Pegylated Bisacycloxypropylcysteine, a Diacylated Lipopeptide Ligand of TLR6, Plays a Host-Protective Role against Experimental Leishmania major Infection

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Pegylated Bisacycloxypropylcysteine, a Diacylated Lipopeptide Ligand of TLR6, Plays a Host-Protective Role against Experimental Leishmania major Infection

Surya Prakash Pandey,* Himanshu Singh Chandel,* Sunit Srivastava,* Sathishkumar Selvaraj,* Mukesh Kumar Jha,* Divanshu Shukla,* Thomas Ebensen, † Carlos A. Guzman, † and Bhaskar Saha*

TLRs recognize pathogen-expressed Ags and elicit host-protective immune response. Although TLR2 forms heterodimers with TLR1 or TLR6, recognizing different ligands, differences in the functions of these heterodimers remain unknown. In this study, we report that in Leishmania major-infected macrophages, the expression of TLR1 and TLR2, but not TLR6, increased; TLR2–TLR2 association increased, but TLR2–TLR6 association diminished. Lentivirus-expressed TLR1–short hairpin RNA (shRNA) or TLR2–shRNA administration reduced, but TLR6–shRNA increased TLR2 association increased, but TLR2–TLR6 association diminished. Corroboratively, Pam3CSK4 (TLR1–TLR2 ligand) and peptidoglycan (TLR2 ligand) increased macropage activation and parasite control, the protozoan devises immune evasion strategies either by a TLR may be followed by macrophage activation and parasite con- after activation of parasitology-normal immune system that, in turn, elicits pathogen-specific adaptive immune response (6, 7). Leishmania major, a protozoan parasite living as aflagellate amastigotes within macrophages, causes cutaneous lesions in a susceptible host such as a BALB/c mouse. Whereas recognition of Leishmania by a TLR may be followed by macrophage activation and parasite con- (8), the protozoan deviates immune evasion strategies either by direct interference of the IFN-γ–secreting T cell generation (9) or by direct interference through lipopolysaccharide.

Toll-like receptor 2 is a unique member among the TLRs, as it forms heterodimers with TLR1 or TLR6 and modulates downstream signaling pathways (1–3). TLR1–TLR2 and TLR2–TLR6 heterodimers recognize triacylated or diacylated lipopeptides, respectively (4, 5). Following recognition of pathogen such as Leishmania, TLRs activate innate immune system that, in turn, elicits pathogen-specific adaptive immune response (6, 7). Leishmania major, a protozoan parasite living as aflagellate amastigotes within macrophages, causes cutaneous lesions in a susceptible host such as a BALB/c mouse. Whereas recognition of Leishmania by TLR2 may be followed by macrophage activation and parasite con- (8), the protozoan deviates immune evasion strategies either by indirect interference of the IFN-γ–secreting host-protective T cell generation (9) or by direct interference through lipopolysaccharide.

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Abbreviations used in this article: ATP2F, activating transcription factor 2; BPPcysMPEG, pegylated bisacycloxypropylcysteine; DC, dendritic cell; iNOS, inducible NO synthase; PGN, peptidoglycan; shRNA, short hairpin RNA; TIRAP, Toll/IL-1R adaptor protein; TRAF6, TNFR-associated factor 6; Treg, regulatory T.

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L. major (11) and L. donovani (23) infections. Although TLR1–
TLR2 and TLR2–TLR6 heterodimers are known for recognition of
triacylated lipopeptides or diacylated lipopeptides, respectively (4,
5), whether they function differently is not known.

Antileishmanial host-protective T cell response is limited by
regulatory T (Treg) cells (24–28), as described earlier (11).

In this study, we report that BPPcysMPEG increased TLR9 ex-
pression in BALB/c-derived uninfected and L. major-infected mac-
rophages and induced a higher IL-12:IL-10 ratio than that induced by
Pam3CSK4 and peptidoglycan (PGN). TLR1 short hairpin RNA
(shRNA) or TLR2 shRNA reduced, but TLR6 shRNA aggravated
L. major infection in BALB/c mice. Pam3CSK4 and PGN augmented,
whereas BPPcysMPEG reduced amastigote number in macrophages.
BPPcysMPEG induced higher p38MAPK and activating transcription
factor 2 (ATF2), whereas Pam3CSK4 and PGN induced more of
ERK1/2 and ELK-1 activation. These contrasting effects of TLR2
heterodimers with TLR1 or TLR6 reveal a TLR2 functional duali-

Materials and Methods
Parasites, mice, peritoneal macrophages, and reagents

L. major (strain MHOM/Su73/5ASKH) was maintained in RPMI 1640
with 10% FCS (Life Technologies-BRL, Grand Island, NY). The parasites
were passed through BALB/c mice to maintain the virulence. C57BL/6,
inducible NO synthase (iNOS)–deficient (C57BL/6 background), BALB/c,
and IL-12–deficient (BALB/c background) mice, originally from The
Jackson Laboratory (Bar Harbor, ME), were maintained in the experi-
mental animal facility of the National Centre for Cell Science. Animals
were used according to the Institute’s ethical animal use committee-approved
animal use protocol.

The elicited peritoneal macrophages were isolated as adherent cells
from C57BL/6, inos-deficient, IL-12–deficient, and BALB/c mice 5 d after
thioglycolate injection (3% thioglycolate, 2 ml; i.p.). Purity of macrophages
was tested routinely, as described earlier (31), and it was always between 97
and 98.5% (data not shown). Macrophages were cultured in RPMI 1640
supplemented with 10% FCS. After 6 h, nonadherent cells were washed out
and cells were incubated for 36 h at 37˚C containing 5% CO2 and humidified
atmosphere.

