Visceral leishmaniasis is characterized by severe immunosuppression of the host cell, resulting in loss of the proinflammatory response. Toll-like receptor 2 (TLR2) is involved in myriad disease forms, including visceral leishmaniasis. During *Leishmania donovani* infection, the parasite modulates TLR2 to suppress interleukin 12 production, indicating the possible involvement of TLR2 in regulation of the immune response against *L. donovani* infection. Arabinosylated lipoarabinomannan (Ara-LAM) possesses immunomodulatory properties and induces proinflammatory responses via induction of TLR2-mediated signaling. Here, we found that pre-treatment of *L. donovani*-infected macrophages with Ara-LAM caused a significant increase in TLR2 expression along with the activation of TLR2-mediated downstream signaling, facilitating active nuclear translocation of nuclear factor κB. These events culminated in up-regulation of the proinflammatory response, which was abrogated by treatment with TLR2-specific small interfering RNA. In vivo experiments were also suggestive of Ara-LAM playing a long-term protective role. This study demonstrates that Ara-LAM confers protection against leishmanial pathogenesis via TLR2 signaling–mediated induction of the proinflammatory response.
in the recognition of its ligand [9]. Other observations suggest that TLR2-stimulated IL-12 is down-regulated during L. donovani infection, indicating the possible involvement of TLR2 in regulation of the immune response against Leishmania infection [10].

The strategy adopted by us to circumvent Leishmania-induced regulation of TLR2 was the application of a novel TLR2-using molecule, arabinosylated lipoarabinomannan (Ara-LAM). It is a cell wall glycolipid of Mycobacterium smegmatis and uses TLR2 during lung inflammation [11]. Other reports from our laboratory have shown that Ara-LAM is involved in the induction of proinflammatory cytokines and confers protection against tuberculosis [12] and leishmaniasis [13]. However, it has yet to be ascertained whether the immunomodulatory properties of Ara-LAM can induce the TLR2-mediated signaling that in turn can trigger a proinflammatory response in both in vitro and in vivo models of visceral leishmaniasis.

In the present study, we have demonstrated that pretreatment with Ara-LAM (3 μg/mL) could induce TLR2 expression in L. donovani-infected macrophages, thereby triggering its downstream signaling via myeloid differentiation primary response gene 88 (MyD88) and its associated signaling molecules and leading to the activation of nuclear factor κB (NF-κB), ultimately culminating in significant release of proinflammatory cytokines, chemokines, and nitric oxide. In in vitro experiments, TLR2 silencing significantly abrogated the protective immune response generated by Ara-LAM. In vivo experiments were also suggestive of Ara-LAM playing a long-term protective role, as was seen by significantly reduced levels of liver and spleen parasitemia along with induction of specific CD4+ T cells that control chronic infection. Thus, these findings might provide crucial cues in understanding the immunomodulatory role played by Ara-LAM in conferring protection against visceral leishmaniasis.

**METHODS**

**Animals and parasites.** BALB/c and C57BL/6 mice were purchased from the National Centre for Laboratory Animal Sciences, India. For each experiment 8–10 mice (4–6 weeks old) were used, regardless of sex. L. donovani organisms (strain MHOM/IN/1983/AG-83) were maintained in Medium 199 (Sigma) plus 10% fetal calf serum (Gibco). Amastigotes were prepared as described elsewhere [14]. Stationary-phase promastigotes obtained by suitable transformation were used for experiments. All experimental protocols were given prior approval by the institutional animal ethics committee.

**Isolation and purification of Ara-LAM.** Ara-LAM was isolated as described elsewhere [15]. Lipopolysaccharide contamination was checked by the Limulus test and was <25 ng/mg in Ara-LAM. The noncytotoxic dose of Ara-LAM was 3 μg/mL [13].

**Peritoneal macrophage preparation.** Peritoneal macrophages from thioglycolated BALB/c and C57BL/6 mice were cultured for 48 h, as described elsewhere [16]. Adherent macrophages were infected with Leishmania promastigotes (stationary phase) at a ratio of 1:10.

