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Kleij, D. van der; Latz, E.; Brouwers, J.F.; Kruize, Y.C.M.; Schmitz, M.; Kurt-Jones, E.A.; ... ; Yazdanbakhsh, M.

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A Novel Host-Parasite Lipid Cross-talk

SCHISTOSOMAL LYSO-PHOSPHATIDYLSERINE ACTIVATES TOLL-LIKE RECEPTOR 2 AND AFFECTS IMMUNE POLARIZATION*

Desiree van der Kleij‡§, Eicke Latz¶‖, Jos F. H. M. Brouwers§, Yvonne C. M. Kruize‡, Marion Schmitz§, Evelyn A. Kurt-Jones¶, Terje Espevik, Esther C. de Jong‡‡, Martien L. Kapsenberg‡‡, Douglas T. Golenbock¶, Aloysius G. M. Tielens§, and Maria Yazdanbakhsh‡§§**

*From the ‡Department of Parasitology, Leiden University Medical Center, 2300RC Leiden, The Netherlands, §Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, The Netherlands, ¶Department of Medicine, Division of Infectious Diseases, University of Massachusetts Medical School, Worcester, Massachusetts 01655, **Institute of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, 7489 Trondheim, Norway, and ‡‡Department of Cell Biology and Histology and Department of Dermatology, Academic Medical Center, University of Amsterdam, 1100 DD Amsterdam, The Netherlands*

Schistosome infections are characterized by prominent T cell hyporesponsiveness during the chronic stage of infection. We found that schistosome-specific phosphatidylserine (PS) activated TLR2 and affected dendritic cells such that mature dendritic cells gained the ability to induce the development of IL-10-producing regulatory T cells. Using mass spectrometry, schistosomal lysophosphatidylserine (lyso-PS) was identified as the TLR2-activating molecule. This activity appears to be a unique property of schistosomal lyso-PS, containing specific acyl chains, because neither a synthetic lyso-PS (16:0) nor PS isolated from the mammalian host activates TLR2. Taken together, these findings provide evidence for a novel host-parasite interaction that may be central to long term survival of the parasite and limited host pathology with implications beyond parasitology.

Schistosomes are trematodes that cause schistosomiasis, a chronic blood-vascular disease that is associated with a Th2 response (1), but at chronic stages of infection also with enhanced IL-10 production and suppressed T cell proliferation to parasite antigens (2). The anti-inflammatory responses induced by helminths seem to enable parasite survival within the host with limited inflammatory responses that might otherwise be destructive to the host tissues. This controlled immune response, central to chronic helminth infections, may arise from signals received from the pathogen, as the chronic presence of metabolically active helminths is mirrored by persistent challenge of the immune system with an array of molecules associated with parasite metabolism, reproduction, and attrition.

Recognition of an invading microorganism by cells of the immune system involves pathogen-associated molecular patterns that bind specific germline-encoded receptors on the host cells. Toll-like receptors (TLRs)[1] with extracellular leucine-rich domains and intracellular IL-1 receptor homology domain are important members of such germline-encoded receptors and actively participate in the stimulation of innate immune responses. To date, ten TLR homologs have been found in humans, and ligands have been identified for several TLRs, most of which are of bacterial origin (3–5).

Instructions for development of specific immune responses are largely mediated by dendritic cells, which are present in peripheral tissues such as sentinel dendritic cells, and upon activation migrate to the draining lymph nodes to activate naive T cells, not only by presenting antigen but also by providing signals that determine polarization of T cell development toward a Th1 (6, 7), Th2 (6), or T regulatory phenotype (8). In this way, dendritic cells can play a central role in providing information on the nature of the invading pathogen by integrating signals received and conveying them to T cells by expressing a variety of factors that will determine differentiation of T cells into polarized subsets (9).

Dendritic cells express several TLRs, depending on their developmental stage and lineage (10, 11). Recently, several studies have shown that bacterial products induce maturation of dendritic cells via TLRs (12, 13). Furthermore, activation of TLR2 or TLR4 in immature dendritic cells can lead to expression of distinct cytokine profiles (14). However, it is still unclear to what extent activation of TLRs on dendritic cells influences the T cell phenotype associated with infectious diseases.

Several bacterial products considered as pathogen-associated molecular patterns have been shown to contain lipid moieties that are essential for activation of TLRs (15–17). Indeed, growing interest in lipids and their receptor biology has generated insights into interaction of this class of molecules with the immune system and into the role they may play in immunopathologies (18). A variety of lipid moieties can bind to specific receptors on the cells of the innate immune system and thereby play a role in immune regulation. For example, the

[1] The abbreviations used are: TLR, Toll-like receptor; PS, phosphatidylserine; SEA, water-soluble egg antigen; MF, neutral maturation factors; IL, interleukin; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate; HKLM, heat-killed *Listeria monocytogenes* .

binding of lysophosphatidylcholine to its receptor appears to have important immunomodulatory function because the deletion of the murine gene encoding the lysophosphatidylcholine-R (that is constitutively expressed in immune cells) resulted in adult-onset autoimmune disease similar to human systemic lupus erythematosus (19), α-galactosylceramide prevents onset or recurrence of autoimmune diabetes when presented in the context of the surface molecule CD1d (20), and the platelet activating factor receptor induces the production of pro- or anti-inflammatory mediators when activated by PAF or oxidized phosphatidylcholine (21).

Here, we have concentrated on the role of schistosome lipids in interaction with the innate immune system. We stimulated dendritic cells with lipid classes derived from *Schistosoma mansoni* eggs and adult worms and found that the fraction containing phosphatidylserine (PS) polarized the maturation of dendritic cells, resulting in Th2 skewing and the development of T regulatory cells. The activation of TLR2 on dendritic cells by PS is essential for induction of development of IL-10-producing regulatory T cells. Using HPLC and tandem mass spectrometry, a unique schistosomal lysophosphatidylserine (lyso-PS) was identified as the TLR2-activating molecule. Thus, specific lyso-PS species from schistosomes act on dendritic cells via TLR2 to modify their T cell-stimulating property in such manner that regulatory T cells are induced.