Pam3CSK4, PGN, and CpG ODN1826 were purchased from Invivogen
(San Diego, CA). BPPcysMPEG was synthesized at the Helmholtz Centre
for Infection Research (Braunschweig, Germany). The primers for TLRs
and cytokines (Table I) were from Integrated DNA Technologies (San
Diego, CA). PCR reagents and Treg cell analyses

For immunoprecipitation, cells were lysed in lysis buffer by incubation on ice
for 1 h. Equal amounts of proteins (50 µg) were incubated with 1 µg anti-
TLR-2 Ab for 8–10 h at 4˚C and pulled with protein AG–agarose beads
(Pierce, Rockford, MI) at 4˚C for 3 h. Immunoprecipitates were washed
twice in cold lysis buffer and once in cold TBST. Pellets were resuspended
in 20 µl 2× SDS sample buffer and subjected to Western blot analysis (32).

For Western blotting, cells were treated with indicated reagents and lysed
with lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% (w/v) Nonidet
P-40, 1 mM EDTA, 1 mM EGTA, and 1% Nonidet P-40), protease inhibitor
mixture (Roche Applied Science, Mannheim, Germany), and phosphatase inhibitor
mixture (Pierce, Rockford, Michigan). Equal amount of protein was loaded
on SDS-PAGE, and resolved protein was transferred to polyvinylidene
fluoride (PVDF) membrane (Millipore, Billerica, MA) for 3 h at 30 V.

Lysates were subjected to Western blot analysis. Membranes were incubated with primary Ab at 4˚C
overnight, washed with TBST, and incubated with HRP-conjugated
secondary Ab. Immunoreactive bands were visualized with the Luminol
reagent (Santa Cruz Biotechnology, Santa Cruz, CA) (32). Densitometric analyses of
bands were performed using Quantity One software.

FACS reagents and Treg cell analyses

Anti-CD3–PE-Cy7 (553062), anti-CD25–allophycocyanin-Cy7 (557688),
anti-GITR–PE (12-5874-82), and anti–IL-10–FITC (554466) Abs were pur-
buched from BD Biosciences (San Jose, CA); anti-CD127–allophycocyanin
(CD127-8121-82) Ab was purchased from Biolegend (San Diego, CA); and anti-
CD4–PerCP-Cy5.5 (100344) and anti-Foxp3–Pacific Blue (126409) Abs were purchased
from Biolegend (San Diego, CA). For multiplex FACS analyses,
cells were stained (after blocking with 5% FCS) with fluorocyanated
Abs anti-CD3–PE-Cy7, anti-CD4–PE-Cy5, anti-CD25–allophycocyanin
Cy7, anti-GITR–PE, and anti-CD127–allophycocyanin for 45 min at 4˚C
and dark washed twice with FACS buffer (1× PBS, 10 mM HEPES
buffer, and 3% FCS). Intracellular cytokine staining was performed using a Cytofix/Cytoperm-Plus Kit with GolgiPlug (550530; BD Pharmingen), as per the manufacturer’s instructions. Cells were acquired in the CD3+CD4+
CD127dimCD25+ gate by FACSComp II flow cytometer (BD Biosciences)
and analyzed for expression of GITR and Foxp3 or Foxp3 and IL-10 using
BD FACS Diva software (version 5.2; BD Biosciences) (28).

Macrophage–T cell coculture

BALB/c-derived peritoneal macrophages from naive mice were infected with
L. major promastigotes at a ratio of 1:10 for 6 h. The extracellular
parasites were washed out, and macrophages were treated with the indicated
doses of BPPcysMPEG for 24 h. The popliteal lymph node was
infected from the 21-d L. major-infected BALB/c mice. Infected macro-
phages were cocultured with lymph node CD4+ T cells at 1:3 ratio
(macrophage:T cells). After 60 h, supernatants were collected for cytokine
assays and cells for Treg analyses. For amastigote count, macrophages
were washed, fixed with chilled methanol, stained with Giemsa stain,
and counted under a light microscope (E-600; Nikon).

Production of lentiviral particles for TLR shRNA and control shRNA

TLR1 and TLR6 shRNA (GenBank accession number NM_030682,
http://www.ncbi.nlm.nih.gov/nucleotide/NM_030682, and NM_011604,
In vitro lentiviral transduction

Peritoneal macrophages were transduced with lentivirally expressed TLR1 shRNA (TLR1-Lv), TLR2 shRNA (TLR2-Lv), TLR6 shRNA (TLR6-Lv), and control shRNA (control-Lv) particles (2 transduction units/cell) for 8 h in RPMI 1640 supplemented with FCS (0.5%) and polybrene (8 μg/ml) (Sigma-Aldrich). Cells were washed to remove residual viral particles. After 48-h transduction, uninfected and L. major-infected cells were treated with BPPcysMPEG and CpG or left untreated for 48 h for amastigote count and ELISA (32).

Generation of bone marrow monocyte–derived dendritic cells

Dendritic cells (DCs) were cultured from bone marrow progenitor cells using a modified protocol of a previously described method (33). In brief, femoral cells were fractionated on a density gradient (Histopaque-1077; Sigma-Aldrich) to isolate mononuclear cells, which were incubated for 90 min. Nonadherent cells were washed out with 1× PBS. DC culture medium (RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml GM-CSF, and 10 ng/ml IL-4) was added to the adhered monocytes (1 × 10^6 cells/ml). Culture medium was replaced with fresh culture medium every 3 d. After 48-h transduction, uninfected and L. major-infected cells were treated with BPPcysMPEG and CpG or left untreated for 48 h for amastigote count and ELISA (32).

