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Phosphoinositide 3-Kinase-Dependent Inhibition of Dendritic Cell Interleukin-12 Production by *Giardia lamblia*▽

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Dendritic cell interactions with pathogenic microbes initiate and direct the development of subsequent adaptive responses. The protozoan pathogen *Giardia lamblia* infects the mammalian small intestine, leading to nutrient malabsorption and diarrhea but rarely causing inflammation. In order to begin to understand how the innate immune system responds to this parasite and shapes the eventual adaptive response, we examined the interaction between parasites and murine bone marrow-derived dendritic cells (DCs). DCs incubated with live parasites or parasite extracts displayed enhanced levels of CD40. The expression of CD80 and CD86 also increased, but less than was seen with lipopolysaccharide-activated DCs. Small amounts of interleukin-6 (IL-6) and tumor necrosis factor alpha were secreted by these DCs, whereas no IL-10 or IL-12 could be detected. Coincubation of DCs with parasite extracts along with known Toll-like receptor (TLR) ligands resulted in enhanced secretion of IL-10 and reduced secretion of IL-12. The levels of major histocompatibility complex class II, CD80, and CD86 were also reduced compared to DCs stimulated with TLR ligands alone. Finally, studies with an extracellular signal-regulated kinase 1/2 pathway inhibitor, a phosphoinositide 3-kinase (PI3K) inhibitor, and anti-IL-10 receptor antibody revealed that the PI3K pathway is the dominant mechanism of inhibition in DCs incubated with both lipopolysaccharide and *Giardia*. These data suggest that this parasite actively interferes with host innate immunity, resulting in an immune response able to control the infection but devoid of strong inflammatory signals.

Interactions between invading pathogens and cells of the innate immune system, especially dendritic cells (DCs), are indispensable for the induction of immune responses. The DC phenotype that results from their activation by antigens in turn determines the type of T-cell differentiation and thus the type of adaptive immunity that is induced. The resulting DC signals that influence T-cell differentiation include presentation of the antigen to T cells in the context of major histocompatibility complex (MHC) receptors, expressed costimulatory molecules, and cytokine production by the DCs. Although interactions between DCs and the vast majority of prokaryotic pathogens have been studied, only a few studies have investigated DC interactions with eukaryotic pathogens. For example, products of malaria parasite infection (hemozoin) have been shown to activate DCs through Toll-like receptor 9 (TLR9) (13) and profilinlike molecules in *Toxoplasma gondii* activate DCs through TLR11 (48), while heat shock protein 70 from the same parasites activates DCs through TLR4 (3). TLR2, -4, and -9 have been shown to recognize lipophosphoglycans and DNA from *Entamoeba histolytica* (23, 31). TLR2 and TLR4 have also been shown to be important in innate responses to *Cryptosporidium parvum* (11, 35), and recently this parasite was shown to enhance TLR4 expression via a microRNA-mediated mechanism (12). Interestingly, some studies have shown an ability of parasites to manipulate host immune responses. Antigens from *T. gondii* (7, 34) and *Schistosoma mansoni* (10, 24, 49) have been shown to suppress production of proinflammatory cytokines such as interleukin-12 (IL-12) from TLR-activated antigen-presenting cells. Secreted *Trypanosoma cruzi* molecules have been shown to activate DCs through TLR4, while infection inhibits DC maturation and responses to lipopolysaccharide (LPS) (44, 45). Finally, interactions between dendritic cells and *Leishmania spp*. can produce a range of both activation and inhibition depending on the particular systems analyzed (42). Parasite modulatory responses likely occur to create a balance between better host exploitation or prevention of parasite death and immunopathology that could compromise host survival and that of the parasite by extrapolation (20).

Giardia lamblia is a flagellated protozoan that infects the small intestine of humans and several other vertebrates, causing nutrient malabsorption, cramps, and diarrhea. It is transmitted principally by food and water contaminated with cysts shed from infected hosts. Estimates of human infections range from 0.2 to 1.0 billion per year, including ~2.5 million cases per year in the United States (19). Most *Giardia* infections result in no overt symptoms to the host. One study determined that 60 to 80% of infected children in day care centers and their household contacts had asymptomatic giardiasis (27). Subjects with symptomatic giardiasis present with fatty diarrhea, abdominal cramps, and a malabsorption syndrome, severe forms of which result in weight loss and interference with normal mental and physical development in children (15). Symptomatic disease is not associated with overt inflammation, and the resultant diarrhea is thought to be due to a combination of nutrient malabsorption, epithelial barrier defects, and ion secretion (6, 15, 32, 41). Adaptive immune responses have been shown to be crucial for the control of this infection (16, 36). Recently, it was shown that epithelial cells cultured with *Giardia* released CCL20, a chemokine able to recruit DCs and T cells to the intestinal mucosa (36). However, no studies have

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yet been reported concerning the direct interactions between DCs and *Giardia* or their role during infection.