EXPERIMENTAL PROCEDURES

Lipid Isolation—Lipids were isolated from *S. mansoni* adult worms and eggs and from the liver of a non-infected hamster and were fractionated using a TEAE-cellulose column as detailed before (22). Briefly, *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. Total lipids were isolated according to the method described by Bligh and Dyer (23) and were separated into different classes using TEAE-cellulose column chromatography as described by Rouser *et al.* (24). The fractions containing PS were used for stimulation of dendritic cells and HEK 293 cells. Synthetic lyso-PS (16:0) was purchased from Sigma.

Peripheral Blood Mononuclear Cell Isolation and Dendritic Cell Generation and Culture—Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers by density centrifugation on Ficoll. For peripheral blood mononuclear cell stimulation, cells were seeded in 96-well flat-bottom plates at 1×10^6 cells/well in RPMI medium as detailed before (25) in the presence of 5% FCS (Greiner) and were stimulated with 100 ng/ml lipopolysaccharide (Sigma) or 10 μg/ml schistosomal lipids that were dissolved by water bath sonication in RPMI containing 0.2% Me$_2$SO.

For generation of dendritic cells, monocytes were isolated from peripheral blood mononuclear cells using a Percoll gradient as described previously (6) and were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (500 units/ml, specific activity 1.11×10^7 units/mg, a gift from Schering-Plough, Uden, The Netherlands) and human rIL-4 (250 units/ml) (R&D Systems). At day 3, the culture medium including the supplements was refreshed. At day 6, CD1a$^+$CD14$^-$ immature dendritic cells were matured with maturation factors (MF) (either LPS (100 ng/ml) or a combination of IL-1β (10 ng/ml) (Strathmann Biotechnology, Hannover, Germany) and tumor necrosis factor α (50 ng/ml) (Strathman biotech) in the presence of IFN-γ (10^3 units/ml), which induces the development of dendritic cells that stimulate the polarization of naive T cells into Th1 (6); PGE2 (10 μM), which induces the development of dendritic cells that stimulate the polarization of naive T cells into Th2 (6); schistosome water-soluble egg antigen (SEA) (100 μg/ml), a parasite extract that induces the development of dendritic cells that stimulate the polarization of naive T cells into Th2 (6); or phosphatidylserine isolated from schistosome eggs or adult worms (10 μg/ml). No differences in the level of maturation of dendritic cells exposed to the various compounds were found, as detected by CD83, CD80, CD86, and HLA-DR expression (data not shown), and therefore differential maturation could not play a role in the polarizing effects observed. In blocking experiments, 10 μg/ml of the anti-TLR2 antibody TL2.1 (26) or a mouse IgG2 control antibody (CLB, Amsterdam, The Netherlands) was added during dendritic cell maturation. After 48 h, mature CD1a$^+$ CD83$^+$ dendritic cells were obtained. To measure cytokine production, 2×10^4 dendritic cells were co-cul-

tured with 2×10^4 CD40L-expressing mouse fibroblasts (J558 cells; a kind gift from Dr. P. Lane, University of Birmingham, Birmingham, UK). Levels of IL12p70 were determined in 24-h supernatants by ELISA using monoclonal antibodies 20C2 (BD Biosciences) and biotinylated mouse-anti-hu IL-12 C8.6 (BD Biosciences) as coating and detection antibodies, respectively. Levels of IL-8 were determined using a commercial ELISA kit (CLB, Amsterdam, The Netherlands) following the manufacturer's recommendations.

Determination of Naive Th Cell Polarization by Dendritic Cells—Highly purified CD4$^+$CD45RA$^+$CD45RO$^-$ naive Th cells (>98% as assessed by flow cytometry) were purified from peripheral blood mononuclear cells using a human CD4$^+$/CD45RO$^-$ column kit (R & D Systems). 2×10^4 naive Th cells were co-cultured with 5×10^3 mature dendritic cells in the presence of superantigen *Staphylococcus aureus* enterotoxin B (Sigma) at a final concentration of 100 pg/ml in 96-well flat-bottom culture plates (Costar). At day 5, rhuIL-2 (10 units/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded. On day 12, the quiescent Th cells were restimulated with immobilized CD3 mAb (CLB-T3/3, CLB, Amsterdam, The Netherlands) and soluble CD28 mAb (CLB-CD28/1, CLB), and IL-10 was measured in 24-h supernatants using a commercial kit (CLB) following the manufacturer's recommendations. To measure the frequency of IL-4- and IFN-γ-producing cells, Th cells were restimulated with PMA (Sigma) and ionomycin (Sigma) in the presence of brefeldinA (Sigma) for 6 h. To detect intracellular production of IL-4 and IFN-γ, cells were stained using anti-hu-IL-4-PE (BD Biosciences) and anti-hu-IFN-γ-FITC (BD Biosciences).

To assess regulatory capacity, the effect of T cells (grown in the presence of dendritic cells that were matured with MF and during maturation exposed to IFN-γ, PGE2, SEA, PS from eggs or PS from worms) on proliferation of autologous Th cells was measured. T cells grown in the presence of unpolarized dendritic cells (matured with MF) were used as target cells for regulation. Effector T cells (5×10^4 cells/well) were co-cultured with target cells (5×10^4 cells/well) in the presence of unpolarized mature dendritic cells (MF dendritic cells) (5×10^3 cells/well). Cultures were done in triplicates. [^{3}H]Thymidine was added after 3 days of culture, and incorporation was measured after a 16-h pulse. The background proliferation of effector T cells (*i.e.* T-effector + dendritic cells) was subtracted from the proliferation when target cells were added (*i.e.* T-effector + dendritic cells + T-target).

Stimulation of Mouse Peritoneal Macrophages—Wild-type and TLR2 knockout mice (27, 28) were inoculated intraperitoneally with 2.5 ml of 3% thioglycolate solution. Peritoneal exudate cells were harvested with cold RPMI 1640 medium containing 10% FCS and 10 μg/ml ciprofloxacin (gift from Miles Pharmaceuticals, West Haven, CT) 72 h post-inoculation. The cells were washed and seeded at a density of 0.5×10^6 cells/well in 96-well flat-bottom plates. After 24 h, non-adherent cells were removed by washing, and the adherent cells were stimulated with repurified LPS (100 pg/ml) from *Escherichia coli* K 235 (gift from S. Vogel, Uniformed Services University of the Health Sciences, Bethesda, MD), heat-killed *Listeria monocytogenes* (HKLM) (10^6 cells/ml) (29), and schistosomal egg and adult worm PS (10 μg/ml). Tumor necrosis factor α production was measured after 20 h using a commercial DuoSet ELISA kit (R & D Systems).