DC and T cell coculture

Bone marrow–derived DCs were infected with L. major promastigotes with a ratio of 1:10 for 3 h. The extracellular parasites were washed out, and DCs were treated with the indicated doses of BPPcysMPEG for 24 h. The popliteal lymph node was collected from the 21-d L. major-infected BALB/c mice. Infected DCs were cocultured with lymph node CD4+ T cells at a 1:3 ratio (DC:T cells). After 60 h, supernatants were collected and assayed for cytokine responses by ELISA (32).

Table 1. The forward and reverse primers used in RT-PCR and real-time PCR

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence (5'-3')</th>
<th>Gene</th>
<th>Primer Sequence (5'-3')</th>
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<td>GAPDH</td>
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<td>F: CACCAAAACCACTGACCTGTT</td>
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<td></td>
<td>R: CCCCTTCAACCCCTTCCTG</td>
<td></td>
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<tr>
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<td>TLR7</td>
<td>F: TCCACACGTGATGTGCTGTG</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>R: TTTTGGCTCTTGTCCTGCTG</td>
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<tr>
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<td>R: TTGTGGCGCCCTGCTTGTAAT</td>
<td></td>
<td>R: ATGGCCAAAGCTGACTGCTTT</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.

In vivo L. major infection

BALB/c and IL-12–deficient mice were infected in the left footpads with L. major promastigotes (s.c., 10^6 in 50 μl saline/mouse). Mice were treated with BPPcysMPEG (1 μg/mouse) for alternating 3 d starting from the third day postinfection. In some experiments, 2 d prior to L. major infection, mice were pretreated with 5 × 10^6 transduction units (in 50 μl HBSS; Life Technologies, Grand Island, NY) of TLR1 shRNA, TLR2 shRNA, TLR6 shRNA, or control shRNA expressing lentiviruses in the same footpad to be infected. Some mice were treated with BPPcysMPEG (1 μg/mouse) or CpG (10 μg/mouse) or both (s.c. into the footpad for 3 d alternatively starting from the third day postinfection). Seven mice were used in each group. The footpad swelling was scored using a digital micrometer (Mitituyo). Mice were sacrificed 5 wk after the infection; popliteal lymph nodes were collected for assessing parasite load (34) and for cytokine production by ELISA (32).

Testing the adjuvanticity of BPPcysMPEG in antileishmanial immunization

Three groups of mice, each containing 10 BALB/c mice, were immunized s.c. into the hind footpad with 1 × 10^6 formalin-fixed L. major. Fixation of the parasites was confirmed by the failure of parasites to grow in vitro. Immunized mice were treated twice with either Pam3CSK4 (1 μg/mouse) or BPPcysMPEG (1 μg/mouse) into the same footpad, on alternate days, starting from the first day of immunization, and one group was left untreated as a control. After 5 wk, all immunized and control mice were infected with 2 × 10^6 L. major promastigotes. Five weeks after the infection, mice were sacrificed and popliteal lymph nodes were collected for parasite enumeration (34), cytokine production assay, and Treg cell analyses (28).

Statistical analysis

The in vitro cultures were set in triplicates. We used 5–10 mice per group for in vivo experiments, as indicated. Error bars signify SEM from one representative from multiple sets of experiments. Student’s t test was used to assess the significance of the differences between the mean values for control and experimental groups.
Results

TLR1, TLR2, and TLR6 are differentially expressed on L. major-infected macrophages

Because several TLRs are implicated in the control of Leishmania infection (11, 12, 14, 23, 35–39), we checked the expression of all TLRs in uninfected and L. major-infected macrophages by RT-PCR (Table I). We observed an increased expression of TLR1 and TLR11/TLR12, but decreased expression of TLR4 and TLR9 (Fig. 1A). Among these altered TLRs, TLR4 and TLR9 promote host-protective antileishmanial immune responses (23, 40–42) and TLR11 and TLR12 are not yet implicated in Leishmania infection. However, the role of TLR1 in Leishmania infection remained to be elucidated. As TLR1 functions as a heterodimer with TLR2, which can also bind to TLR6, we first confirmed TLR1, TLR2, and TLR6 expression in uninfected and L. major-infected macrophages. The real-time PCR and Western blot data confirmed that TLR1 expression was increased in L. major infection (Fig. 1B).

Next, we tested the association between TLR1 and TLR2 and between TLR2 and TLR6 in uninfected and L. major-infected macrophages. It was observed that TLR1–TLR2 and TLR2–TLR6 associations were enhanced, but the TLR2–TLR6 association was reduced in L. major-infected macrophages (Fig. 1C). Because the ligands can promote heterodimerization of receptors, we tested whether providing extraneous TLR2–TLR6-ligand BPPcysMPEG would restore TLR2–TLR6 association in the infected macrophages. It was observed that BPPcysMPEG enhanced the association between TLR2 and TLR6 in both uninfected and L. major-infected macrophages (Fig. 1D). These results suggest that L. major selectively augments TLR1 expression; the parasite enhances TLR1–TLR2 and TLR2–TLR6 association, but reduces TLR2–TLR6 association. It is plausible that the parasite reduces the TLR2–TLR6 association as an immune evasion strategy. In that case, befitting the principle of parasitism, the reduced association between TLR2 and TLR6 implies a host-protective role in L. major infection.