**Preparation of TLR2-specific small interfering RNA.** We synthesized TLR2-specific small interfering RNA (siRNA) (forward, 5′-AAAGAGAAGTACTTACTGCACCTGTC-3′; reverse, 5′-AATGCAATGACTTCTCTCTGGTCT-3′) using the Silencer siRNA Construction kit (Ambion). A nonspecific scrambled siRNA was generated with same GC content.

**Flow cytometry.** Macrophages were stained with fluorescein isothiocyanate (FITC)–labeled anti–TLR2 and anti–TLR4 antibodies. For intracellular cytokine staining, isolated spleen cells were stimulated with soluble leishmanial antigen (SLA) for 48 h. Brefeldin A (10 μg/mL) was added for 4 h, fixed, and permeabilized (0.1% saponin) and then stained with anti–mouse CD4–FITC and anti–mouse interferon γ (IFN-γ)–phycocerythrin antibodies (Santa Cruz Biotech). Cells were analyzed using a FACScalibur flow cytometer (Becton Dickinson).

**Coimmunoprecipitation.** Coimmunoprecipitation experiments were conducted using a protocol for anti–TLR2 and anti–MyD88, anti–MyD88 and anti–interleukin 1 receptor–associated kinase 1 (IRAK-1), and anti–IRAK-1 and anti–interleukin 1 receptor–associated kinase M (IRAK-M) antibodies that has been described elsewhere [17].

**Preparation of cell lysate and immunoblot analysis.** Cell lysates were prepared as described elsewhere [18]. Equal amounts of protein (50 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotting was performed as described elsewhere [19].

**Preparation of nuclear extracts.** Cells were collected after incubation for indicated periods by gentle scraping, and nuclear extraction was performed as described elsewhere [19].

**Electrophoretic mobility shift assay.** NF-κB DNA binding was analyzed by electrophoretic mobility shift assay, as described elsewhere [19].

**Cytokine enzyme-linked immunosorbent assay.** Culture supernatants were analyzed using a sandwich enzyme-linked immunosorbent assay kit (Quantikine M; R&D Systems), in accordance with the manufacturer’s instructions.

**Isolation of mRNA and real-time polymerase chain reaction.** Total RNA extracted from macrophages (TRI reagent; Sigma) was reverse transcribed using Revert Aid M-MuLV reverse transcriptase (Fermentas). Real-time polymerase chain reaction (PCR) was performed using SYBR Green mix and the ABI 7500 real-time PCR system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. Sequences of the PCR primers are listed in Table 1. The reaction conditions consisted of an initial activation step (5 min at 95°C) and cycling step (denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C) for 40 cycles. Each PCR mixture contained 0.5 μM of each primer, 2× SYBR Green mix, and 2 mM MgCl2 in a final volume of 20 μL. The expression levels were normalized with GAPDH expression and are presented relative to control.
Nitrite generation. Nitrite level in culture was measured using the Nitric Oxide Colorimetric Assay kit (Boehringer Mannheim Biochemicals) [20]. Cell-free supernatants were collected from different experimental sets at different time points of infection, and nitrite levels were estimated in accordance with the manufacturer’s instructions. Data were expressed in micromoles of nitrite.

In vivo experiments. BALB/c mice (8 weeks old) were divided into the following groups: (1) control mice (administered phosphate-buffered saline); (2) L. donovani–infected mice; and (3) Ara-LAM–treated infected mice (injected with 30 µg of Ara-LAM 2 days before L. donovani infection) [12]. Mice were killed on days 1, 7, 14, 28, and 56 after infection. The course of visceral infection was determined macroscopically using stained liver and spleen tissue imprints after Giemsa staining, and spleen and liver parasite burdens were determined (expressed in Leishman-Donovan units) [21]. Isolated splenocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium plus 10% fetal calf serum.