Transfection of HEK 293 Cells—HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% FCS (Hyclone) and 10 μg/ml ciprofloxacin. The cells were transiently transfected at a density of 0.2×10^6 cells/well in 12-well plates using PolyFect transfection reagent (Qiagen) with 0.5 μg/transfection TLR-expression plasmid and 0.5 μg/transfection pELAM-luc, a reporter construct that transcribes firefly luciferase from an NF-κB-dependent promoter, as described previously (30).

FLAG-tagged human TLR1 and TLR2 were provided by Tularic (San Francisco, CA), FLAG-tagged TLR3 was PCR-cloned from a cDNA (DNAX, Palo Alto, CA) into pFLAG-CMV1. Non-tagged human TLR4 (hTOLL) in pcDNA3 was a gift from C. Janeway and R. Medzhitov (Yale University, New Haven, CT) and was co-transfected with human MD-2 (0.25 μg DNA/transfection of TLR4 and MD-2), a gift from K. Miyake (University of Tokyo, Japan), FLAG-tagged TLR7 was cloned from genomic DNA into pFLAG-CMV1 and FLAG-tagged TLR9 was a gift from S. Akira (Osaka University, Japan). 24 h after transfection, cells were washed with phosphate-buffered saline and stimulated with IL-1β (100 ng/ml) (Genzyme Pharmaceuticals, Cambridge, MA), HKLM (10^5 bacteria/ml), poly(IC) (100 μg/ml) (Amersham Biosciences), repurified LPS (100 ng/ml), phosphothioate-stabilized CpG oligodeoxynucleotides (10 μM, 2006–TCGTCGTTTTGTCGTTTTGTCGTT, gift from K. Heeg, Institute for Microbiology, Marburg, Germany) or schistosomal adult worm or egg PS fraction (10 μg/ml). Six hours after stimulation, cells were lysed in reporter lysis buffer (Promega, Madison, WI), and lucif-

erase activity of the cellular lysate was measured using an assay kit from Promega (Madison, WI) per the manufacturer's protocol.

Cell Lines—HEK 293-CD14 and HEK 293-CD14/TLR2 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 10 μg/ml ciprofloxacin, and 5 μg/ml puromycin. For stimulation experiments, cells were seeded at 0.2×10^6 cells/well in 96-well flat-bottom plates and were stimulated the next day with PS fractions from schistosomes or hamster liver (5 μg/ml). IL-8 production was measured in supernatants after 20 h using a commercial kit (CLB) following the manufacturer's recommendations.

Structural Identification of TLR2-activating Molecules—The schistosomal PS preparations were separated by HPLC using a 5-μm Lichrosphere diol normal-phase column (Merck). Elution was performed at a flow rate of 1 ml/min by a gradient from 95% eluent A (hexane/isopropanol/acetone (82:17:1)) and 5% eluent B (isopropanol/water/acetone (85:14:1)) to 60% A and 40% B in 30 min, followed by additional elution with the latter solvent for 10 min. Fractions were collected manually, evaporated to dryness, and used to stimulate HEK-CD14/TLR2 cells.

Both the total PS preparations and the TLR2-activating HPLC fraction were analyzed using mass spectrometry. Mass spectrometry was performed on an API-365 triple quad mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) equipped with an electrospray ionization source. Negative ionization spectra were recorded at a scan rate of 200 atomic mass units (amu) per second, using an ion spray voltage of -4500 V, a declustering potential (cone voltage) of 30 V, and a focusing potential of 210 V. Alternatively, PS and lyso-PS were analyzed by recording neutral loss spectra of 87 amu in the negative ionization mode at ion spray-, decluster-, and focusing potentials mentioned above and with a collision energy of 34 V (r). Nitrogen was used as collision- and curtain gas and air as the nebulizing gas.

For digestion with phospholipase C, 100 μg of the schistosomal adult worm PS fraction was incubated in 2 ml of diethyl ether and 1 ml of Tris-HCl buffer (pH 7.2) supplemented with 5 mM CaCl$_2$ and 1.3 units/ml phospholipase C (Sigma) during 3 h while shaking. As a control, schistosomal PS was incubated in the same buffer in the absence of phospholipase C. After hydrolysis, the ether was evaporated and lipids were extracted according to Bligh and Dyer (23).

Statistical Analysis—Data were analyzed for statistical significance using a paired *t* test.

