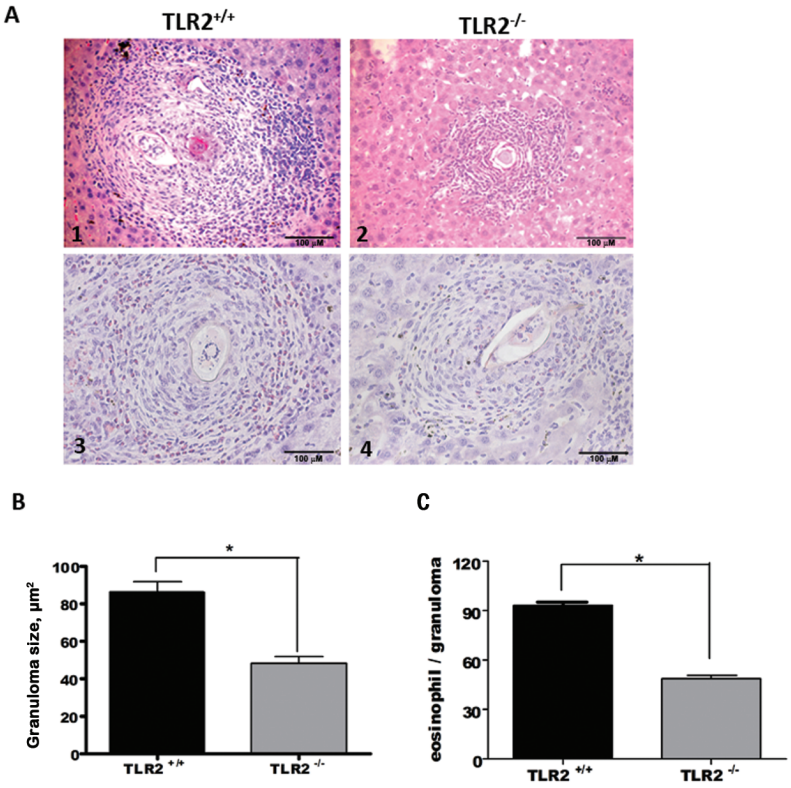

Figure 1. Size and eosinophil composition of liver granulomata developed on *Schistosoma mansoni* infection are dependent on Toll-like receptor (TLR)–2. Slide sections from infected mice were examined under bright microscope, and images were captured and analyzed. *A*, Representative granuloma in liver sections from wild-type (*TLR2*$^{+/+}$) and *TLR2*$^{-/-}$ mice (hematoxylin-eosin [*A1* and *A2*] and Sirius Red [*pH* = 10.2] [*A3* and *A4*]). Original magnification, × 400. Individual granuloma area (*B*) and eosinophils per granuloma (*C*) were calculated from images of 10–20 granuloma acquired from ⩾5 mice per group. Statistical differences between *TLR2*$^{+/+}$ and *TLR2*$^{-/-}$ groups are represented by *asterisks*. *$P \leqslant .05$.

dose-dependent TNF production by macrophages (Figure 6*A*). This effect was comparable with that induced by schistosomal lipid extracts from adult worm and required a functional TLR2 (Figure 6*B*), suggesting that schistosomal lyso-PC are major TLR2-activating molecules within schistosomal lipid extract.

To confirm purity of schistosomal lyso-PC, this lipid fraction was analyzed by mass spectrometry. The analysis of the mass spectra on both positive and negative modes (data not shown) confirmed the purity of the lyso-PC fraction. Schistosomal lyso-PC were assigned as containing majorly the fatty acids palmitic acid (m/z 518.3 = 16:0-LysoPC $[M + Na]^+$; m/z 496.3 = 16:0-LysoPC $[M + H]^+$) or stearic acid (m/z 546.3 = 18:0-LysoPC $[M + Na]^+$; m/z 524.3 = 18:0-LysoPC $[M + H]^+$) (Figure 5*E*). The schistosomal lyso-PC preparations were also found to be devoid of endotoxin contamination by means of a kinetic chromogenic LAL test (not shown).

To further define lyso-PC as the active component, lyso-PC were treated with either phospholipase C or phospholipase A_2. Digestion of lyso-PC with phospholipase C, which cleaves onto the monoacylglycerol and phosphocholine head group, caused a significant reduction of TNF-α secretion (Figure 6*C*). The lyso-PC activity was not affected by treatment with phospholipase A_2, which cleaves onto fatty acid on the position sn-2 in phospholipid and therefore is not effective on lyso-PC (Figure 6*C*). The platelet-activating factor (PAF) was used as a control of an active phospholipid that is affected by both phospholipase C and phospholipase A_2, showing the effectiveness of enzyme treatments.