In the present study, we sought to characterize the DC responses induced by their interaction with *G. lamblia* by coincubating bone marrow-derived DCs with *Giardia* extracts. We show that *G. lamblia* is a weak activator of murine bone marrow-derived DCs, since extracts induce only small amounts of IL-6 and tumor necrosis factor alpha (TNF-α), compared to stimulation of DCs by LPS. *Giardia* does not induce DC production of IL-12 nor IL-10. Interestingly, *Giardia* extract potently inhibits the production of IL-12 and the expression of costimulatory molecules by TLR-activated DCs, while augmenting IL-10 production by these same cells. Finally, we show that IL-12 inhibition is primarily dependent on phophoinositide 3-kinase (PI3K) activity, since inhibition of this enzyme by its specific inhibitor, wortmannin, restored substantial amounts of *Giardia*-inhibited IL-12 produced by TLR-stimulated DCs.

MATERIALS AND METHODS

Parasites and extracts. The GS(M)/H7 clone of *G. lamblia* is a human isolate that was adapted to axenic culture in 1987 (1) and first shown to readily infect adult animals from several strains of laboratory mice in 1994 (8). Trophozoite forms were propagated in vitro in Keister's modified TYI-S-33 medium (26). Parasites were harvested by chilling on ice, collected by centrifugation, and washed three times with endotoxin-free phosphate-buffered saline (PBS). Extract was made by three freeze-thaw cycles and stored in aliquots at −70°C. The total protein concentration was measured by converting absorbance, with an A_{280} of 1 defined as 1 mg/ml.

Antibodies and reagents. Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was obtained from PeproTech, Rocky Hill, NJ. Fluorescent antibodies for CD80, CD86, CD40, CD11c, CD11b, MHC class II (MHC-II) (A^d), and CD8a were obtained from Pharmingen, San Diego, CA. Anti-IL-10 receptor antibodies were kindly provided by Nancy Noben-Trauth of George Washington University, Washington, DC. LPS from *Salmonella enterica* serovar Typhimurium was obtained from Sigma-Aldrich, and ligands for TLR2, -3, and -9—PAM3CSK4 {*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[R]-Cys-[S]-Serl-[S]-Lys(4) trihydrochloride}, poly(I:C), and CpG DNA, respectively—were obtained from InvivoGen, San Diego, CA. Polymyxin B was obtained from Calbiochem, San Diego, CA. Aliquots of 1 mg/ml were made by dilution with sterile, pyrogen-free water and stored at −20°C until used. Rabbit polyclonal antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated ERK were obtained from Cell Signaling, Inc., San Diego, CA. Wortmannin was obtained from Calbiochem, San Diego, CA, while the MEK1/2 inhibitor, U0126, was obtained from Cell Signaling, Inc. Drugs were dissolved in dimethyl sulfoxide (DMSO) at 100 μM, and aliquots were stored at −20°C until used. In experiments requiring drugs, DCs were pretreated for 30 min with the drugs before the other treatments were performed.

Cells and cell cultures. BALB/cJ and C57BL/6J female mice were obtained from Jackson Laboratories, Bar Harbor, ME, and housed at the Georgetown University Comparative Medicine Animal Facilities. All mouse experiments were approved by the Georgetown University Animal Care and Use Committee. DCs were generated from mouse bone marrow according to a previously described protocol (30). Briefly, bone marrow from the femurs and tibias of mice was harvested in DC culture medium (RPMI 1640, 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 20 ng of GM-CSF/ml) and grown in 100-mm petri dishes. Four million cells were seeded per dish in a total initial volume of 10 ml. Cultures were maintained in 5% CO_2 atmosphere incubators at 37°C. Fresh medium with 20 ng of GM-CSF/ml was supplemented on day 3, while half of the culture medium was replaced with fresh medium and GM-CSF on days 6 and 8. Nonadherent cells were harvested on day 10 of culture and used as DCs. Cells were typically determined to be 80 to 90% $CD11c^+$ by flow cytometry (data not shown). DCs were stimulated with *Giardia* extracts and/or TLR ligands in 24-well plates at a density of 10^6 DCs/well in 1 ml of medium for 18 h. Supernatants were then collected and stored at −20°C for cytokine measurements by enzyme-linked immunosorbent assay (ELISA), while cells were stained with fluorescent antibodies for surface molecule analysis by flow cytometry.