Proliferation assay. The T cell proliferation assay was performed as described elsewhere [22]. Single-cell suspensions of splenocytes from different experimental groups of Ara-LAM–treated BALB/c mice 4 weeks after infection were plated in complete RPMI 1640 medium in triplicate at 1 × 10^5 cells/well in 96-well plates and allowed to proliferate for 3 days at 37°C in a 5% CO₂ incubator in the presence or absence of SLA (5 μg/mL). Eighteen hours before they were harvested, cells were pulsed with 1 μCi (6.7 Ci/mmol) of [³H]thymidine/well (JON-AKI, DAE). [³H]thymidine uptake, as an index of proliferation, was measured using a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

Statistical analysis. In vitro cultures were done in triplicate, and a minimum of 4 mice were used per group for in vivo experiments. Data, shown as means ± standard deviations, are from 1 experiment performed at least 3 times. The Student t test was used to assess the significance of differences between the mean values for control and experimental groups. A difference with P<.05 was considered significant, and a difference with P<.001 was considered highly significant.

**RESULTS**

Specific induction of TLR2 expression in Leishmania-infected macrophages by Ara-LAM. Ara-LAM has been reported to use TLR2 during lung inflammation [11]. We studied the effect of Ara-LAM on other TLRs (TLR4, TLR5, TLR6, TLR7, and TLR9) as well as TLR2. Ara-LAM was found to specifically induce expression of TLR2. Moreover, it was found to be a specific ligand for TLR2 by distinct association between TLR2 and Ara-LAM, as observed during coimmunoprecipitation experiments (Figure 1A). Furthermore, treatment of parasitized macrophages with Ara-LAM led to a significant increase in TLR2 surface expression, compared with that for infected macrophages (Figure 1B). At the mRNA level, pretreatment of infected macrophages with Ara-LAM resulted in an increase in TLR2 by an average of 5-fold over unstimulated infected macrophage (Figure 1D). Ara-LAM showed more potent induction of TLR2 in infected macrophages, compared with that of the known TLR2 agonist Pam3CSK4 (Figure 1B and 1D).

In addition to TLR2, TLR4 plays an important role in regulating the immune response against Leishmania infection [23]. Experiments with Ara-LAM showed that it had no effect on TLR4 expression in uninfected and Leishmania-infected macrophages (Figure 1C and 1D). Thus, Ara-LAM specifically upregulated TLR2 expression in infected macrophages and had no effect on TLR4 during Leishmania infection.

Activation of the TLR2 signaling cascade during Leishmania infection by Ara-LAM. Down-regulation of TLR2-dependent IL-12 production during Leishmania infection might be due to inactivation of downstream signaling cascades [10]. Moreover, Ara-LAM is known to induce inflammatory reactions in lungs by induction of TLR2 signaling [11]. We studied whether Ara-LAM was able to significantly modulate the functional status of TLR2-mediated downstream signaling in Leishmania-infected macrophages. It is known that TLR2 activation depends on the association between TLR2 and MyD88, an adaptor molecule located immediately downstream of TLR2 [24], and this event is crucial for the initiation of additional signals. Coimmunoprecipitation experiments showed a strong association between TLR2 and MyD88 in Ara-LAM–pretreated L. donovani–infected macrophages, compared with that in infected macrophages (Figure 2A). Because CD14 has been shown to be involved in the corecognition of various TLR ligands by TLR2 [9], we explored whether CD14 plays any role in the corecognition of Ara-LAM by TLR2. We observed that pretreatment with CD14 monoclonal antibody followed by Ara-LAM led to a significant inhibition of the association between TLR2 and MyD88 in parasitized macrophages, compared with that in Ara-LAM–pretreated parasitized macrophages (Figure 2A).
Figure 1.  

A. Effect of arabinosylated lipoarabinomannan (Ara-LAM) on Toll-like receptor (TLR) 2, 4, 5, 6, 7, and 9. Peritoneal macrophages were cultured and then subjected to Ara-LAM pretreatment (3 μg/mL) for 3 h. Changes in messenger RNA (mRNA) expression of TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 were determined by real-time polymerase chain reaction (PCR) analysis. Results are presented as changes (n-fold) relative to control cells. The experiment was repeated 3 times, yielding similar results; data are expressed as means ± standard deviations. Ara-LAM binds with TLR2 and induced TLR2 expression in Leishmania-infected macrophages. Ara-LAM–stimulated murine peritoneal macrophage cell lysates were subjected to immunoprecipitation with anti-TLR2 or anti–Ara-LAM antibody. The precipitates were subjected to Western blotting and were further probed with anti–Ara-LAM or anti-TLR2 antibody, respectively. 