Previous studies demonstrated that lyso-PC bind and activate G protein–coupled receptor (GPCR) [30]. To test the involvement of GPCR on lyso-PC signaling, peritoneal macrophages from both wild-type and *TLR2*$^{-/-}$ mice were treated with the Gαi inhibitor, pertussis toxin (100 ng/mL), for 30 min before treatment with lyso-PC. Treatment with pertussis toxin did not affect the TNF-α production induced by lyso-PC (Figure 6*D*). These results indicate that the immunomodulatory activity of schistosomal lyso-PC is not mediated by pertussis toxin-sensitive Gαi.

Schistosomal lyso-PC induce cytokine production in vivo. To investigate the properties of schistosomal lyso-PC in triggering immune response in vivo, schistosomal lyso-PC were intraperitoneally administered to wild-type and *TLR2*$^{-/-}$ mice.

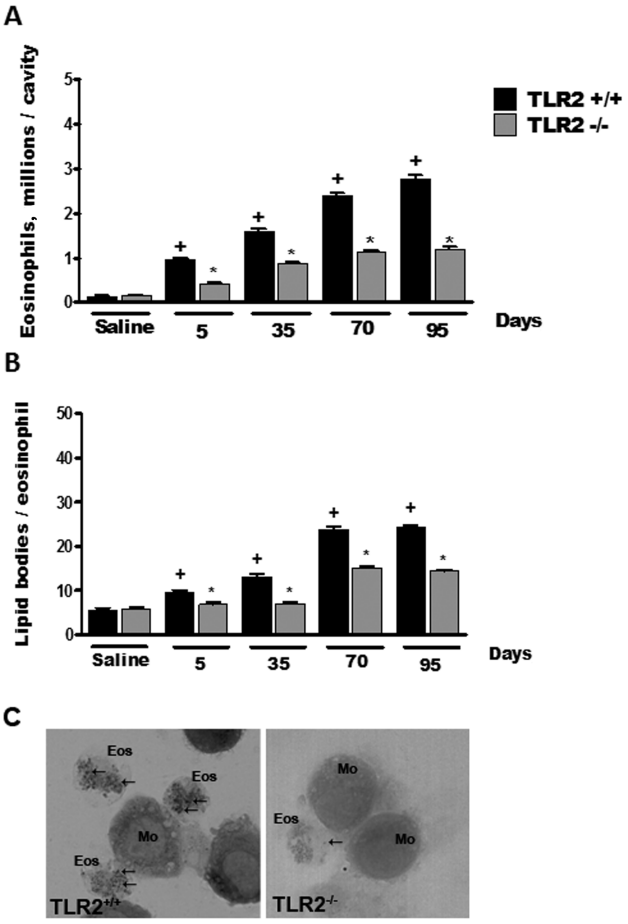

Figure 2. Eosinophil activation and recruitment is dependent on Toll-like receptor (TLR)–2 during *Schistosoma mansoni* infection in vivo. Time course analysis of peritoneal eosinophil accumulation (*A*) and eosinophil lipid body formation (*B*) after *S. mansoni* infection. Each bar represents means (± standard error of the means) from 6–8 animals; + represents statistical differences between control and infected groups. Statistical differences between $TLR2^{+/+}$ and $TLR2^{-/-}$ groups are represented by *asterisks*. $*P \leq .05$. *C*, Representative images of lipid body osmium staining in peritoneal leukocytes after 35 days of *S. mansoni* infection, as observed by light microscopy. Original magnification, × 100. Eos, eosinophils.

Lyso-PC administration significantly triggered eotaxin, IL-5, and IL-13 production within 24 h. As shown in Figure 7, eotaxin, IL-5, and IL-13 production induced by lyso-PC was largely dependent on functional TLR2, because their concentrations were greatly reduced in $TLR2^{-/-}$ infected mice, compared with the corresponding concentrations in wild-type mice (Figure 7*A*–*C*). Like cercaria, schistosomal lyso-PC were capable of inducing eosinophil recruitment and activation mediated by TLR2, as demonstrated by the decreased number of eosinophils recruited to the peritoneal cavity (Figure 7*D*) and the decreased number of lipid bodies in eosinophils (Figure 7*E*) in $TLR2^{-/-}$ mice, compared with those of wild-type mice.

DISCUSSION

In the present study, we showed that TLR2 contributes to the immunopathogenesis of *S. mansoni* infection by the regulation of cytokine production, including IL-5, IL-13, and eotaxin, and by controlling the eosinophil recruitment and activation. Moreover, our findings suggest that TLR2 signaling is not required to control *S. mansoni* infection at the acute phase but may contribute to pathogenesis and lethality at the chronic phase of infection.