TLR1 and TLR2 shRNA reduced L. major infection, but TLR6 shRNA increased the infection

To test the plausible roles for TLR1, TLR2, and TLR6 in L. major infection, the expression of each of these TLRs was silenced using respective shRNA, followed by L. major infection of the BALB/c mice. We observed that TLR1 and TLR2 shRNA reduced the parasite load in macrophages (Fig. 2A); a part of the TLR2 shRNA-induced reduction in parasite load could be due to less internalization (Fig. 2B). In BALB/c mice, TLR1 shRNA and TLR2 shRNA reduced parasite burden, whereas TLR6 shRNA-treated BALB/c mice increased the parasite load compared with that observed in control shRNA-treated mice (Fig. 2C). These

FIGURE 1. L. major infection differentially regulates the expression of TLR1, TLR2, and TLR6 and association of TLR1 or TLR6 with TLR2. BALB/c-derived thioglycolate-elicited peritoneal macrophages were kept either uninfected (UIM) or infected with L. major promastigotes at 1:10 ratio for 72 h (IM). (A) The cells were lysed and the mRNA levels were analyzed for all TLRs by RT-PCR. Densitometric quantitation was performed using the Quantity One software (Bio-Rad), and the values for genes were normalized against β-actin (right panel) (A). (B) cDNA from the above experiments was used to check the expression of TLR1, TLR2, and TLR6 by real-time PCR. mRNA expression levels of the target genes were normalized against GAPDH and expressed as relative fold change compared with uninfected controls. The cells from the above experiments were lysed, and proteins were used for Western blotting for TLR1, TLR2, and TLR6 (lower panel) (B). (C) Equal amount of protein was immunoprecipitated with anti-TLR2 Ab, and the association of TLR1, TLR2, and TLR6 was checked by immunoblotting using respective Abs. Densitometric values for TLR1, TLR2, and TLR6 expression were normalized against the values of β-actin (right panel) (C). (D) Macrophages were stimulated with BPPcysMPEG (100 ng/ml) for 15 min and lysed; equal amount of protein was immunoprecipitated with anti-TLR2, and the association of TLR6 was checked by immunoblotting. Data shown are densitometric unit for TLR6 (right panel) (D). From the same set of the experiment, equal amount of protein was resolved and blotted for β-actin to ensure the equal loading. The experiments were performed thrice. Results from one representative experiment are shown. The densitometric values represent mean ± SEM. *p < 0.05. **p < 0.01. ***p < 0.001. D-Units, densitometric units.
observations suggest that TLR1 and TLR2 promote *L. major* infection, whereas TLR6 plays an antileishmanial role.

Because TLR2–TLR6 association was reduced in *L. major*-infected macrophages, but restored by its ligand BPPcysMPEG, we tested the effect of its ligand on parasite growth in BALB/c-derived peritoneal macrophages and compared with the effects of Pam3CSK4, a ligand for TLR1–TLR2 heterodimer, and PGN, a ligand for TLR2 on the infection. It was observed that Pam3CSK4 and PGN increased the infection, but BPPcysMPEG reduced the infection in a dose-dependent manner (Fig. 2D). Corroborating with this observation, among these three ligands, BPPcysMPEG appeared to be the strongest inducer of IL-12 (Fig. 2E), a host-protective cytokine (13, 22); iNOS (Fig. 2F), the enzyme that catalyzes the generation of NO, which kills *Leishmania* (43); and TLR9 (Fig. 2G), which is also reported to play a host-protective role by inducing IL-12 (44). Thus, BPPcysMPEG is observed to enhance key host-protective functions in vitro.

**BPPcysMPEG activates higher p38MAPK and ATF2**

Because Pam3CSK4, PGN, and BPPcysMPEG, the ligands for TLR1, TLR2, and TLR6, respectively, induced differential effector functions in macrophages, we examined the signaling induced by these ligands. As TLR2 signals through the adaptor molecules MyD88, TIRAP, and TRAF6 (44), TLR2 was immunofluorescently double stained with an antibody against TLR2 and the indicated antibodies. The images were analyzed for the intensity of fluorescence using ImageJ software. The results showed that TLR2 signals through the adaptor molecules MyD88, TIRAP, and TRAF6 (44).