B-D. Induction of TLR2 but not TLR4 expression by Ara-LAM in Leishmania donovani–infected macrophages from BALB/c mice. Peritoneal macrophages isolated from BALB/c mice were cultured and then subjected to Ara-LAM pretreatment (3 μg/mL) for 3 h, followed by Leishmania challenge for 24 h. Macrophages were stained with either anti-TLR2–fluorescein isothiocyanate (FITC) or anti-TLR4–FITC antibody and then analyzed by flow cytometry for TLR2 (FL1-H) (B) and TLR4 (FL1-H) (C) expression. Changes in expression of TLR2 and TLR4 mRNA were determined by real-time PCR analysis (D). Results are presented as changes (n-fold) relative to uninfected control cells. The experiment was repeated 3 times, yielding similar results; data are expressed as means ± standard deviations. *P < .001 and **P < .005 for the comparison with infected macrophages.
Figure 2. A, Facilitation of Toll-like receptor 2 (TLR2)–mediated downstream signaling and the TLR2–myeloid differentiation primary response gene 88 (MyD88) association by arabinosylated lipoarabinomannan (Ara-LAM) in *Leishmania donovani*–infected macrophages. Murine macrophages (6 × 10⁶) were treated as described in the Figure 1 legend. After 24 h of incubation, cell lysates were subjected to immunoprecipitation with anti–TLR2 antibody, and the blots were probed with anti-MyD88 antibody. B, Abrogation of the Ara-LAM–induced TLR2-MyD88 association in *L. donovani*–infected macrophages under CD14-blocking conditions. Macrophages were neutralized using anti-CD14 monoclonal antibody followed by Ara-LAM treatment, after which cells were infected with *Leishmania* parasites. The TLR2-MyD88 association was then analyzed by coimmunoprecipitation, as described in Methods. C–K, Induction of nuclear factor κB (NF-κB) nuclear translocation through modulation of signaling molecules by Ara-LAM. Murine macrophages (6 × 10⁶) were cultured and treated as described in the Figure 1 legend. After 24 h of incubation, macrophages were subjected to coimmunoprecipitation to analyze the association between MyD88–interleukin 1 receptor–associated kinase 1 (IRAK-1) (C) and IRAK-1–interleukin 1 receptor–associated kinase M (IRAK-M) (D), and Western blot analysis was performed to analyze the expression of IRAK-1 (E), IRAK-M (F), tumor necrosis factor receptor–associated factor 6 (TRAF-6) (G), IκB kinase α (IKK-α) (H), and inhibitor of NF-κB (IKB-α) (I). The electrophoretic mobility shift assay using differently treated nuclear extracts was performed to analyze the nuclear translocation of NF-κB in *L. donovani*–infected macrophages (J and K). The blots shown are representative of triplicate experiments. The autoradiograms are representative of 3 independent experiments that had identical results. lpp, immunoprecipitation; LPS, lipopolysaccharide; Wb, Western blot.
Expression of IRAK-1, which is crucial for activation of downstream kinases [25], was found to be significantly elevated in Ara-LAM–treated sets (Figure 2E) and also showed a distinct association with MyD88 (Figure 2C). IRAK-M, a negative regulator of TLR2 signaling [26], was abrogated (Figure 2F) and did not show any association with IRAK-1 in Ara-LAM–treated sets (Figure 2D). Furthermore, the level of TNF-α, which plays a crucial role in TLR-induced NF-κB activation [27], was enhanced in Ara-LAM–treated sets (Figure 2G), abrogating the expression of inhibitor of NF-κB (Figure 2F) along with the induction of IκB kinase α (Figure 2H) and ultimately leading to nuclear translocation of NF-κB (Figure 2J and 2K), a potent transcription factor for proinflammatory cytokines [28].