The involvement of TLRs in *S. mansoni* infection is still highly controversial. Innate immune receptors, including TLR2, have important roles in the establishment of innate response to infection as well as provide signals to the establishment and shape of the adaptive immune response. Here we demonstrated that TLR2 participates in the mechanism of type 2 cytokine production, eosinophil infiltration, and granuloma formation during *S. mansoni* infection. Accordingly, it has been demonstrated that because of the lack of TLR2 stimulation, CD4+CD25+ T cells fail to expand during *S. mansoni* infection, and in turn this completely unbalances the immunopathology [19]. In vitro investigations have shown that when dendritic cells are matured with the schistosomal lyso-PS, they can drive naive T cells into a regulatory phenotype, and interestingly this process was shown to be dependent on TLR2 being present on the antigen-presenting cell [22]. In contrast, Kane et al [31] evaluated the role of TLR2, TLR4, and MyD88 in *S. mansoni* SEA–induced dendritic cell activation through the use of cells derived from gene-deleted animals, to conclude that TLRs are not involved in SEA-induced immunomodulatory responses. Of note, SEA is generated as the PBS-soluble egg extracts; as such, it is unlikely that SEA contains significant amounts of lipid molecules that have immunomodulatory functions through TLR2. The lack of antigen-specific Th1 response alters granuloma formation and composition in *S. mansoni*–infected $Myd88^{-/-}$ mice, whereas a diminished granuloma formation has occurred in the absence of MyD88 [32], indicating that other TLRs, such as TLR4 [33], may participate in the recognition of schistosomal antigens. Parasite-derived factors released by schistosome larvae act partly through a TLR4-dependent pathway to induce production of a limited repertoire of cytokines [34]. Besides, TLR3-deficient dendritic cells showed a reduced response to schistosome eggs, relative to the response of wild-type cells, indicating that dsRNA from a nonviral pathogen may act as an inducer of the innate immune system through TLR3 [20].

The TLR2-dependent mechanisms involved in activation and recruitment of eosinophils in the *S. mansoni* mouse model of infection appear to be regulated indirectly through the regulation of chemokine and cytokine production. Indeed, it has been demonstrated that the TLR4 ligand LPS and the TLR2-dependent mycobacterial activation induce eosinophil accumulation in vivo through mechanisms that are largely depen-