ELISAs. Cytokines in cell culture supernatants were measured by using the Pharmingen ELISA kits for mouse IL-12p70, IL-10, IL-6, and TNF-α. Aliquots of standards were prepared by diluting with 10% fetal calf serum in PBS at manufacturer-recommended concentrations and stored at −80°C. We strictly adhered to the manufacturer's recommended procedure. All samples were analyzed in duplicate. Optical densities were measured at 450 nm with a background correction at 630 nm, using the ELx 800 reader and the KCjunior software (Bio-Tek Instrumenets, Inc., Winooski, VT). The optical densities were converted to cytokine concentrations by using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Flow cytometry. Staining buffer for flow cytometry consisted of 4% adult bovine serum and 0.05% sodium azide in PBS. DCs were incubated with phycoerythrin-, fluorescein isothiocyanate-, allophycocyanin-, or phycoerythrin/Cy5 fluorochrome-conjugated antibodies against DC surface molecules in staining buffer for 20 min at 4°C. Cells were washed twice with 10 times the staining volume with PBS and fixed with 4% formaldehyde before analysis on a FACSAria flow cytometer (Becton Dickinson, San Jose, CA). Only CD11c-expressing cells were analyzed.

Western blots. DCs were cocultured with *Giardia* extract, LPS, or both in six-well plates at cell concentrations of 5×10^6/ml. Cells were harvested and washed twice with PBS. Total protein was extracted by using mammalian protein extraction reagent (Pierce, Rockford, IL) and stored at −20°C until used. Samples of 20 μg of protein/lane were separated on 12% NuPage Bis Tris gels as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes, blocked with 1% bovine serum albumin in PBS, and incubated with anti-phospho-ERK1/2 and then horseradish peroxidase-conjugated anti-rabbit antibodies according to the specifications of the antibody manufacturer (Cell Signaling, Inc., San Diego, CA). Membranes were then incubated at room temperature for 1 to 2 min with Western blotting detection reagent (Amersham Biosciences, United Kingdom) before exposure to X-ray films. Blots were stripped with Restore-Western blot stripping buffer (Pierce) and reprobed with total ERK1/2 antibodies.

Statistical analyses. Data were analyzed by using Prism software v4.0 (GraphPad Software). Means of groups were compared by using Student *t* tests. *P* values of <0.05 were considered significant.

RESULTS

***G. lamblia* weakly activates DCs.** Activation of the adaptive immune system is required for effective control of *Giardia* infections (8). We therefore sought to determine the nature of the innate response to *Giardia* by exposing bone marrow-derived DCs to parasite antigens. DCs were therefore incubated with *Giardia* extract, and the expression of the costimulatory surface molecules CD80, CD86, and CD40 was determined by flow cytometry. CD40 was induced by 1 mg of *Giardia* extract/ml to a similar extent as by 10 ng of LPS/ml (Fig. 1A). Although CD80 and CD86 were also induced, the level of induction was not as robust as seen with LPS (Fig. 1B and C). Stimulation with greater amounts of extract never resulted in induction of CD80 or CD86 to levels seen with LPS (data not shown). Thus, we concluded that *Giardia* weakly induces activation of DCs.

Poor activation of DCs was even more apparent when we analyzed cytokines present in the supernatants of DCs incubated with *Giardia* extracts. Although LPS potently activated the secretion of IL-12, IL-10, IL-6, and TNF-α, *Giardia*-stimulated DCs produced no IL-10 or IL-12 and relatively small amounts of IL-6 and TNF-α (Fig. 1D to G). Combining data from multiple experiments utilizing different DC cultures and batches of extract, the median level of IL-6 produced by DCs stimulated with *Giardia* extract was 96 pg/ml (range, 38 to 359 pg/ml, $n = 13$), while unstimulated DCs failed to produce detectable IL-6. A similar analysis of TNF-α production found a median of 99.5 pg/ml (range, 0 to 630 pg/ml, $n = 10$). To determine whether the observed responses were due to the