**FIGURE 2.** TLR1 and TLR2 increased, whereas TLR6 reduced *L. major* infection. BALB/c-derived thioglycolate-elicited peritoneal macrophages were transduced with lentivirally expressed TLR1, TLR2, TLR6 shRNA-Lv, or control shRNA-Lv (two transduction units per cell) for 48 h. (A) Untransduced or TLR-specific lentivirally transduced cells were infected with *L. major* for 6 h. Extracellular parasites were washed out, and parasite burden was assessed by Giemsa staining. RT-PCR was performed to check the inhibition of TLR1, TLR2, and TLR6 expression by respective TLR shRNA Lv or control shRNA Lv, transduced in macrophages (lower panel) (A). (B) Internalization of *L. major* parasites in peritoneal macrophages was checked 1 h postinfection. (C) BALB/c mice (*n* = 7) were injected s.c. with 5 × 10⁶ transduction units (in 50 μl HBSS) TLR1, TLR2, TLR6 shRNA Lv, or control shRNA Lv into hind footpad and were infected s.c. with 2 × 10⁷ *L. major* in same hind footpad 3 d after lentiviral treatment. Some mice left untreated were infected with *L. major* and were kept as controls. Footpad thickness was measured weekly to assess the severity of disease (upper panel). Five weeks after the infection, mice were sacrificed and parasites were enumerated from the popliteal lymph node cells (lower panel), as described earlier. The experiments were repeated twice. The error bars represent mean ± SEM. (D and E) Thioglycolate-elicited BALB/c-derived macrophages were either left uninfected (UIM) or infected (IM) with *L. major* promastigotes at a ratio of 1:10 for 12 h, followed by washing out extracellular parasites and incubation for an additional 12 h. The cells were treated with the indicated doses of Pam3CSK4 (P3C), PGN, or BPPcysMPEG (BPP) for another 48 h. (D) Parasite burden was assessed by Giemsa staining. (E) IL-10 and IL-12 (p70) from the culture supernatants of uninfected and *L. major*-infected macrophages treated with the indicated doses of P3C, PGN, or BPP were assessed by ELISA. The significance tests were performed to compare the IL-12 productions induced by BPPcysMPEG and the other two ligands. (F and G) Uninfected and 64-h *L. major*-infected macrophages were treated with the indicated doses of P3C, PGN, or BPP for another 8 h, followed by assessment of iNOS expression by RT-PCR (F) and TLR9 expression by RT-PCR and real-time PCR (G). For real-time PCR, doses used were P3C (100 ng/ml), PGN (10 μg/ml), or BPP (100 ng/ml) (right panel) (G). The experiments were performed three times. The error bars show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
noprecipitated from the Pam3CSK4-, PGN-, and BPPcysMPEG-stimulated macrophages, followed by immunoblotting for MyD88, TIRAP, and TRAF6. We observed that TLR2 recruited more MyD88 in response to PGN or BPPcysMPEG than to Pam3CSK4; only BPPcysMPEG drastically increased the TIRAP recruitment to TLR2, whereas Pam3CSK4 showed a tendency to increased recruitment of TRAF6 to TLR2 (Fig. 3A). As signals from membrane receptors are taken to the nucleus via p38MAPK and ERK1/2 (45, 46) and transcription factors, it was possible that the observed differences between TLR2 ligands might occur due to differential phosphorylation and activation of these signaling intermediates. We observed that Pam3CSK4, PGN, and BPPcysMPEG also differed in their ability to activate p38MAPK, ERK1/2, and transcription factors. Compared with Pam3CSK4 and PGN, BPPcysMPEG activated more p38MAPK and ATF2 than ERK1/2 and ELK1 (Fig. 3B). It was reported that the L. donovani-infected THP-1 cells, a macrophage-like cell line, responded to Pam3CSK4 by increasing higher ERK1/2 and IL-10 production (14). Taken together, these observations suggest that BPPcysMPEG signals through p38MAPK and ATF2, whereas Pam3CSK4 and PGN signaled primarily through ERK1/2 and ELK1.

The expression of TLR1, TLR2, and TLR6; the associations between these three TLRs; and the effects of the respective TLR shRNAs and ligands on various effector functions, including the L. major infection, identify TLR2–TLR6 and its ligand BPPcysMPEG as the host-protective receptor–ligand pair. Therefore, we further assessed the mechanism of the host-protective function of BPPcysMPEG.

**BPPcysMPEG induces T cell–mediated Th1 response both in vitro and in a susceptible host**

As BPPcysMPEG showed antileishmanial effects in macrophages in vitro, we tested its effect on the induction of T cell–mediated host protection in a macrophage–T cell coculture system. When L. major-infected macrophages were cocultured with the lymph node T cells from L. major-infected BALB/c mice, the amastigote numbers increased in macrophages, but BPPcysMPEG treatment reduced the parasite count (Fig. 4A). A lower dose of BPPcysMPEG increased both IL-4 and IFN-γ production, but IL-4 production was reduced, whereas IFN-γ production increased, with the higher doses of BPPcysMPEG (Fig. 4B); a resultant effect of these changes was significantly enhanced IFN-γ production (Fig. 4B, inset). These observations suggested that BPPcysMPEG enhanced the antileishmanial effect even in presence of the disease-exacerbating T cells, which predominated in the draining lymph node of a L. major-infected susceptible host. Therefore, we next examined the antileishmanial effect of BPPcysMPEG treatment on L. major infection in BALB/c mice, which received three BPPcysMPEG treatments on alternate days starting from the third day postinfection. We observed a significant reduction in parasite load in the lymph node of the BPPcysMPEG-treated BALB/c mice (Fig. 4C, inset). This antileishmanial effect was accompanied by heightened IFN-γ.

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**FIGURE 3.** BPPcysMPEG activates higher p38MAPK and ATF2. (A) Thioglycolate-elicited BALB/c-derived macrophages were treated with the indicated doses of Pam3CSK4, PGN, or BPP for 15 min and lysed, and an equal amount of protein was immunoprecipitated with anti-TLR2 Ab and blotted with Abs against MyD88, TIRAP, and TRAF6. Densitometric values were normalized against the values of β-actin (lower panel) (A). Densitometry shown is the collated data from two experiments. Error bars show mean ± SEM. (B) Cell lysates were immunoblotted for p-p38MAPK, p-ERK1/2, p-IKKα/β, p-NF-κB, p-ATF2, p-ELK1 or total p38MAPK, ERK1/2, IKKα, NF-κB, ATF2, and ELK1. Densitometric values were normalized against the values of respective total proteins, as indicated (lower panel) (B). The data from two repeat experiments are shown in the densitometry. The ratios of p-p38/ERK1/2 or p-ATF2/p-ELK1 were calculated from the mean values of their respective lanes.
production, but lowered IL-4 production (Fig. 4D). An apparent difference in the profile of BPPcysMPEG-induced changes in IL-4 and IFN-γ productions in vitro (Fig. 4B) and in BALB/c mice (Fig. 4D) was reconciled as the ratio of IFN-γ to IL-4 (Fig. 4B, inset), which showed a drastic increase in IFN-γ production compared with IL-4. Another plausible explanation for the apparent discrepancy is more complex interaction between APCs and T cells in vivo than that observed in defined macrophage–T cell coculture. Thus, the TLR2/TLR6 ligand, BPPcysMPEG, establishes host-protective T cells to exert its antileishmanial functions.