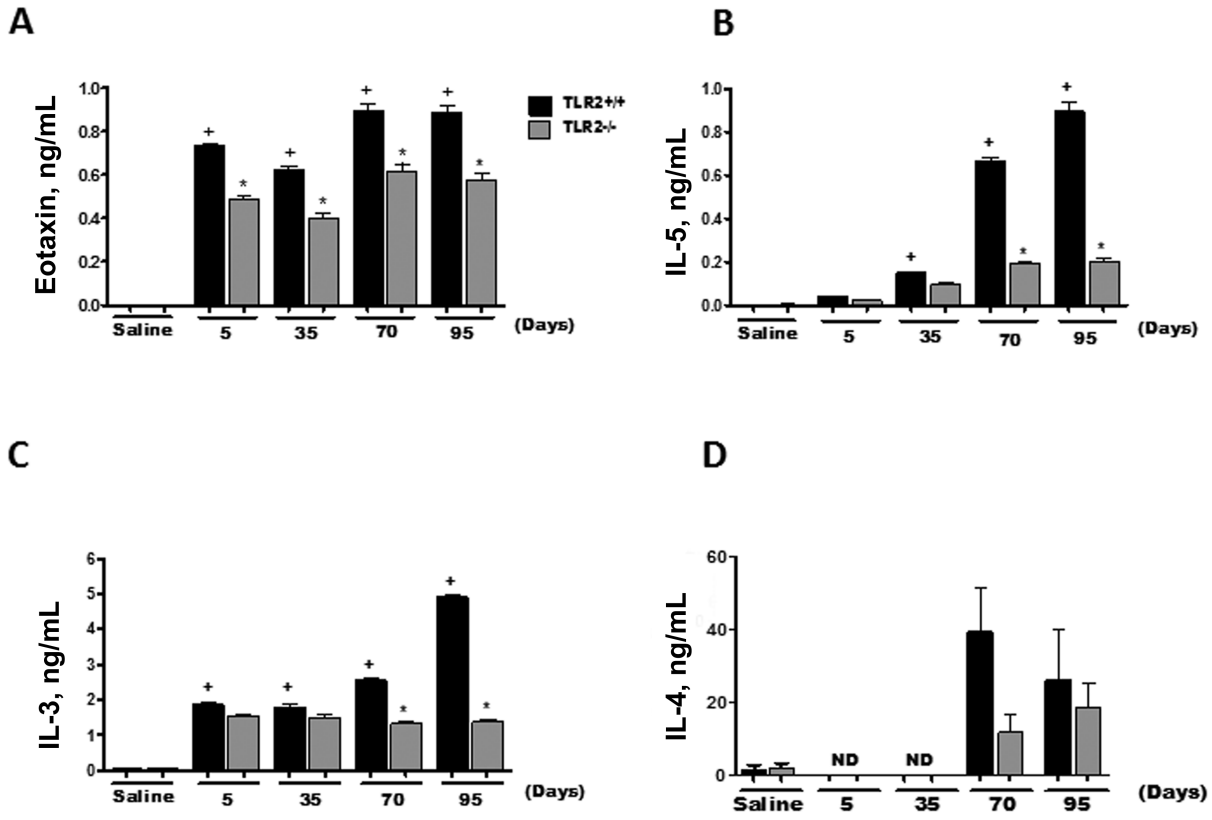

Figure 3. Toll-like receptor (TLR)–2–dependent Th2 cytokine production during *Schistosoma mansoni* infection in vivo. Cytokine production was analyzed in peritoneal fluid by enzyme-linked immunosorbent assay at different times after *S. mansoni* infection. Data represent means (± standard error of the means) from 6–8 animals; + represents statistical differences between control and infected groups. Statistical differences between $TLR2^{+/+}$ and $TLR2^{-/-}$ groups are represented by *asterisks*. $*P \leq .05$.

dent on monocyte/macrophage and lymphocyte-derived cytokine production [35–37]. The roles of IL-5 and IL-13 in eosinophil accumulation induced by *S. mansoni* infection have been broadly demonstrated [6, 38]. The involvement of TLR2 in IL-5 and IL-13 synthesis after *S. mansoni* infection was analyzed. $TLR2^{-/-}$ mice produced reduced amounts of IL-5 and IL-13, compared with the amount produced by wild-type mice, in different periods of infection. Likewise, eotaxin levels were decreased in infected $TLR2^{-/-}$ mice. Eotaxin is a key mediator in the eosinophil trafficking. In addition, eotaxin-mediated activation is a potent inducer of lipid body formation in eosinophils [39]. Our results suggest that the inhibition of eosinophil recruitment and activation observed in *S. mansoni* infected $TLR2^{-/-}$ mice is due to the important reduction in the production of eosinophil-activating cytokines. Whether schistosomal lipids acting through TLR2 have additional direct roles in eosinophil activation will need to be investigated.

Of note, although we observed a significant reduction in eosinophil recruitment and activation in $TLR2^{-/-}$ infected mice, we could not observe differences in parasite fecundity and worm burden during *S. mansoni* infection (not shown) or mouse survival rates during the initial phase of infection when $TLR2^{-/-}$ and wild-type mice were compared. Accordingly, it has been demonstrated that TLR2 is not required to control *S. mansoni* infection assessed by worm burden and egg deposition within 50 days after infection, although it was important to shape the

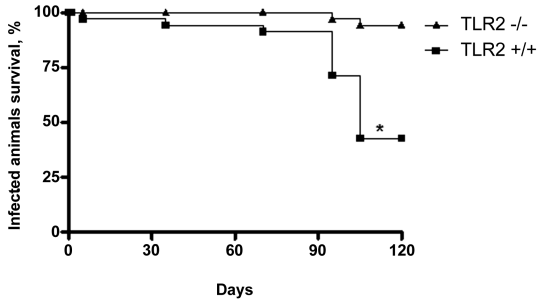

Figure 4. Toll-like receptor 2 ($TLR2$)$^{-/-}$ mice infected with *Schistosoma mansoni* have increased survival rates, compared with the survival rates of infected wild-type mice. Survival was analyzed up to 120 days after *S. mansoni* infection. $TLR2^{-/-}$ mice are represented by *triangles* and $TLR2^{+/+}$ mice by *squares*. The *asterisk* indicates that, according to the log-rank test, a statistically significant difference ($P \leq .05$) exists between these survival curves. Each group contained 40 mice at the beginning of the experiment.