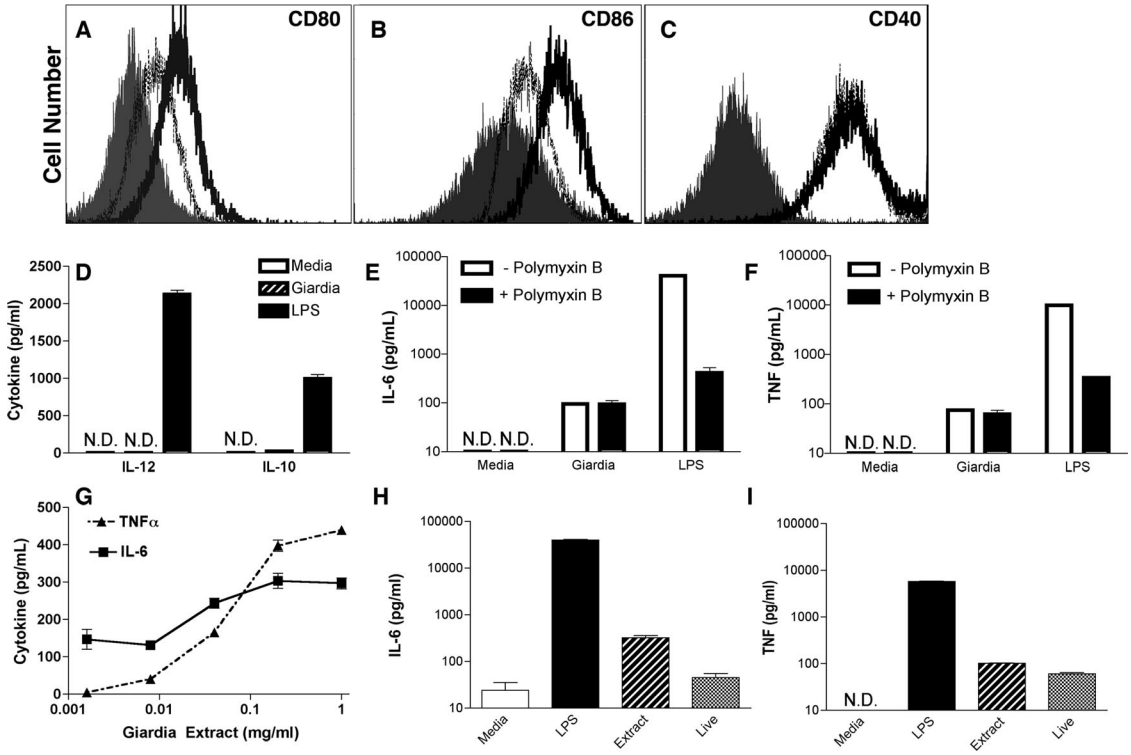

FIG. 1. Response of mouse bone marrow DCs to *G. lamblia*. One million DCs were incubated overnight with medium alone (filled curves), 1 mg of *Giardia* extract/ml (dashed lines), or 10 ng of LPS/ml (solid lines). $CD11c^+$ cells were analyzed for CD80 (A), CD86 (B), and CD40 (C) expression by flow cytometry. Overlap in the LPS and *Giardia* stimulated cells for CD40 expression obscures the *Giardia* trace. Culture supernatants were analyzed for IL-12p70 and IL-10 (D). For analysis of IL-6 (E) and TNF-α (F), DCs were stimulated with 1 mg of *Giardia* extract/ml or 10 ng of LPS/ml in the presence or absence of 100 μg of polymyxin B/ml. IL-6 and TNF-α production were also measured in response to titrated amounts of *Giardia* extract (G) or to live *Giardia* trophozoites (H and I) at a 10:1 parasite/DC ratio. This ratio was equivalent to a total protein concentration of 1 mg/ml, as was used for parasite extracts. Medium alone and 10 ng of LPS/ml were also used as controls. N.D., none detected. Flow cytometry data are representative of four independent experiments. Cytokine data are presented as means ± the standard error of the mean (SEM) of duplicate cultures and are representative of three or more independent experiments each.

presence of small amounts of contaminating LPS, DCs were stimulated with either *Giardia* extract or LPS in the presence of polymyxin B. Although polymyxin B inhibited >95% of the IL-6 and TNF-α produced in response to LPS ($P < 0.05$), no inhibition of cytokine production in response to *Giardia* was observed, indicating that LPS contamination was not present (Fig. 1E and F). To define the active components of the extract, we stimulated DCs with extracts that had been heated to 65°C for 15 min. No decrease in activation was observed (data not shown), indicating that a conformational protein determinant may not be involved in DC activation. Similarly, the addition of up to 40 mM soluble *N*-acetylglucosamine (GlcNAc), a sugar found on some *Giardia* proteins and recognized by the innate immune system, also failed to block the effect of the extract on DCs (data not shown).