RESULTS

PS-activated Dendritic Cells Induce the Development of Polarized T Cells—To study whether lipids from helminths are involved in interaction with the immune system, lipid classes derived from *S. mansoni* eggs and adult worms were fractionated on an anion exchange column and were screened for activity by their capacity to stimulate cytokine production (IL-6, IL-8, IL-10, or tumor necrosis factor α) by peripheral blood mononuclear cells isolated from non-exposed individuals. We found that the lipid fraction containing PS was an inducer of cytokine production (data not shown), and we proceeded to investigate its effects on dendritic cells and subsequent T cell polarization. So-called dendritic cells type-1 (DC1) have previously been shown to induce development of Th1 cells, whereas DC2 cells have been found to promote Th2 development (6). Human monocyte-derived dendritic cells were exposed to schistosomal PS, schistosomal SEA, a parasite antigen preparation that has previously been shown to induce DC2 development (6), and as controls, IFN-γ (which induces the development of DC1) or PGE2 (which stimulates the development of DC2) in combination with neutral maturation factors (MF). After several washing steps, the fully matured dendritic cells were tested for cytokine production and used to prime naive T cells. We found that as reported before (6), upon stimulation with CD40-ligand (a molecule expressed on T cells that activates dendritic cells), the IFN-γ exposed dendritic cells produced high levels of IL-12p70 (IFN-γ-dendritic cells, 6.2 ± 0.4 ng/ml) compared with mature control dendritic cells (2.1 ± 0.3 ng/ml) and enhanced the development of Th1 cells (Fig 1*A*), whereas PGE2- and SEA-exposed dendritic cells had suppressed IL-12p70 production (PGE2-dendritic cells, 0.3 ± 0.2 ng/ml and SEA-dendritic cells, 0.3 ± 0.1 ng/ml) and increased frequency of IL-4 produc-
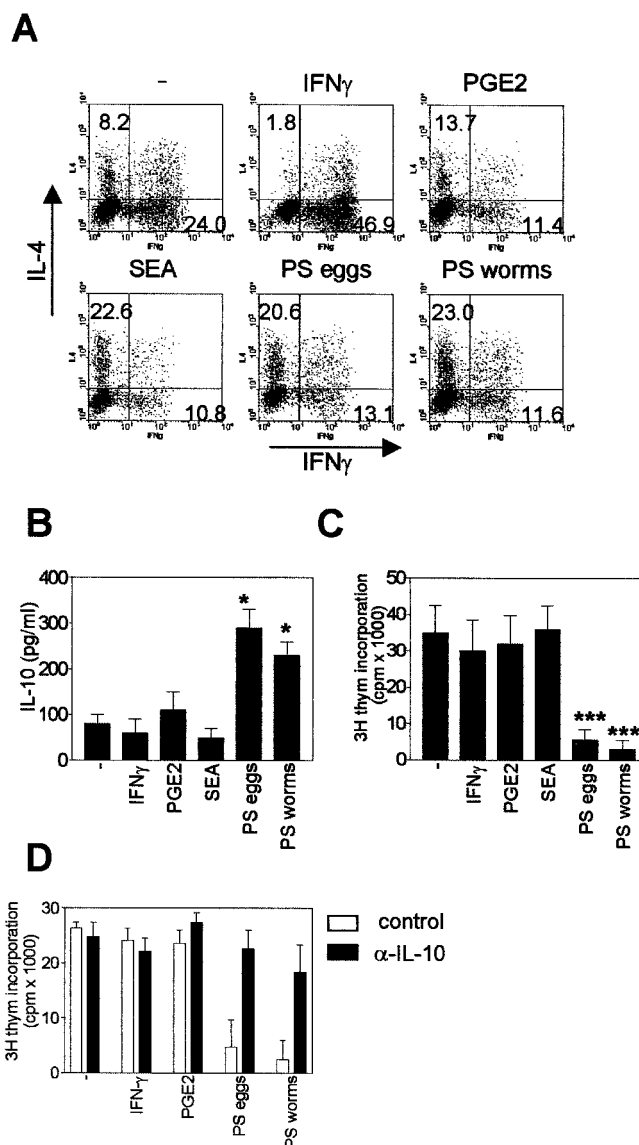

FIG. 1. **Polarization of naive T cells by mature dendritic cells.** Human immature monocyte-derived dendritic cells were matured with neutral maturation factors in the presence of IFN-γ, PGE2, SEA (100 μg/ml), or phosphatidylserine isolated from schistosome eggs or adult worms (10 μg/ml). Subsequently, mature dendritic cells were co-cultured with naive T cells for 12 days and the T cells were further analyzed. *A*, T cells restimulated with PMA and ionomycin were examined for intracellular IL-4 and IFN-γ by flow cytometry. One representative of 5 independent experiments is shown. *B*, IL-10 production was measured in 24-h supernatants of T cells restimulated with CD3/CD28. Results of 5 independent experiments are shown as mean $\pm$ S.D., *, $p < 0.05$. *C*, to assess regulatory capacity, the effect of T cells grown in the presence of dendritic cells that were matured with MF and during maturation exposed to medium, IFN-γ, PGE2, SEA, PS eggs, or PS worms was determined on the proliferation of autologous Th cells. The result of 5 independent experiments is shown as mean $\pm$ S.D., ***, $p < 0.001$. Similar results were obtained using schistosomal PS in the absence of neutral maturation factors, although maturation was slightly lower (lower CD83 expression, data not shown). Because immature dendritic cells have also been described to affect T cell development (48), experiments performed in the presence of neutral maturation factors that did not affect T cell polarization are shown. *D*, to study the role of IL-10, neutralizing antibodies were used. The effect of T cells grown in the presence of dendritic cells, which were matured with MF and during maturation exposed to medium, IFN-γ, PGE2 and PS eggs, on proliferation of autologous Th cells was tested in the presence of a blocking IL-10 antibody or a control antibody. Results of 3 independent experiments are shown.

ing T cells (Th2 cells) with decreasing numbers of IFN-γ producing T cells (Fig 1*A*). The schistosome fraction containing PS extracted from adult worms or eggs polarized the maturation of
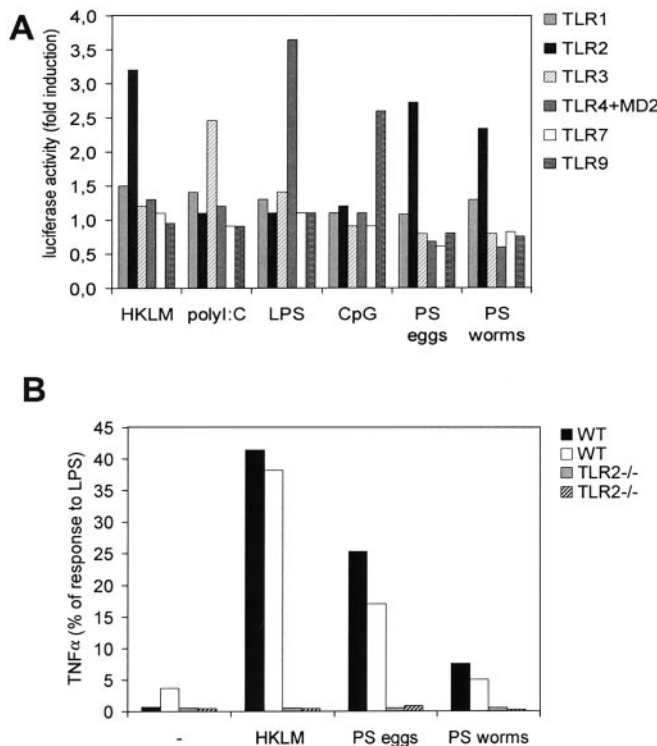
FIG. 2. **Activation of TLR-2 by schistosome PS.** *A*, HEK 293 cells were transiently transfected with human TLR1, TLR2, TLR3, TLR4+MD2, TLR7, and TLR9, together with an ELAM-luciferase reporter construct and were stimulated with schistosomal PS from eggs and adult worms (10 μg/ml) and HKLM as positive control for TLR-2, poly(IC) for TLR-3, lipopolysaccharide (LPS) for TLR-4, and CpG DNA for TLR9. Luciferase activity was determined 6 h after stimulation. *B*, peritoneal macrophages from TLR2−/− mice (*n* = 2) and wild-type mice (*n* = 2) were stimulated with LPS (taken as 100%), culture medium (−), HKLM, and schistosome PS extracted from eggs and adult worms (10 μg/ml), and tumor necrosis factor α production was measured in 24-h supernatants.