**TLR6 shRNA significantly abrogated the antileishmanial effects of BPPcysMPEG**

Because BPPcysMPEG, a TLR2/6 ligand, increased TLR9 expression, we studied the synergism between TLR2/TLR6 and TLR9. BALB/c-derived thioglycolate-elicited macrophages were transduced with TLR6 shRNA or control shRNA-expressing lentivirus, followed by L. major infection and treatment with BPPcysMPEG and CpG, or left untreated as control. It was observed that TLR6 shRNA-treated cells reversed the antileishmanial response to BPPcysMPEG or CpG alone or in combination (Fig. 5A). The TLR6 shRNA-transduced macrophages produced less IL-12, but higher IL-10 in response to these ligands (Fig. 5B). Next, BALB/c mice were treated with lentivirally expressed TLR6 shRNA or control shRNA, followed by L. major infection and treatment with BPPcysMPEG or CpG, or were left untreated. It was observed that TLR6 shRNA significantly abrogated the antileishmanial effect of BPPcysMPEG and CpG (Fig. 5C). Whereas BPPcysMPEG and CpG increased IFN-γ production in these mice, TLR6 shRNA reduced IFN-γ production, but increased IL-4 production (Fig. 5D). These results clearly suggested that the observed antileishmanial effects of BPPcysMPEG were indeed due to TLR6 and that CpG and BPPcysMPEG synergize to effect the observed antileishmanial functions.

**BPPcysMPEG reduced Treg cells in cocultures with macrophages or DCs**

Treg cells promote *Leishmania* survival and growth in a susceptible host, as the numbers of Treg cells increase in progressive infection (24–28), but depletion of Treg cells reduces the disease (26, 47). As BPPcysMPEG reduced L. major infection in BALB/c mice (Fig. 4C), we examined whether BPPcysMPEG would decrease the number of Treg cells. To test the hypothesis, we cocultured the BPPcysMPEG-treated or untreated, L. major-infected macrophages or bone marrow–derived DC with CD4+ T cells from the draining popliteal lymph nodes of 21-d L. major-infected BALB/c mice. We observed that the BPPcysMPEG treatment reduced the number of CD3+CD4+CD127lowCD25+GITR−Foxp3+ cells in both macrophage–CD4+ T cell and DC–CD4+ T cell cocultures (Fig. 6) and reduced the number of IL-10+ cells in DC–CD4+ T cell cocultures (Fig. 6B). The culture supernatants were collected from DC–CD4+ T cell cocultures, and the levels of IL-12, IL-10, IL-4, and IFN-γ were assessed. We observed that a reduced number of Treg cells was associated with higher IL-12 and IFN-γ,

![FIGURE 4.](https://www.jimmunol.org/)

**BPPcysMPEG elicits host-protective immune response.** BALB/c-derived peritoneal macrophages were infected with *L. major* for 72 h. After washing out the extracellular parasites, macrophages were treated with the indicated doses of BPP and were cocultured with the CD4+ T cells isolated from the lymph nodes of *L. major*-infected (21-d–infected) and naive BALB/c mice at 1:3 ratio (APC:T cells). (A) After 72-h infection, macrophages were washed, fixed, stained with Giemsa stain, and evaluated for the amastigotes per 100 macrophages. (B) IL-4 and IFN-γ were assessed from culture supernatants, as described in Materials and Methods. (B, inset) Fold over change in cytokine IL-4 and IFN-γ was determined from the values of BPP treated against the values of infected macrophages cocultured with infected T cells. Data shown are the representative of one experiment, repeated twice. Error bars show mean ± SEM. (C) BALB/c mice were infected s.c. in hind footpad with 2 × 10^6 *L. major* promastigotes. Mice were treated with BPP (1 μg/mouse) into the *L. major*-infected footpad alternatively for 3 d starting from the third day postinfection or were infected with sterile PBS as control. Disease progression was scored weekly by evaluating the net footpad swelling (the thickness of the uninfected left footpad subtracted from the infected right footpad) by digital micrometer for 5 wk. (C, inset) Mice were sacrificed 5 wk after the infection, and parasite burden in lymph node cells was assessed. (D) Lymph node cells from mice, as indicated, in each group (n = 7) were pooled and stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (2 μg/ml) for 60 h. Culture supernatants were assessed for the production of the cytokines IL-4 and IFN-γ, as described in Materials and Methods. The experiment was repeated twice. Data shown are the representative of one experiment, and error bars show the mean ± SEM. *p < 0.05, **p < 0.01.
but reduced IL-10 and IL-4 levels (Fig. 6B, inset), suggesting that BPPcysMPEG-induced host protection was associated with decreased Treg cells and increased IL-12 induction. BPPcysMPEG-induced host protection is IL-12 dependent

Because BPPcysMPEG increased the production of IL-12 that played a host-protective role against Leishmania infection (13, 22), the uninfected and L. major-infected macrophages from IL-12–deficient or BALB/c mice were treated with BPPcysMPEG. It was observed that BPPcysMPEG reduced the number of amastigotes in wild-type, but not IL-12–deficient macrophages (Fig. 7A), commensurating with its effect on iNOS expression (Fig. 7B). Corroborating with these observations, the antileishmanial effect of BPPcysMPEG was significantly reduced in iNOS-deficient mice (Fig. 7C). Together these observations indicate that the antileishmanial effect of BPPcysMPEG is partly iNOS mediated. To confirm the role of IL-12 in BPPcysMPEG-induced antileishmanial defense, we tested the effects of BPPcysMPEG treatment on parasite load, IL-4/IFN-γ production, and Treg cell numbers in IL-12–deficient and wild-type BALB/c mice. We observed that BPPcysMPEG treatment reduced the severity of the disease (Fig. 7D) and parasite load in BALB/c mice, but not in the IL-12–deficient mice (Fig. 7E); the antileishmanial effect of BPPcysMPEG was accompanied by high IFN-γ, but less IL-4 productions (Fig. 7E) and reduced Treg cell numbers (Fig. 7F) in BALB/c, but not in IL-12–deficient mice. These observations indicate that the BPPcysMPEG-induced antileishmanial defense is IL-12 dependent, but Treg sensitive.