Titrations of the extract were then used to stimulate DCs to determine the concentration-specific dynamics of DC cytokine secretion upon *Giardia* stimulation. Levels of IL-6 and TNF-α were detectable above background using as little as 40 μg of extract/ml. The production of both cytokines reached a plateau at between 0.2 and 1 mg of total *Giardia* protein/ml (Fig. 1G). In separate experiments, concentrations of up to 10 mg of total *Giardia* protein/ml induced cytokines in amounts similar to those induced by 1 mg/ml (data not shown). Given that the IL-6 and TNF-α responses to *Giardia* were relatively weak, we considered that live parasites might provide a better stimulus, as has been seen for the response of intestinal epithelial cells to *Giardia* (37). However, the addition of live trophozoites to DC cultures at a 10:1 parasite/DC ratio, such that total parasite protein concentration in culture was equivalent to those used in experiments with parasite extracts, produced cytokine responses no better than what was seen using parasite extracts (Fig. 1H and I). All further studies were therefore performed using parasite extracts since these provided better consistency among experiments. Together, these data suggest an intrinsic ability of *Giardia* extracts to only moderately activate DCs.

***Giardia* inhibits IL-12 production by LPS-activated DCs.** We considered the possibility that the selective and weak activation of DC responses might be due to the presence of inhibitory molecules in *Giardia* extracts. To address this possibility, titrated amounts of *Giardia* extract were added to DCs stimulated with LPS (Fig. 2). If an inhibitory component were present in the *Giardia* extract, we would expect to see a reduction in the responses to LPS. IL-6 production was inhibited in a dose dependent fashion (Fig. 2A). In five separate experiments, the average reduction in IL-6 production by 1 mg of extract/ml was 48% ($P < 0.001$, $n = 12$). The effect of 0.1 mg of extract/ml approached statistical significance ($P = 0.059$,

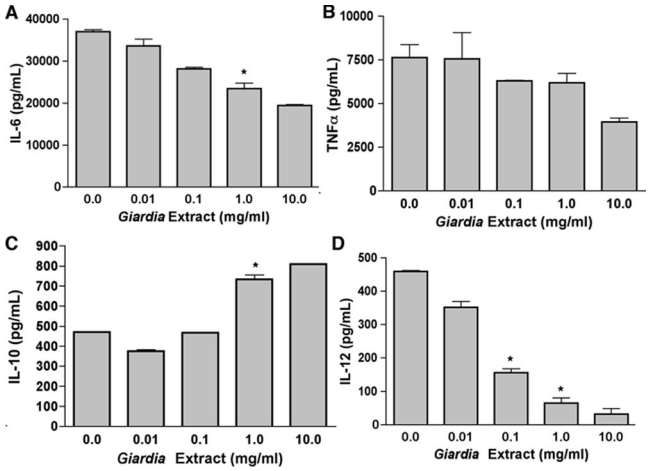

FIG. 2. Inhibition of DC responses to LPS by *Giardia* extracts. DCs were cultured with the indicated amounts of *Giardia* extract, followed by addition of 10 ng of LPS/ml. IL-6 (A), TNF-α (B), IL-10 (C), and IL-12p70 (D) were assayed in the supernatant by ELISA in duplicates. Cytokine data are presented as means ± the SEM of duplicate cultures. These data represent two independent experiments, and the effect of 1 mg of extract/ml was tested in at least four additional experiments with similar results. ∗, $P < 0.05$.