dendritic cells, resulting in low IL-12p70 release (PS eggs-dendritic cells, 0.4 ± 0.2 ng/ml, PS worms-dendritic cells, 0.4 ± 0.1 ng/ml) and not only in Th2 skewing (Fig 1*A*) but in contrast to SEA and PGE2, also in the development of IL-10 producing T cells (Fig 1*B*). T cells that develop in co-culture with dendritic cells, which were matured in the presence of schistosomal PS, are capable of suppressing proliferation of autologous Th cells significantly at a T_{reg}:T_{target} cell ratio of 1:1 (Fig 1*C*). Inhibition of proliferation was reduced to 50% at a T_{reg}:T_{target} cell ratio of 1:2, and no inhibition was detectable at a 1:4 ratio. The observed suppression of proliferation was abrogated when a blocking IL-10 antibody was present in the co-culture of regulatory T cells (T_{reg}) and target T cells (Fig. 1*D*), suggesting that the T_{reg} cells exert their inhibitory function via IL-10.

The low IL-12p70 production was not caused by PS toxicity, because production of another cytokine (IL-8) by dendritic cells was unaffected or even enhanced and also no apoptotic cells were detected as determined using the method described by Nicoletti *et al.* (31) (data not shown). Taken together, the maturation of dendritic cells in the presence of schistosome egg- or adult worm-PS leads to the development of dendritic cells capable of inducing T_{reg} cells. This is specific for schistosomal PS, as dendritic cells matured in the presence of SEA or PGE2 (which polarize toward Th2) lacked the capacity to stimulate the development of the IL-10-producing T_{reg} cells. The mechanism whereby dendritic cells matured in the presence of schistosomal PS induce IL-10-producing T cells is currently being investigated. Preliminary experiments show that whereas dendritic cell-derived soluble factors present in supernatants me-
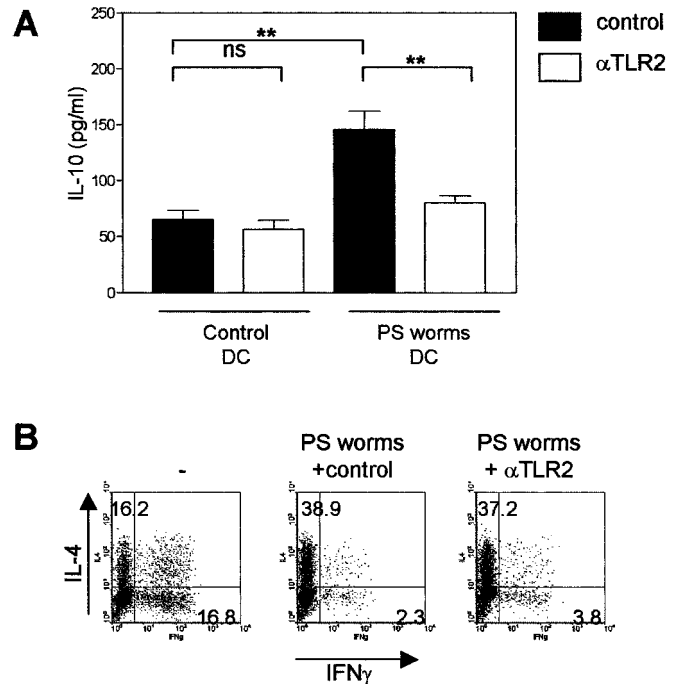

FIG. 3. **TLR2 activation on dendritic cells is essential for the development of IL-10 producing T cells.** Dendritic cells were matured with MF alone (−) or with MF and PS worms (10 μg/ml) in the presence of a TLR2 blocking antibody or a control antibody. After maturation, dendritic cells were co-cultured with naive T cells, which after 12 days were restimulated with immobilized CD3 and soluble CD28 (*A*) to measure IL-10 in 24-h supernatants (results of 3 independent experiments are shown as mean ± S.D., **, *p* < 0.01) or PMA and ionomycin in the presence of brefeldin A (*B*) and examined for intracellular IL-4 and IFN-γ (one representative of 3 independent experiments is shown).

diate Th2 development, cell-cell contact is required for T_{reg} cell induction.

Schistosomal PS Activates Toll-like Receptor 2—We transiently transfected HEK 293 cells with human TLR1, TLR2, TLR3, TLR4+MD2, TLR7, and TLR9 to elucidate the mechanism by which schistosomal PS interacts with cells of the innate immune system. On stimulation with schistosomal PS, only cells transfected with TLR2 were activated (Fig. 2*A*). These results were confirmed by using peritoneal macrophages of TLR2 −/− mice, which were unresponsive to schistosomal PS, whereas macrophages of the control mice showed a clear response (Fig. 2*B*). When an anti-TLR2 antibody was added during dendritic cell maturation in the presence of schistosomal PS, the development of IL-10-producing T cells was diminished (Fig 3*A*) whereas Th2 polarization was not affected (Fig. 3*B*), indicating that TLR2 plays a role in specifically promoting the development of regulatory T cells, but not Th2 cells.