**Induction of proinflammatory responses via TLR2 by Ara-LAM in Leishmania-infected macrophages.** We explored whether Ara-LAM could also enhance expression of proinflammatory cytokines, such as TNF-α (Figure 3A, 3H, and 3I) and IL-12 (Figure 3B, 3H, and 3J), as well as down-regulate anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) (Figure 3E and 3K) and IL-10 (Figure 3F and 3J), at both the protein and mRNA level in *L. donovani*–infected macrophages isolated from BALB/c and C57BL/6 mice. Because proinflammatory cytokines play a critical role in the induction of iNOS2 from host cells during infection [29], we studied the effect of Ara-LAM pretreatment on nitrate generation and iNOS2 expression in *L. donovani*–infected macrophages. Our data suggested that pretreatment with Ara-LAM followed by *L. donovani* infection led to a significant release of nitric oxide (Figure 3G) along with a prominent increase in iNOS2 mRNA expression (Figure 3H and 3I), compared with that in infected macrophages. A protective immune response during *Leishmania* infection is also associated with a strong chemokine response [30]. Therefore, along with cytokines we also studied the expression of such chemokines as macrophage inflammatory protein 1α (MIP-1α) and monocyte chemoattracting protein 1 (MCP-1). Comparative chemokine profiles clearly revealed that a significant inhibition of MIP-1α and MCP-1 expression occurred in infected macrophages, compared with that in uninfected macrophages, but pretreatment of parasitized macrophages with Ara-LAM induced MIP-1α (Figure 3C, 3H, and 3I) and MCP-1 (Figure 3D, 3H, and 3I) expression at both the protein and mRNA level. To establish the involvement of TLR2 in the generation of a host-protective immune response by Ara-LAM, we studied Ara-LAM–mediated induction of proinflammatory cytokines as well as chemokine and nitrite generation under TLR2-silenced condition. There was a significant abrogation of expression of proinflammatory cytokines and chemokines along with a significant reduction in iNOS2 at the mRNA level, thereby highlighting the role played by TLR2 in the Ara-LAM–induced proinflammatory response in both BALB/c- and C57BL/6-derived peritoneal macrophages during *Leishmania* infection (Figure 3H and 3I).

**Ara-LAM confers protection via the proinflammatory response in Leishmania-infected mice.** The in vitro experiments clearly reiterated that Ara-LAM activated the TLR2 signaling cascade, leading to significant induction of proinflammatory mediators; this prompted us to study whether Ara-LAM could abrogate immune suppression in an in vivo murine model of visceral leishmaniasis. Pretreatment with Ara-LAM could confer significant long-term protection against leishmanial challenge. Seven days after infection, Ara-LAM–pretreated BALB/c mice showed 80% suppression of infection in the liver and 76% suppression of infection in the spleen (Figure 4A and 4B); 14 days after infection, Ara-LAM pretreatment led to 82% suppression of infection in the liver and 80% suppression of infection in the spleen (Figure 4A and 4B). Interestingly, Ara-LAM–pretreated mice showed a marked decrease in parasite burden day 28 after infection, by ~90% in the liver and ~87% in spleen (Figure 4A and 4B), and this protection was seen to persist until 56 days after infection. It has been noted that *Leishmania* infection of the susceptible host results in apoptosis of T cells, leading to impairment of cell-mediated immunity [31]; in this regard, we observed that Ara-LAM could restore the impaired T cell proliferation in *Leishmania*-infected mice (Figure 4C).