$n = 4$). In contrast, TNF-α production (Fig. 2B) was inhibited only slightly ($P = 0.15$, $n = 10$). IL-10 production in response to LPS (Fig. 2C) was significantly enhanced by the addition of *Giardia* extract, but only with doses of ≥1 mg/ml. The average increase in IL-10 production by DCs stimulated with *Giardia* extract and LPS together was 84% compared to LPS alone ($P < 0.01$, $n = 8$). Finally, IL-12 production (Fig. 2D) was reduced ~66% with as little as 0.1 mg of *Giardia* extract/ml ($P < 0.05$, $n = 4$) and was >90% reduced with 1 mg of extract/ml ($P < 0.01$, $n = 12$). Similar inhibition of IL-12 and enhancement of IL-10 production were seen using live parasites, as well as heat-treated extracts or in the presence of GlcNAc (data not shown). Thus, the absence of IL-12 and the low amounts of IL-6 in supernatants of DC cultures stimulated with parasite extract alone likely reflects the presence of both inhibitory and activating molecules in these preparations.

***Giardia* inhibits DC activation initiated by other TLR ligands.** A possible explanation for the ability of *Giardia* extracts to inhibit cytokine production by LPS activated DCs could be that the *Giardia* extract was binding to the LPS, LPS binding protein, or the signaling molecule TLR4. This would suggest the *Giardia* response is LPS/TLR4 specific. To clarify this point, DCs were stimulated with other selected TLR ligands in the presence of *Giardia* extracts (Fig. 3). Similar to its effect on the response to LPS, 1 mg of *Giardia* extract/ml had a significant inhibitory effect on the production of IL-6 (Fig. 3A) in response to ligands of TLR2 (PAM3CSK4, $P < 0.001$, $n = 8$), TLR3 [poly(I:C), $P < 0.05$, $n = 8$)], and TLR9 (CpG DNA, $P < 0.01$, $n = 6$). *Giardia* extract led to a significant inhibition of TNF production (Fig. 3B) only after stimulation with CpG ($P < 0.01$, $n = 6$). As was seen with the TLR4 agonist LPS, *Giardia* extract also enhanced IL-10 production (Fig. 3C) from DCs stimulated with agonists of TLR2 ($P < 0.05$, $n = 8$). Changes in IL-10 production in conjunction with agonists of TLR3 and TLR9 agonist were not statistically significant in repeated experiments (Fig. 3C and data not shown). Finally, a significant inhibitory effect on IL-12 production (Fig. 3D) was seen in DCs stimulated by ligands of TLR2 ($P < 0.05$, $n = 8$) and TLR4 and TLR9 ($P < 0.05$, $n = 6$), while no consistent effect on TLR3 signaling was seen. Together, these data suggest that it is much more likely that *Giardia* extract interacts with signaling pathways common to these four TLRs, rather than specifically interfering with recognition of each of the particular ligands.

Since *Giardia* induced only moderate upregulation of the DC costimulatory molecules CD80 and CD86 (Fig. 1A and B), DCs from the experiments described above were also analyzed to determine whether expression of these costimulatory mol-

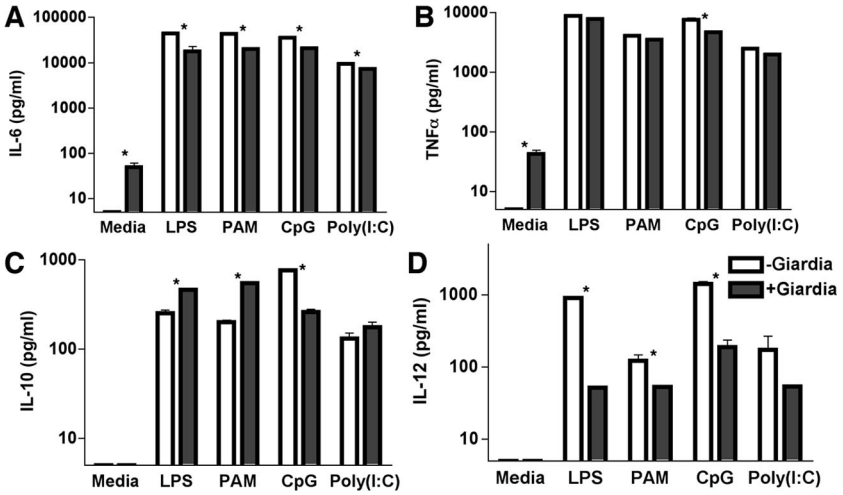

FIG. 3. Inhibition of DC responses to multiple TLR agonists by *Giardia* extracts. DCs were incubated with or without 1 mg of *Giardia* extract/ml, followed by the addition of medium alone, 10 ng of LPS/ml (TLR4), 0.5 μg of PAM3CSK4/ml (TLR2), 25 μg of poly(I:C)/ml (TLR3), or 5 μg of CpG/ml containing oligodeoxynucleotide (TLR9). IL-6 (A), TNF-α (B), IL-10 (C), and IL-12p70 (D) were assayed in the overnight supernatant in duplicates. Cytokine data are presented as means ± the SEM of duplicate cultures, and the data shown are representative of six or more independent experiments. ∗, $P < 0.05$ comparing cultures with or without *Giardia* extract.