Structural Characterization of the TLR2-activating PS Molecule—To confirm that the active molecule present in the schistosomal PS fractions is indeed a phospholipid and not a minor compound not detectable by mass spectrometry, the schistosomal PS fraction was treated with phospholipase C, which removes the phosphoserine head group from the acylglycerol moiety, and subsequently HEK CD14/TLR2 stably transfected cells were stimulated with the resulting lipid fraction. On phospholipase C digestion, the TLR2-stimulating activity was significantly reduced (Fig. 4*A*), indicating that the phosphoserine head group is required for TLR2 activation. Phosphoserine alone did not have any TLR2-stimulating activity on HEK CD14/TLR2 cells (Fig. 4*B*), demonstrating that both the acyl group and the phosphoserine moieties of PS form a combined epitope for activity. In addition, HEK 293 cells stably trans-
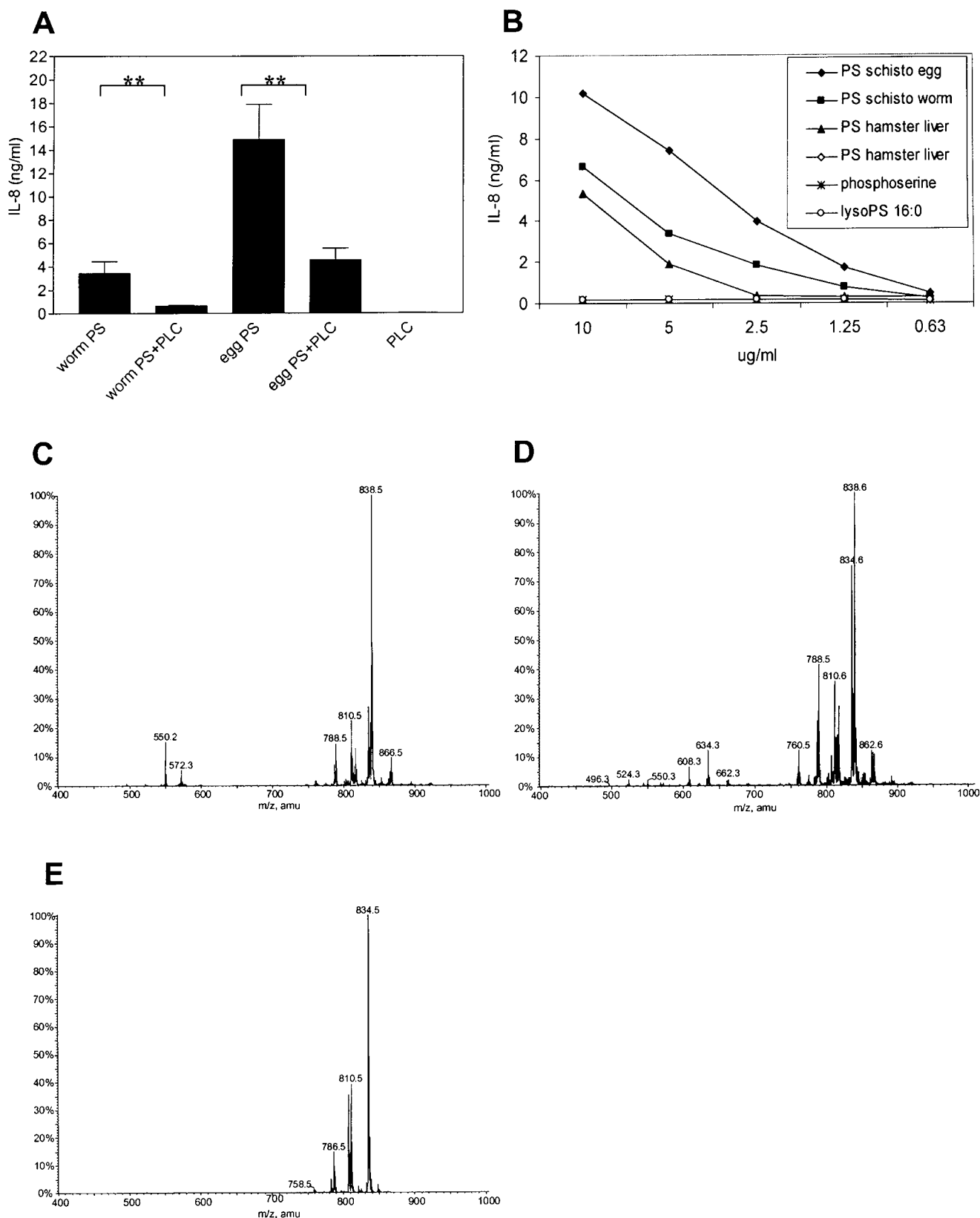
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FIG. 4. **Structural analysis of PS preparations.** *A*, PS worms and PS eggs were digested with phospholipase C, and the TLR2 stimulating activity of the resulting lipids was determined by stimulating HEK 293 CD14/TLR2 transfectants with an amount equivalent to a final concentration of 10 μg/ml undigested material. IL-8 was measured 20 h after stimulation. Results of 3 independent experiments are shown as mean $\pm$ S.D., **, $p < 0.01$. *B*, HEK 293 cells stably transfected with CD14 or CD14 and TLR2, which responded to HKLM but not to LPS, were stimulated with various concentrations of PS extracted from schistosome eggs, schistosome adult worms or PS isolated from hamster liver, phosphoserine (without any acyl moieties), and commercially available lyso-PS 16:0. IL-8 production was measured after 20 h. A representative experiment of 3 independent experiments is shown. PS fractions from schistosome adult worms (*C*, PS worms), schistosome eggs (*D*, PS eggs), and hamster liver (*E*) were analyzed using tandem MS in the negative mode. Neutral loss scans of 87 amu, corresponding to the loss of serine from the phospholipid, are given.
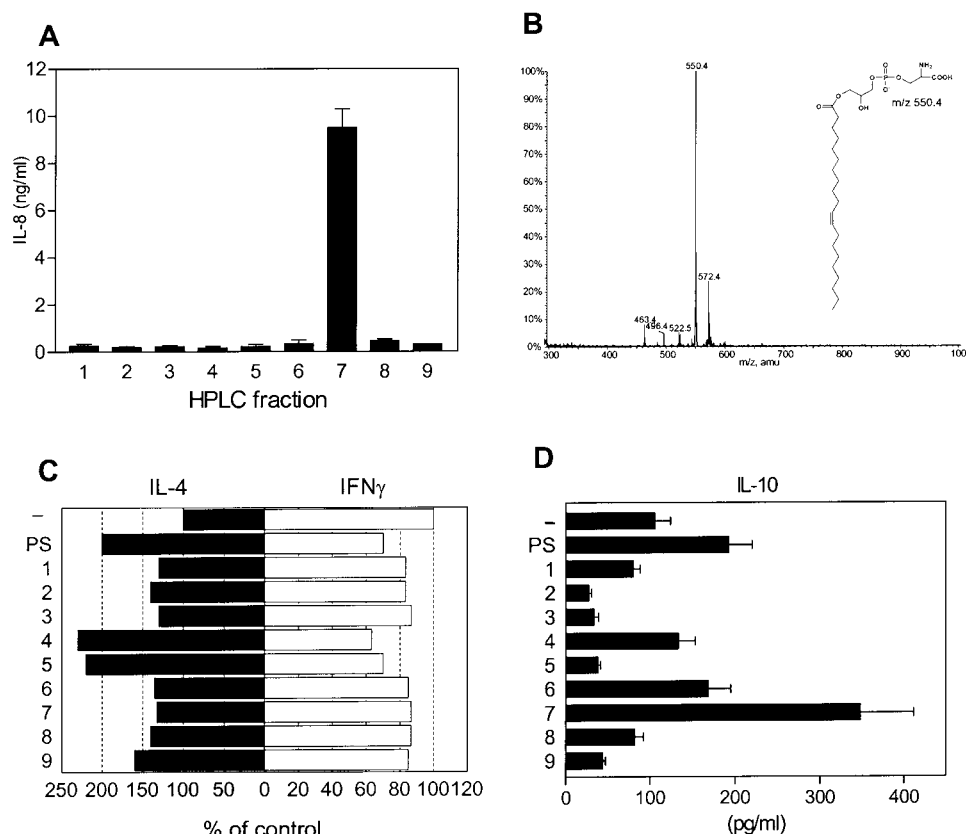
FIG. 5. **Structural identification of TLR2-activating molecules.** The schistosome adult worm preparation was fractionated by HPLC and the resulting fractions were used to stimulate HEK 293 CD14/TLR2 transfectants (*A*). Fraction 7, which had TLR2-stimulating activity, was analyzed by MS. Fractions 4 and 5 contained PS molecules with 2 acyl chains (confirmed by MS, data not shown). A negative mode mass spectrum of fraction 7 is depicted, and the structure of the major component lyso-PS 20:1 (*m/z* 550.4) is indicated (*B*). Dendritic cells (0.5 ml) were matured in the presence of the PS HPLC fractions (derived from 5 µg of unfractionated material) and were used to prime the development of naive T cells. T cell polarization was monitored by intracellular staining of IL-4 and IFN-γ upon PMA/ionomycin stimulation of T cells (percentage of cytokine positive T cells grown in the presence of DC matured with neutral MF (= control) was set at 100% (*C*), and by measurement of IL-10 in 24-h supernatants of CD3/CD28-stimulated T cells (*D*).