**T helper type 1 (Th1) cytokines (such as TNF-α, IL-12, and IFN-γ) are involved in the clearance of *Leishmania* parasites** [32], whereas Th2 cytokines (such as TGF-β and interleukin 10 [IL-10]) assist in the survival of the parasite. Twenty-eight days after infection, we analyzed cytokine production in spleen cells from Ara-LAM–treated infected mice at both the protein and mRNA level. The results showed that there was significant inhibition of TNF-α, IL-12, and IFN-γ expression and high expression of TGF-β and IL-10 in the splenocytes of infected mice, compared with that in uninfected mice. Interestingly, Ara-LAM pretreatment of *L. donovani*–infected mice caused significant induction of TNF-α (Figure 5A and 5G), IL-12 (Figure 5B and 5H), and IFN-γ (Figure 5C and 5I) expression at the protein and mRNA level, compared with that in infected mice. In contrast, Ara-LAM–treated mice showed significant abrogation of TGF-β (Figure 5E and 5K) and IL-10 (Figure 5F and 5L) expression at the protein and mRNA level, respectively, compared with that in untreated infected mice. Because the release of proinflammatory cytokines such as TNF-α strongly induces the production of reactive nitrogen intermediates either alone or with IFN-γ [33], we determined whether up-regulation of Th1 cytokines by Ara-LAM treatment led to the up-regulation of nitric oxide in the splenocytes of infected BALB/c mice. We found a 14-fold increase in nitrite generation in the splenic cell supernatant of Ara-LAM–treated mice, compared with that in infected con-
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Figure 3. A–G, Induction of the proinflammatory response by arabinosylated lipopolysaccharide (Ara-LAM) via Toll-like receptor 2 (TLR2) in Leishmania donovani–infected macrophages from both BALB/c and C57BL/6 mice. Ara-LAM induced proinflammatory cytokine (tumor necrosis factor α [TNF-α] and interleukin 12 [IL-12] [p70] and chemokine (macrophage inflammatory protein 1α [MIP-1α] and monocyte chemotactic protein 1 [MCP-1]) production and dampened anti-inflammatory cytokine (transforming growth factor β [TGF-β] and interleukin 10 [IL-10]) expression in L. donovani–infected macrophages from both BALB/c and C57BL/6 mice. Peritoneal macrophages (2 × 10⁶ cells/mL) from BALB/c and C57BL/6 mice were pretreated separately with Ara-LAM and then infected with L. donovani, as described in the Figure 1 legend, for 24 h. Levels of TNF-α (A), IL-12 [p70] (B), MIP-1α (C), MCP-1 (D), TGF-β (E), and IL-10 (F) in the culture supernatant were evaluated by sandwich enzyme-linked immunosorbent assay (ELISA), as described in Methods. ELISA data are expressed as means ± standard deviations of values from triplicate experiments that yielded similar observations.

Macrophages were cultured and treated as described above. Culture supernatants were collected 48 h after infection, and nitrite levels were determined using the Nitric Oxide Colorimetric Assay kit, as described in Methods (G). Results are expressed as means ± standard deviations of values from 3 replicate experiments that yielded similar results. Asterisks indicate a statistically significant increase or decrease (*P < .001 and **P < .005) in cytokine release, chemokine release, and nitrite generation, compared with that in infected macrophages.

H–K, Ara-LAM–mediated increase in proinflammatory cytokine, inducible nitric oxide synthase 2 (iNOS2), and chemokine mRNA expression by TLR2. In separate experimental sets, cells isolated from both BALB/c and C57BL/6 mice were transfected with control small interfering RNA (siRNA) or TLR2-specific siRNA for 24 h in medium containing a low quantity of fetal bovine serum, washed, and then treated with Ara-LAM followed by L. donovani for 3 h. Then they were collected in Trizol for RNA extraction, and real-time polymerase chain reaction (PCR) analysis was performed to determine proinflammatory cytokine, chemokine, and iNOS2 mRNA expression. The real-time data presented were the best representable among the 3 independent experiments that showed similar results, whereas the bar diagram depicting the fold change in mRNA quantification shows means ± standard deviations of values from triplicate experiments that yielded similar observations. A separate set of cells (2 × 10⁶) was collected in Trizol for mRNA extraction and real-time PCR (see Methods). Levels of TGF-β (K) and IL-10 (J) mRNA expression were determined by quantitative real-time PCR. Results are presented as changes (n-fold) relative to uninfected control cells. Data are means ± standard deviations of values from 3 independent experiments that yielded similar results (*P < .001 and **P < .005).

trols (Figure 5D). Because the generation of nitric oxide is dependent on activation of iNOS2, we studied its expression and observed that there was a 21-fold increase in iNOS2 mRNA expression in Ara-LAM–treated mice, compared with that in infected mice (Figure 5F).