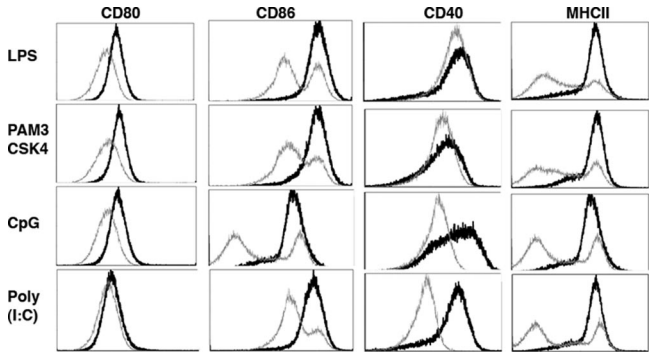

FIG. 4. Inhibition of DC surface molecule expression after treatment with *Giardia* extract and LPS. DCs from BALB/c bone marrow were cultured overnight with TLR agonists with (gray lines) or without (black lines) *Giardia* extracts as in Fig. 3. The cells were then stained with fluorochrome-conjugated antibodies to CD80, CD86, CD40, and MHC-II (A^d) and analyzed by flow cytometry. Only $CD11c^+$ cells were analyzed. The data are representative of three independent experiments.

ecules was also being inhibited. We also analyzed CD40 and MHC-II expression in the present study since TLR activation induces these molecules as well. Stimulation of DCs with *Giardia* extract in addition to TLR agonists reduced the level of CD80 expression on DCs activated with LPS, PAM3CSK4, and CpG DNA (Fig. 4). No inhibition of CD80 expression was seen on DCs stimulated with poly(I:C), possibly because poly(I:C) alone failed to induce high levels of CD80. Coincubation with *Giardia* extract also reduced the proportion of DCs that upregulated expression of CD86 and MHC-II compared to DCs stimulated with each of the four TLR agonists alone (Fig. 4). Finally, while *Giardia* extract did not reduce CD40 induction on DCs stimulated with either LPS or PAM3CSK4, expression of CD40 was reduced compared to DCs stimulated with poly(I:C) or CpG DNA (Fig. 4). Given that *Giardia* extract alone induced as much CD40 expression as LPS (Fig. 1C), inhibition of CD40 induction was not expected. Although the precise level of inhibition of surface molecule expression varied among the TLRs tested, these results clearly support our conclusion that *Giardia* extract interferes with signaling through all four TLRs.

***Giardia* inhibition of TLR-activated DC production of IL-12 is both IL-10 and PI3K dependent.** Inhibition of IL-12 production by DCs activated with LPS has been seen before using soluble egg antigen (SEA) from the helminth parasite *S. mansoni* (2, 24). In one study, IL-10 was further shown to contribute to this inhibition, although IL-10 independent mechanisms were also observed (24). In the other study, SEA was shown to activate the mitogen-activated protein kinase ERK1/2, although the role of ERK1/2 in SEA-mediated IL-12 regulation was not directly tested (2). The ERK1/2 pathway has also been shown to be important for the production of IL-10 (25, 29). We therefore examined the ability of *Giardia* extract to affect ERK1/2 phosphorylation in DCs. Immunoblot studies demonstrated that *Giardia* extract mediates little if any ERK1/2 phosphorylation in DCs compared to the response to LPS (Fig. 5A). Addition of *Giardia* extract along with LPS did not appear to have a major effect on ERK1/2 phosphorylation levels compared to LPS activation alone (Fig. 5A). We also pretreated