fected with TLR2 responded to schistosomal PS in a dose-dependent manner, but did not respond to any concentration of PS isolated from the liver of a non-infected mammalian host (a golden hamster) (Fig. 4*B*). In agreement with the observation that mammalian PS did not activate TLR2, it also did not affect dendritic cell maturation (data not shown).

To identify the structure of the TLR2-activating molecule within the schistosomal PS preparations, we analyzed the contents of the PS preparations extracted from schistosome adult worms and eggs and hamster liver using HPLC and tandem mass spectrometry (Fig. 4, *C*, *D* and *E*, respectively). The PS-containing fractions comprised a variety of molecules, but they all showed the typical loss of a serine head group (with a mass of 87 Da) and showed a great variety in the number of C atoms and presence of double bonds in the acyl chains. Strikingly, both in the schistosomal worm and egg PS fractions, PS molecules containing only one acyl chain (so-called lyso-PS molecules) were found, whereas such molecules could not be detected in the hamster liver PS extract (Fig. 4, *C–E*). Many different lyso-PS species were detected in the schistosome PS preparations, with fatty acyl chains containing up to 30 carbon atoms that were mostly either saturated or monounsaturated.

The worm PS preparation was fractionated by HPLC, and the fraction containing the TLR2-activating component was identified by measuring the activation of HEK 293 CD14/TLR2 transfected cells (Fig. 5*A*). The active fraction (fraction 7) was analyzed by MS and found to contain lyso-PS molecules, whereas PS molecules having two acyl chains could not be

detected (Fig. 5*B*), indicating that TLR2-stimulating activity resides in the lyso-PS molecules. HPLC fractions 4 and 5 contained PS molecules with 2 acyl chains but no lyso-PS (confirmed by MS; data not shown), and did not activate CD14/TLR2 transfected cells (Fig. 5*A*). When dendritic cells were matured in the presence of these HPLC fractions, we found that Th2 polarization was induced by components in fractions 4 and 5 (Fig. 5*C*), whereas IL-10-producing T cells primarily developed in the presence of DC that were matured with fraction 7 (Fig. 5*D*), indicating that Th2 polarization is induced by diacylated PS, whereas monoacylated lyso-PS induces development of IL-10-producing T cells. Collision-induced dissociation spectra of the ions found in fraction 7 generated product spectra from which the associated fatty acyl moieties could be identified as carboxylate ions (data not shown). In this way, the fatty acids present in lyso-PS from adult schistosomes (molecular ions *m/z* 550 and *m/z* 572) were identified as 20:1 and 22:4, respectively (Figs. 4*C* and 5*B*), whereas the most abundant lyso-PS species in eggs were 26:1 (*m/z* 634), 24:0 (*m/z* 608), 18:0 (*m/z* 524), 26:2 (*m/z* 632), and 26:0 (*m/z* 636) (Fig. 4*D*). The differences in lyso-PS species found in schistosome eggs and adult worms may account for the difference in TLR2-stimulating activity (Fig. 4*B*). Because HEK CD14/TLR2 transfectants failed to respond to commercially available synthetic lyso-PS 16:0, it is clear that the structure of the acyl chain found in schistosomal lyso-PS is critical for TLR2 reactivity (Fig. 4*B*). In agreement with this, lyso-PS 16:0 did not affect polarization of DC maturation (data not shown).