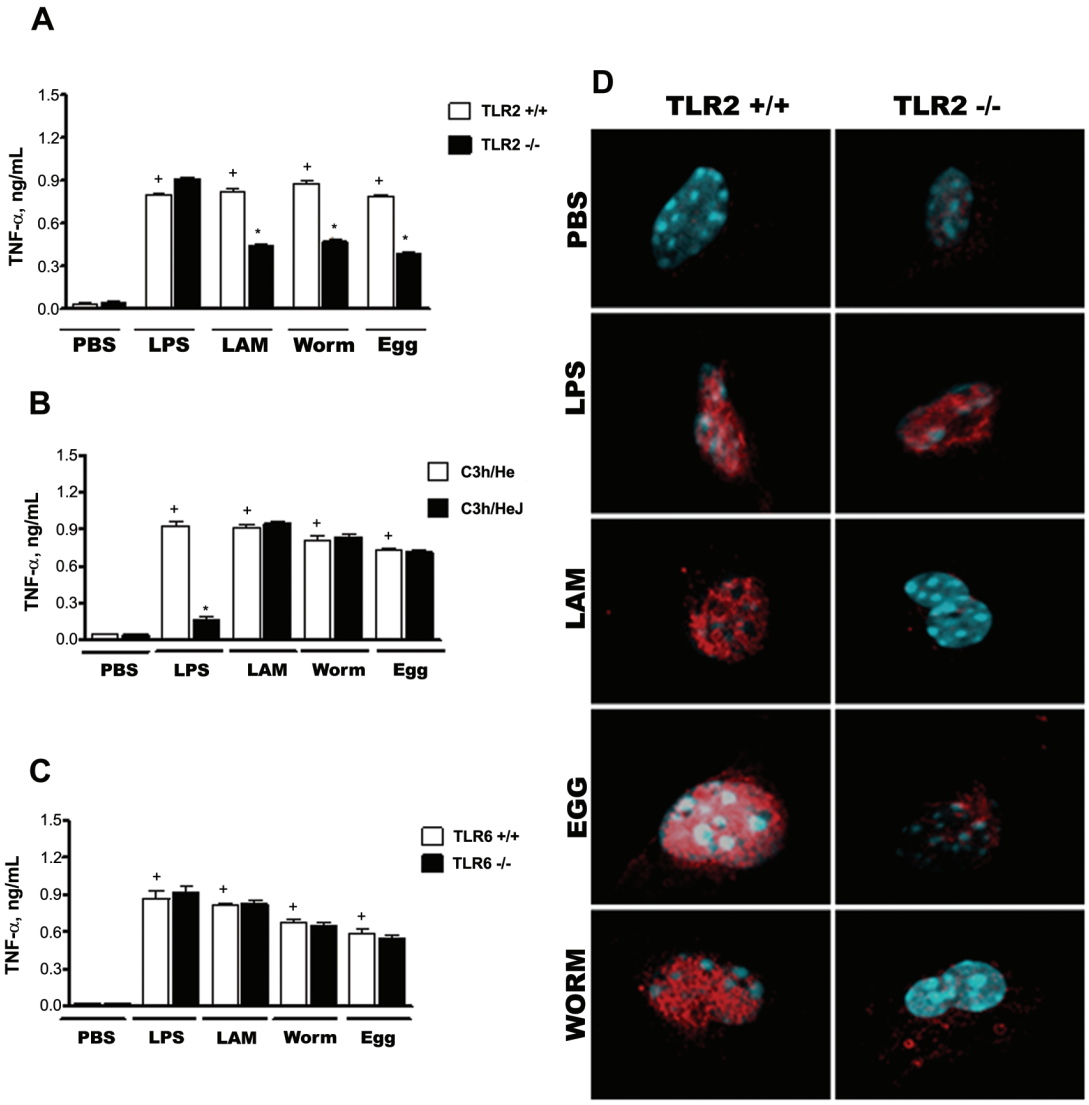

Figure 5. Schistosomal lipid extract–induced Toll-like receptor 2 (TLR2)–dependent cytokine production and NF-κB activation in vitro. Peritoneal macrophages from C57BL/6, *TLR2*$^{-/-}$, C3H/HeJ (*TLR4* deficient), C3HeN, and *TLR6*$^{-/-}$ mice were obtained and stimulated with schistosomal lipids extract from eggs and adult worms from *Schistosoma mansoni*. Tumor necrosis factor α (TNFα) production was analyzed within 24 h (*A–C*). Data represent means (± standard error of the means); + represents statistical differences between control and infected groups; $n = 3$. Statistical differences between *TLR2*$^{+/+}$ and *TLR2*$^{-/-}$ groups are represented by *asterisks*. *$P \leq .05$. *D*, NF-κB (*red*) translocation into the nucleus (*light blue*) induced by schistosomal lipid extract from eggs and adult worm (10 μg/mL), lipopolysaccharide (LPS) (500 ng/mL), and lipoarabinomannan (LAM) (300 ng/mL) were evaluated by confocal laser microscopy analysis after 18 h. PBS, phosphate-buffered saline.