FIGURE 5. TLR6 shRNA significantly abrogated the antileishmanial effects of BPPcysMPEG. Thioglycolate-elicited BALB/c-derived mouse macrophages were transduced with TLR6 shRNA (TLR6 Lv) or control shRNA (control Lv) lentiviral particles. Forty-eight hours later, lentivirally transduced cells were either left uninfected or infected with L. major at a ratio of 1:10, followed by treatment with BPP (50 ng/ml) and CpG (0.12 μM) for a total 72-h infection. (A) Cells were stained with Giemsa, and amastigotes were enumerated. The amastigote numbers counted in the control CpG or control shRNA-Lv were 565.5 ± 27.5 and 684.5 ± 19.5, respectively. (B) Culture supernatants collected were assessed for IL-12 and IL-10 by ELISA. Ratio between IL-12:IL-10 was determined from the respective treatments. The IL-12:IL-10 ratios in the control CpG- and control shRNA-Lv–treated cultures were 0.33 ± 0.03 and 0.25 ± 0.05, respectively. (C and D) TLR6 lentivirus reversed the CpG-induced antileishmanial immune response in susceptible BALB/c mice. Some groups of BALB/c mice were treated with TLR6 shRNA Lv, 2 d later infected with L. major, and treated with BPP (1 μg/mouse) and CpG (10 μg/mouse) together or alone on alternating 3 d, starting from 2 d postinfection. (C) Parasite load was determined wk after the infection. Number of parasites enumerated from control CpG- or control shRNA-Lv–treated mice were (69.75 ± 9.4681) × 10^6 and (88.333 ± 13.4361) × 10^6, respectively. (C, inset) Immunoblot analysis of TLR6 expression from footpad lesion (FP) and lymph node (LN) of L. major–infected BALB/c mice treated with control shRNA-Lv and TLR6 shRNA-Lv 5 wk after L. major infection. (D) Total lymph node cells from various mice groups were stimulated with anti-CD3 Ab (0.5 μg/ml) and anti-CD28 Ab (2 μg/ml) for 60 h. Culture supernatants from different groups were assessed for IL-4 and IFN-γ production by ELISA. The values for IL-4: control CpG (478.5 ± 33.5 pg/ml), control shRNA-Lv (913.0 ± 8.0 pg/ml); the values for IFN-γ: control CpG (384 ± 12 pg/ml), control shRNA-Lv (313.5 ± 90.5 pg/ml). The experiments were performed twice, and one representative data are shown. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
**BPPcysMPEG induces host-protective prophylactic antileishmanial effects**

As BPPcysMPEG reduced parasite load, accompanied by an IFN-γ-dominated response in vitro and in BALB/c mice, we examined the adjuvanticity of BPPcysMPEG in establishing host-protective antileishmanial memory response. The BPPcysMPEG treatment during priming with the fixed *L. major* promastigotes resulted in significantly reduced parasite load in these mice, compared with the untreated controls or unprimed controls, following a challenge *L. major* infection (Fig. 8A). The protection was accompanied by high IFN-γ, but low IL-4 (Fig. 8B). The protection was associated with reduced Treg cells in the BPPcysMPEG-treated group (Fig. 8C). These observations together indicate that BPPcysMPEG possesses a strong adjuvanticity against *L. major* infection.

**Discussion**

This work documents several unique observations. First, the expression of the TLR2 associates, TLR1, TLR2, and TLR6, and their dimerizations is differentially modulated by *L. major* infection in macrophages. The reduced TLR2–TLR6 association in *L. major*-infected macrophages was restored by its synthetic ligand BPPcysMPEG. Second, TLR1 or TLR2 silencing reduced *L. major* infection, but TLR6 silencing increased the infection. Conversely, the ligands Pam3CSK4 and PGN exacerbated the infection, but BPPcysMPEG reduced the infection. Third, these host-protective effects of BPPcysMPEG were accompanied by increased TLR9 expression, enhanced IL-12 production, and higher iNOS induction. Fourth, as a possible mechanism for its host-protective effects, BPPcysMPEG induced leishmanicidal activities in T cells from the *L. major*-infected mice and reduced Treg cell numbers. These host-protective effects were TLR6 mediated, IL-12 dependent, iNOS dependent, and Treg sensitive. Finally, targeting TLR2–TLR6 led to significant reduction in parasite load in both therapeutic and prophylactic modes, accompanied by modulation of T cell responses. Thus, the role for TLR2 in selective heterodimerizations with either TLR1 or TLR6 in *L. major* infection assumes significance, which was indicated by the strong antileishmanial effect of the TLR2–TLR6 ligand, BPPcysMPEG, but not by the ligand of the TLR1–TLR2 heterodimer. Altogether, to our knowledge, this is the first demonstration of TLR2 functional duality, contingent upon its dimerization with either TLR1 or TLR6.