Because the activation and proliferation of T cells (mainly
Figure 4. A and B, Effect of arabinosylated lipoarabinomannan (Ara-LAM) on parasitic load in Leishmania-infected mice. Mice were treated with either phosphate-buffered saline (PBS) (control) or Ara-LAM (30 μg intraperitoneally) for 2 days, after which mice were infected. Treated mice were killed 1, 7, 14, 28, and 56 days after infection. Levels of parasitic burden in liver and spleen are expressed in Leishman-Donovan units (LDUs). Results are for 3 independent experiments and represent the mean values ± standard errors for 5 animals per group per time point. *P<.001 for the comparison with infected mice. C, Enhancement of lymphocyte proliferation in Leishmania donovani–infected BALB/c mice by Ara-LAM treatment. Proliferative responses to soluble leishmanial antigen (SLA) (5 μg/mL) of splenocytes from Ara-LAM–treated L. donovani–infected mice were examined. Proliferation was measured by [3H]thymidine incorporation. At 5 μg/mL SLA, optimal proliferation was obtained, showing high lymphocyte proliferation compared with infected groups of mice. Results are representative of 4 individual experiments, and data are mean values ± standard errors from triplicate wells. The asterisk indicates a statistically significant induction (*P<.001) of T cell proliferation, compared with that in infected mice.

CD4+ T cells) leads to the production of Th1 cytokines, which is essential for long-term protection against leishmaniasis [34], we investigated whether Ara-LAM treatment could induce CD4+ T cells to secrete IFN-γ in infected mice. Splenocytes isolated from Ara-LAM–treated infected mice were stimulated with SLA, and the frequency of IFN-γ–secreting CD4+ T cells was determined by intracellular cytokine staining. Flow cytometry analysis showed a significantly higher frequency of IFN-γ–producing CD4+ T cells in infected mice pretreated with Ara-LAM, compared with that in infected mice (Figure 6).

DISCUSSION

Visceral leishmaniasis, which is characterized by severe immunosuppression due to increased parasite-driven Th2 cytokine production [34], has given rise to a major lacuna resulting from the absence of a suitable immunomodulator. Recent findings have confirmed the role played by TLR2 in initiating a proinflammatory response during visceral leishmaniasis. Ara-LAM, which uses TLR2, has been shown by our group to possess a proinflammatory function [15] and also restores the impaired cell-mediated immune responses in a murine model of tuberculosis [12]. However, it has yet to be ascertained whether Ara-LAM can render protection against Leishmania-induced pathogenesis through the use of TLR2.

In the present study, we have demonstrated that Ara-LAM may be used as a novel immunoprophylactic agent against visceral leishmaniasis. In vitro experiments with Ara-LAM showed that Ara-LAM specifically up-regulated the expression of TLR2 in Leishmania-infected macrophages (Figure 1B and 1D). TLR2 expression is abrogated during visceral leishmaniasis, which affects the production of proinflammatory cytokines by host macrophages [10]. Although TLR4 plays a crucial role in circumventing the parasite-induced immunosuppression [23], Ara-LAM functioned independently of TLR4 (Figure 1C and
Figure 5. Induction of T helper type 1 cytokines and nitric oxide and down-regulation of T helper type 2 cytokines by arabinosylated lipoarabinomannan (Ara-LAM) in *Leishmania*-infected mice. Splenocytes from Ara-LAM–treated mice infected with *Leishmania donovani* were isolated 28 days after infection, plated aseptically (2 × 10⁶ cells/well), and stimulated with soluble leishmanial antigen at 5 μg/ml for 48 h. Tumor necrosis factor α (TNF-α) (A), interleukin 12 (IL-12) p70 (B), interferon γ (IFN-γ) (C), nitric oxide (D), transforming growth factor β (TGF-β) (E), and interleukin 10 (IL-10) (F) in spleen cell culture supernatants for the indicated treatment groups were determined by enzyme-linked immunosorbent assay and the Nitric Oxide Colorimetric Assay kit, respectively. A separate set of splenocytes (2 × 10⁶) was collected in Trizol for mRNA extraction and real-time polymerase chain reaction (PCR) analysis (see Methods). Levels of mRNA expression for TNF-α (G), IL-12p40 (H), IFN-γ (I), inducible nitric oxide synthase 2 (iNOS2) (J), TGF-β (K), and IL-10 (L) were determined by quantitative real-time PCR. Results are presented as changes (n-fold) relative to uninfected control cells. Data represent means ± standard errors for 5 animals per group. *P < 0.001 and **P < 0.005 for the comparison with infected mice.
Neutralized macrophages (Figure 2) was shown by the failure of TLR2 to bind to MyD88 in CD14−A signaling via the TLR2-MyD88 association (Figure 2). Strong proinflammatory cytokine responses (such as TNF-α and IL-12) along with down-regulation of disease-promoting anti-inflammatory cytokines (such as TGF-β and IL-10) in Ara-LAM−pretreated parasitized peritoneal macrophages derived from both BALB/c and C57BL/6 mice, compared with that in infected macrophages (Figure 3).