immune response and cytokine production in response to infection [20]. Moreover, *S. mansoni* infection in eosinophil lineage–ablated dblGATA and TgPHIL mice demonstrated that eosinophils have no impact on worm burden or egg deposition [11]. Similarly, we recently observed that *Mif* deficient mice infected with *S. mansoni* have profound reduction of eosinophils in the blood and in the granulomata but no reduction in worm burden and egg deposition, compared with wild-type animals [40]. Interestingly, we observed a reduction in the granuloma size along with an increased survival rate in *TLR2*$^{-/-}$ mice, compared with the granuloma size and survival rate of wild-type mice, in the chronic phase of infection. The reduction in the granuloma size observed in the present study may involve cytokine-mediated effects that go beyond the effect on eosinophil recruitment. Our data show that *TLR2*$^{-/-}$ animals have decreased concentrations of IL-4, IL-5, and IL-13. Accordingly, decreased granuloma sizes have been observed in the *IL-4Rα* [41, 42], *Stat 6* [43], and *IL-5* [6] gene-deleted animals, suggesting that the TLR2-dependent cytokine production and eosinophil recruitment may participate in the tissue remodeling and damage and may contribute to the pathophysiology of chronic helminth infection. Indeed, IL-5 and IL-13 have been associated with the

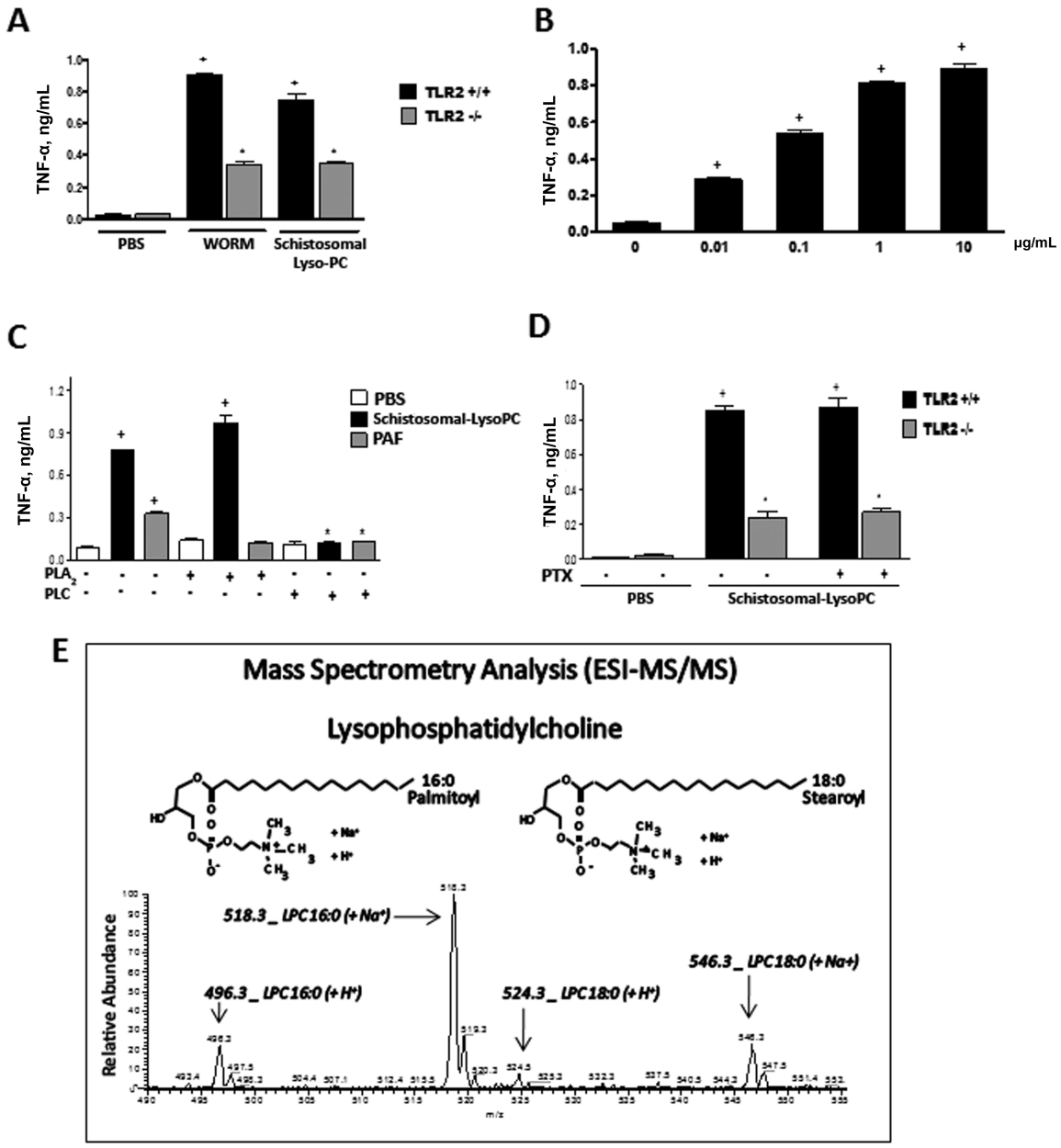