The increased TLR1–TLR2 or TLR2–TLR2 association, but reduced TLR2–TLR6 association, implies selective targeting of TLR2 partners by the parasite. It is possible that due to *Leishmania*-induced changes in membrane anisotropy of the infected macrophages (48), the interaction between the hydrophobic domains of these TLRs (49) is differentially affected, resulting in altered associations in *L. major*-infected macrophages. In contrast, TLR1 or TLR2 silencing reduces, but TLR6 silencing increases, *L. major* infection. It is possible that silencing of one TLR alters the other two TLRs’ heterodimerizing propensity. Thus, the initial difference between TLR1–TLR2, TLR2–TLR2, and TLR2–TLR6...
dimers can be induced either by alteration of the physical properties of the macrophage membrane or by alteration of the ratios among these three TLRs. Through a cascade of events, these initial changes led to contrasting outcomes of *L. major* infection.

The cascade of events may be represented by the differential signaling triggered by the ligand-induced homotypic or heterotypic association of TLR2. For example, Pam3CSK4 and PGN trigger primarily ERK1/2 and ELK1 activation, whereas BPPcysMPEG induces p38MAPK phosphorylation, leading to activation of C-Jun (50) that can dimerize with the BPPcysMPEG-activated ATF2 to induce inflammatory response (51, 52). In contrast, ERK1/2 activates ELK1 via AP-1, which binds to activated c-Fos (53, 54). How these signaling pathways are reciprocally related to the TLR1–TLR2– or TLR2–TLR6–induced contrasting outcomes—disease promotion or host protection—of *L. major* infection awaits elucidation.

One mechanism of the host-protective antileishmanial function of BPPcysMPEG is related to the enhanced expression of TLR9, IL-12, and iNOS. iNOS catalyzes the formation of NO (11, 43), which kills *Leishmania* (11, 32). As TLR6 silencing significantly compromised the antileishmanial function of BPPcysMPEG, the failure of IL-12–deficient macrophages to reduce *L. major* infection in response to BPPcysMPEG treatment suggests a significant role for autocrine IL-12 in TLR2–TLR6-dependent *Leishmania* killing. Finally, as TLR9 recognizes *L. major* DNA and induces IL-12 production (41), it is possible that the leishmanial DNA-activated TLR9-induced IL-12 may exert antileishmanial functions in macrophages. As T cells were absent in all these assays, this mode of antileishmanial function of BPPcysMPEG can be attributed to innate immune mechanisms.

Besides the innate immune mechanisms, BPPcysMPEG is also shown to affect T cells in the coculture with macrophages and DCs. It is possible that the T cell–dependent antileishmanial functions of BPPcysMPEG build up from its innate immune effects. For example, the TLR2–TLR6–activated or TLR9-activated IL-12 can set a Th1 bias, as BPPcysMPEG failed to exert its antileishmanial effects in IL-12–deficient mice. In a different study, BPPcysMPEG, along with IFN-γ, is shown to reduce the allergic airway inflammation (55). Because the allergic manifestations in the airway may indicate exaggerated Th2 response (21), the reduction in the airway allergy by BPPcysMPEG implies possible antagonism of Th2 differentiation. As Th2 responses exacerbate *L. major* infection (56), it is possible that BPPcysMPEG exerts therapeutic and prophylactic effects by facilitating Th1 response (57). The enhanced productions of IL-12 in macrophages in vitro and of IFN-γ in BPPcysMPEG-treated mice indicate the Th1 response-facilitating role for TLR2–TLR6 heterodimers and thereby its host-protective role against *L. major* infection.

In contrast, one of the disease-promoting T cells in *L. major* infection is Treg cells, as the reduction in parasite burden has been correlated with reduced number of Treg cells (24–28). To explore the prophylactic role for TLR2–TLR6 heterodimer, we primed BALB/c mice with fixed *L. major* and BPPcysMPEG, followed by challenge infection with *L. major*. BPPcysMPEG, but not Pam3CSK4, the ligand for the TLR1–TLR2 heterodimer, established a host-protective antileishmanial immune response, accompanied
by significantly reduced Treg cells. Because Treg cell differentiation depends on TGF-β (58), it is possible that PamCSK4 induced much higher TGF-β than that induced by BPPcysMEPG (data not shown). Thus, the effects of BPPcysMEPG on Treg cells reveal a unique facet of TLR2-regulated antileishmanial immune response.

The decreased TLR2–TLR6 association in L. major-infected macrophages indicates a parasite-devised immune evasion strategy unfolding an intriguing TLR2-targeted host–parasite interaction that has not been reported earlier. In case of intracellular pathogens, such as Leishmania, Salmonella, Listeria, and Mycobacterium, the pathogens need to survive within the host cells and also to evade adaptive immune responses that can eliminate these pathogens. Therefore, it was logical to hypothesize that, to ensure their intracellular survival, the pathogen would modulate the expression of the intracellular TLRs as a consequence of their initial interactions with the cell surface TLRs. Indeed, the ligands for the TLR1–TLR2 and TLR2–TLR6 heterodimers differentially modulated the expression of TLR9, an intracellular TLR, clearly suggesting the significance of the selective TLR2 heterodimerization with TLR1 or TLR6. As a novel therapeutic strategy, we demonstrate that a combinatorial target of TLR2, TLR6, and TLR9 can indeed elicit an effective host-protective antileishmanial immune response.

Disclosures
C.A.G. and T.E. are named as inventors in patents covering the immune modulatory properties of BPPcysMEPG.

References


60. Li, C., P. J. Ebert, and Q. J. Li. 2013. T cell receptor (TCR) and transforming growth factor β (TGF-β) signaling converge on DNA (cytosine-5)-methyltransferase to control forkhead box protein 3 (foxp3) locus methylation and inducible regulatory T cell differentiation. J. Biol. Chem. 288: 19127–19139.