TLR2 gene silencing abrogated Ara-LAM−mediated induction of inflammatory responses, in agreement with other observations [36]. These in vitro results clearly suggest that Ara-LAM has the ability to establish a host-protective Th1 immune response in L. donovani−infected macrophages by use of TLR2. Although no substantial report on TLR2 levels in human patients with visceral leishmaniasis exists, experiments with monocytes isolated from human peripheral blood mononuclear cells have also shown the involvement of TLR2 in visceral leishmaniasis [10].

On the basis of the in vitro experiments investigating the role played by Ara-LAM in regulating Th1-Th2 cytokine secretion in infected macrophages by use of TLR2, we further investigated its effect on the regulation of these cytokines in Leishmania-susceptible infected BALB/c mice only, because C57BL/6 mice have been shown to recover from L. donovani infection as a result of a predominant Th1 response [37]. In vivo experiments demonstrated that Ara-LAM treatment could significantly restrict parasitic growth in the spleen and liver 28 days after infection, and this protection was observed until 56 days after infection (Figure 4A and 4B). The depressed T cell response associated with visceral leishmaniasis [31] was found to be recovered by Ara-LAM, given that splenocytes isolated from Ara-LAM−treated mice 28 days after infection showed a strong lymphoproliferative response (Figure 4C). The cytokine profile suggested that Ara-LAM could generate a strong Th1 response in Ara-LAM−treated infected mice (Figure 5). IL-10, an immunosuppressive cytokine, along with TGF-β were found to be abrogated in Ara-LAM−treated infected mice (Figure 5), suggesting that Ara-LAM plays a protective role in mediating the skewing from a Th2 to a Th1 response in L. donovani−infected mice. This protective response of Ara-LAM was found to be further mediated by the induction of iNOS2 gene expression and the generation of nitric oxide (Figure 5 and 5D). Further confirmation that Ara-LAM causes skewing from a Th2 to a Th1 cytokine response was obtained in flow cytometry analysis, which showed a strong induction of IFN-γ-secreting CD4+ T cells (Figure 6). These findings demonstrated that Ara-LAM could confer protection against Leishmania infection in susceptible BALB/c mice through an efficient skew toward a Th1 immune response.

Collectively, these findings from both in vitro and in vivo models of visceral leishmaniasis point toward a novel mechanism of Ara-LAM−mediated immunoprotection. We suggest that this glycolipid antigen isolated from nonpathogenic mycobacteria could be of immense importance as an immunoprophylactic molecule by which disease progression in leishmaniasis could be restricted, because it can induce the proinflammatory responses that create an antipathogenic environ-

**Figure 6.** Induction of the proliferation of interferon γ (IFN-γ)−secreting CD4+ T cells by arabinosylated lipoarabinomannan (Ara-LAM) in Leishmania−infected mice. Intracellular IFN-γ staining of splenocytes was done after in vitro stimulation with soluble leishmanial antigen. Twenty-eight days after infection, different groups of mice were killed, and splenocytes were isolated and cultured. Before harvesting, cells were incubated with brefeldin A (10 μg/mL) for 4 h. Cells were permeabilized and stained with anti–mouse IFN-γ–phycoerythrin, CD4-fluorescein isothiocyanate antibody, and isotype-matched control mouse antibody and were analyzed by flow cytometry. Data are from 1 of 3 experiments conducted in the same way with similar results.
ment in the host cell. To our knowledge, this is the first study to unveil triggering of TLR2-mediated signal transduction as a mechanism of Ara-LAM–mediated protection against leishmanial pathogenesis, thus suggesting that TLR2 signaling could be a potent target in antileishmanial immunoprophylaxis.

References