DISCUSSION

Dendritic cells form a bridge between innate and adaptive immunity, acquiring signals from pathogens at the site of infection and subsequently activating naive T cells in the draining lymph nodes, leading to initiation of a particular immune response desired either by the host to eliminate the invader, or by the pathogen to survive. Here we describe a specific interaction between parasite-derived lipid structures with not only the innate but also the adaptive immune system. Schistosomal phosphatidylserine stimulated innate immune responses in peripheral blood mononuclear cells of naive individuals and polarized maturation of dendritic cells. Polarization of dendritic cell maturation with schistosomal PS led to the development of fully mature dendritic cells that were capable of inducing Th2 (Fig. 1*A*) as well as IL-10-producing (Fig. 1*B*) regulatory T cells (Fig. 1, *C* and *D*), both features characteristic of immune responses in chronic schistosome infections. We previously reported that a water-soluble extract of schistosome eggs (SEA) polarizes DC maturation toward a Th2 phenotype (Fig. 1*A*) (6). However, these DC, in contrast to DC matured in the presence of schistosome PS, do not induce IL-10 producing regulatory T cells (Fig. 1, *B* and *C*).

Use of TLR transfectants as well as gene knockout mice indicated that schistosome PS (Fig. 2) but not SEA (data not shown) was able to stimulate TLR2. An important role for TLR2 in the ability of schistosomal PS to instruct dendritic cells to induce regulatory T cells was demonstrated by using blocking antibodies to TLR2 (Fig. 3*A*). Structural analysis indicated that the number of acyl chains present on schistosomal PS was critical for its activity. The importance of the acyl chains for TLR2 activation has been previously reported for bacterial lipopeptides, in which the removal of two of the three acyl chains from a synthetic triacyl lipopeptide leads to the loss of TLR2 activity (17). For schistosomal PS, the opposite is true; schistosomal PS containing two acyl chains is inactive with respect to TLR-2 activation, whereas lyso-PS containing only one acyl chain has prominent TLR-2-activating capacity (Fig. 5). Interestingly, PS (with two acyl chains), when present during DC maturation, was able to polarize T cell responses toward Th2- but not toward IL-10-producing T cells, whereas lyso-PS specifically induced IL-10-producing T cells (Fig. 5).

This is the first report of a TLR2-active monoacylated structure; reports to date have shown TLR2 activity for triacylated lipopeptides from bacteria and diacylated lipopeptides from mycoplasma (15, 26). The fact that lyso-PS 16:0 (16 C lacking a double bond) failed to activate TLR2-expressing cells indicates that for TLR2 activation, the presence of a specific acyl chain structure is required, which is present in schistosomal lyso-PS. These data suggest a highly specific interaction between a lipid ligand and a hydrophobic region of TLR2, perhaps residing within one or more leucine-rich repeats in its extracellular domain. Because experiments using phospholipase C (Fig. 4*A*) suggest that the phosphoserine head group also plays a role in TLR2 activation, it has to be concluded that both the specific structure of the acyl group and phosphoserine moiety of lyso-PS form a combined epitope critical for activity.

Experiments in MyD88−/− and Rip2−/− mice have indicated that downstream signaling from TLRs is important for the development of Th1 responses typified by high IFN-γ, but not of Th2 responses characterized by IL-4 and IL-5 (32–34). However, the role of these receptors in induction of immunoregulatory responses characterized by high IL-10 or TGF-β has not been analyzed. It is interesting to note that in some experiments performed with human dendritic cells, TLR2 ligands, which were poor stimulators of IL-12 production, stimulated IL-10 transcription (14).

It is becoming clear that TLR2 recognizes a wide range of pathogen-associated molecular patterns with distinct chemical properties ranging from proteins such as heat shock protein 60 (35), bacterial peptidoglycan (28), or lipids, such as shown here. The observation that a single TLR can be activated by ligands that are so diverse may be explained by the ability of the TLRs to form heterodimers as already shown for TLR2, which can form dimers with either TLR1 or TLR6 (36, 37). In addition, it is also clear that although TLRs share common signaling features such as IRAK and downstream NF-κB activation (38), they can express relatively unique signaling (39) that may come from cooperation with other molecules or receptors expressed in the innate immune system. It remains to be determined whether additional molecules take part in the recognition described here. In particular, the recent identification of several G protein-coupled receptors for lysophospholipids opens the way for the elucidation of their role in innate immune responses (18).

Schistosomes do not synthesize fatty acids *de novo* but rely on the host for fatty acid supply. However, the parasite has retained the capability to modify fatty acids by chain elongation, resulting in a fatty acid profile that is clearly distinct from that of the host (40–42). For instance, 20:1 and 22:4 are typical examples of schistosome-specific fatty acids not present in the mammalian host. We have also shown previously that schistosomes display a high rate of deacylation and reacylation, in contrast to host cells (43). Our current findings indicate that this high rate of lipid remodeling could specifically target the immune system via specific receptors actively leading to the development of regulatory T cells involved in immunosuppression. Therefore, considering the relationship between exposure of cells of the innate immune system to PS-bearing apoptotic cells and induction of an anti-inflammatory state (44), it is tempting to speculate that the modulating effect of lyso-PS may be a way in which the parasite exploits the pathways normally used by its host to prevent excessive inflammation and autoimmune reactions in response to changes in cell membrane lipids.

Strong regulatory responses induced by parasite products operative during chronic infections may be responsible for balanced host-parasite interaction whereby host tissue damage is restricted on the one hand and parasite survival is enhanced on the other (45). Identification of molecular structures capable of inducing regulatory T cells and elucidation of the mechanisms by which they do so may contribute to the development of novel therapeutic strategies with implications beyond parasitology (46, 47).

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A Novel Host-Parasite Lipid Cross-talk: SCHISTOSOMAL LYSO-PHOSPHATIDYLSERINE ACTIVATES TOLL-LIKE RECEPTOR 2 AND AFFECTS IMMUNE POLARIZATION

Desiree van der Kleij, Eicke Latz, Jos F. H. M. Brouwers, Yvonne C. M. Kruize, Marion Schmitz, Evelyn A. Kurt-Jones, Terje Espevik, Esther C. de Jong, Martien L. Kapsenberg, Douglas T. Golenbock, Aloysius G. M. Tielens and Maria Yazdanbakhsh

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