Figure 6. Functional and structural characterization of schistosomal lysophosphatidylcholine (lyso-PC). *A*, Peritoneal macrophages from Toll-like receptor 2 (*TLR2*)$^{+/+}$ or *TLR2*$^{-/-}$ mice were stimulated with schistosomal-derived lyso-PC (0.1 μg/mL) or schistosomal lipids extract from adult worm (10 μg/mL). *B*, Peritoneal macrophages from C57BL/6 were stimulated with different concentrations of schistosomal-derived lyso-PC. *C*, Effect of phospholipase C (PLC; 1.3 units/mL), phospholipase A_2 (PLA_2; 1.3 units/mL), or vehicle on schistosomal-derived lyso-PC (100 ng/mL)– or platelet-activating factor (PAF; 100 ng/mL)–induced tumor necrosis factor α (TNFα) production. *D*, Effect of macrophage pretreatment with a GiPCR inhibitor, pertussis toxin (PTX; 100 ng/mL), during 30 min before schistosomal-derived lyso-PC challenge. TNF production was analyzed within 24 h (*A*–*D*). Data represent means (± standard error of the means) from $n = 3$; + represents statistical differences between control and infected groups. Statistical differences between *TLR2*$^{+/+}$ and *TLR2*$^{-/-}$ groups or between treated and untreated groups were represented by *asterisks*. *$P \leq .05$. *E*, Mass spectrometry analysis of schistosomal lyso-PC. ESI-MS/MS, electrospray ionization tandem mass spectrometry; LPC, lyso-PC; PBS, phosphate-buffered saline.

severity of hepatic fibrosis in clinical and experimental studies [6, 44].

The immunosuppressive mechanisms evolved by parasitic helminths are thought to facilitate long-term survival of the parasite [45]. Because many lipids of pathogens, or their degradation products, are known to have potent effects on the immune system of the host, degradation products of other (tegument-specific) schistosomal lipids may have an as-yet-

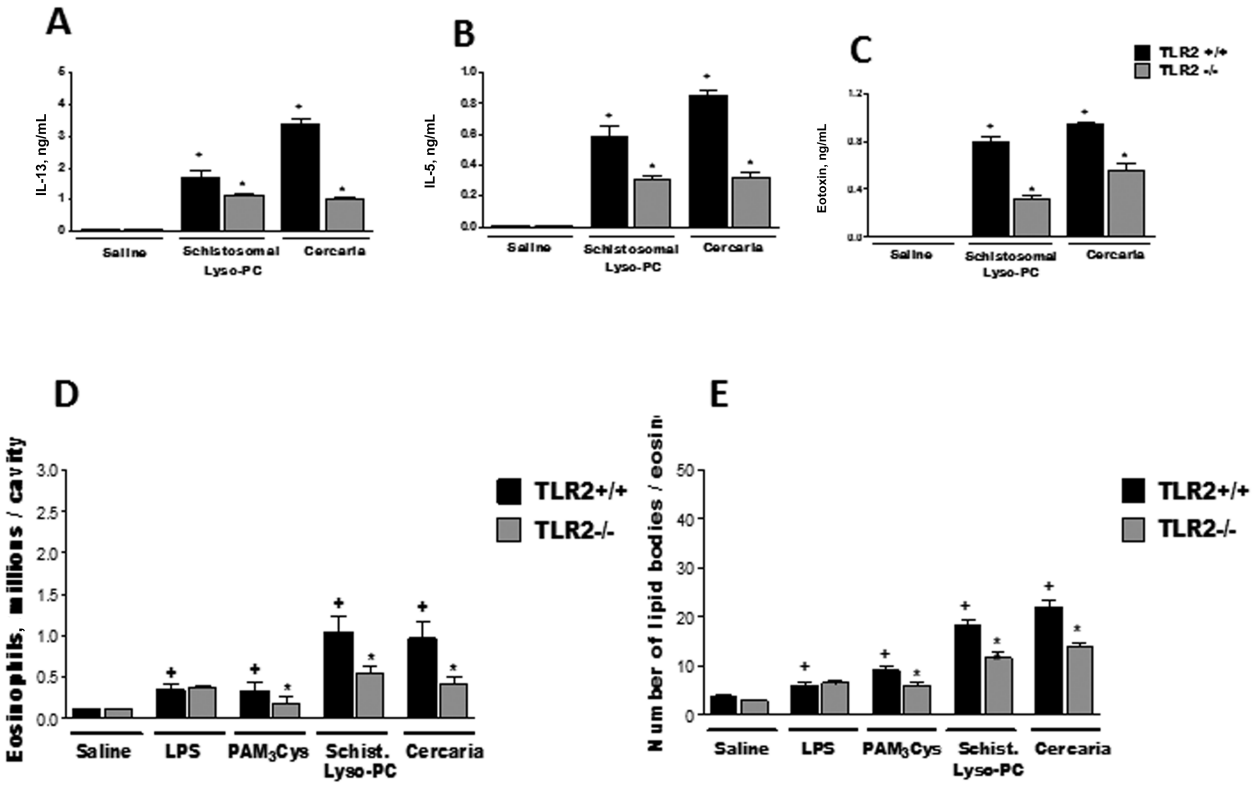

Figure 7. Schistosomal-derived lysophosphatidylcholine (lyso-PC) induce Toll-like receptor 2 (TLR2)–dependent cytokine production and eosinophil recruitment in vivo. *A–C*, Schistosomal-derived lyso-PC (10 μg/cavity), cercaria (70 cercaria/cavity), or phosphate-buffered saline was intraperitoneally administered into *TLR2*$^{+/+}$ and *TLR2*$^{-/-}$ mice. Eotaxin (*A*), interleukin (IL)-5 (*B*), and IL-13 (*C*) production in the peritoneal cavity were quantified 24 h after infection. Peritoneal eosinophil accumulation (*D*) and eosinophil lipid body formation (*E*) after intraperitoneal administration of cercaria (70 cercaria/cavity), schistosomal lyso-PC (10 μg/cavity), PAM_3Cys (50 μg/cavity), or lipopolysaccharide (LPS; 10 μg/cavity) into *TLR2*$^{-/-}$ or wild type (*TLR2*$^{+/+}$) mice were evaluated after 24 h. Data represent means ($\pm$ standard error of the means) from 6–8 animals; + represents statistical differences between control and infected groups. Statistical differences between *TLR2*$^{+/+}$ and *TLR2*$^{-/-}$ groups were represented by *asterisks*. $^*P \leq .05$.

unknown but important effect on the host [46]. Our findings indicate that schistosomal lipid extracts are recognized by the host innate immune system, resulting in TLR2-dependent cytokine production and eosinophil recruitment. We demonstrated that schistosomal lyso-PC significantly induce response through recognition by TLR2, but not TLR4 or TLR6. Lysophospholipids, such as lyso-PC, regulate a wide array of biological processes [47–49]. The presence of lyso-PC was previously demonstrated in *S. mansoni* adult worms, and lyso-PC were also shown to be excreted by schistosomula [50]. Recently, the partial characterization of lipids content of *S. mansoni* was analyzed in a lipidome study that revealed that the phospholipids phosphatidylcholine and phosphatidylethanolamine are the major constituents of the membrane of adult worms, but the composition of the fatty acids present in these phospholipids is very distinct from those found in mammalian blood cells [46]. Another schistosome-derived lysophospholipid class, lysophosphatidylserine (lyso-PS), was shown to activate TLR2 to suppress IL-12 production [22]. Considering that lysophospholipids, including lyso-PC, could signal through GPCRs [47], we investigated whether schistosomal lyso-PC induce immune response triggered by a GPCR. Schistosomal lyso-PC induced TNF-α production levels with no difference between macrophages treated and macrophages untreated with GiPCR inhibitor pertussis toxin, indicating that immunomodulatory activity of schistosomal lyso-PC is not mediated by pertussis toxin–sensitive GiPCR, but at this point we cannot rule out the participation of other GPCRs. Collectively, our results demonstrated that schistosomal lyso-PC trigger TLR2, both in vivo and in vitro, to activate pathways of cytokine production and cell activation.

In conclusion, our findings demonstrate that TLR2-dependent pathways activated by schistosomal-derived lipids play an important immunomodulatory role and are involved in eosinophil recruitment and activation in *S. mansoni* infection. Schistosome-derived lyso-PC activate TLR-2–dependent signaling and recapitulate some of the observed effects on eosinophil recruitment and may participate in *S. mansoni* immunopath-

ology. Moreover, our results suggest that TLR2 signaling, although dispensable to control *S. mansoni* infection, may contribute to the pathogenesis and lethality in the chronic phase of infection.

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