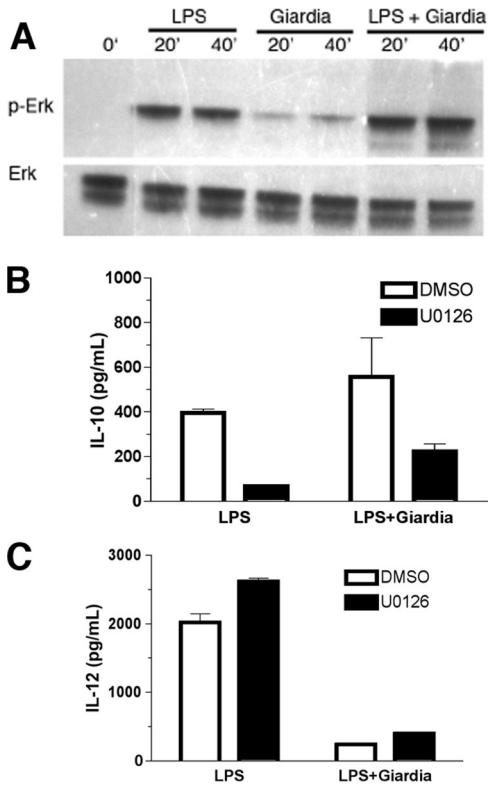

FIG. 5. Effect of ERK activation on DC cytokine production. (A) DCs were incubated with 10 ng of LPS/ml, 1 mg of *Giardia* extract/ml, or both. Protein extracts were made 20 and 40 min after DC stimulation and Western blotted for phosphorylated ERK (p-Erk) or total Erk protein. DCs were pretreated with DMSO or the MEK1/2 inhibitor U0126 (10 μM) for 30 min prior to stimulation with 10 ng of LPS/ml, with or without 1 mg of *Giardia* extract/ml. (B and C) The levels of IL-10 (B) and IL-12p70 (C) were measured in supernatants of overnight cultures. Error bars represent the SEM of duplicate cultures. Western blot data are representative of three independent experiments. Cytokine data are presented as means ± the SEM of duplicate cultures and are representative of two independent experiments each.

DCs with the MEK1/2 inhibitor U0126 to block ERK1/2 phosphorylation and examined the effects on both IL-10 and IL-12 production. U0126 completely blocked ERK1/2 phosphorylation (data not shown). Similar to results shown earlier, the presence of *Giardia* extract enhanced IL-10 production by LPS-activated DCs by 41% without U0126 (Fig. 5B). As expected based on previous reports, inhibition of ERK1/2 phosphorylation greatly reduced IL-10 production in response to LPS. However, *Giardia* extract continued to enhance IL-10 production compared to LPS alone. Thus, the increase in IL-10 production in response to LPS in the presence of *Giardia* is unlikely due to signaling through the ERK1/2 pathway.

We also examined the effect of inhibiting ERK1/2 phosphorylation on IL-12 production by DCs activated by LPS with or without *Giardia* extract. U0126 had little effect on the ability of *Giardia* extract to inhibit IL-12 production (Fig. 5C). *Giardia* reduced IL-12 production 88% in the presence of U0126 and 85% after the addition of vehicle. We noted a 30% increase in IL-12 production by DCs activated by LPS alone with drug treatment compared to IL-12 production without treatment. This increased slightly to ca. 50% when *Giardia* was used in

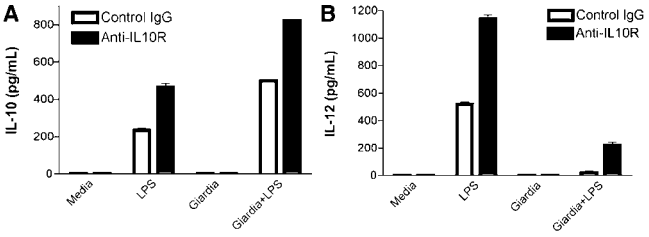

FIG. 6. Effect of IL-10 signaling on DC cytokine production. DCs were cultured with medium alone, with 10 ng of LPS/ml, with 1 mg of *Giardia* extract/ml, or with both LPS and *Giardia* extract in the presence of 10 μg/ml of either anti-IL-10R antibody or isotype control immunoglobulin G/ml. The levels of IL-10 and IL-12p70 were measured in supernatants of overnight cultures. Cytokine data are presented as means ± the SEM of duplicate cultures and are representative of four independent experiments.

addition to LPS in stimulating the DCs (Fig. 5C). Together, these results suggest that inhibition of IL-12 production by LPS-activated DCs was not dependent on phosphorylation of ERK1/2. Moreover, these results also suggest that IL-10 is only moderately involved in the inhibition of IL-12 production by *Giardia* extract since reducing IL-10 production by treatment with U0126 led to only minor increases in IL-12.

To more directly test whether IL-10 was responsible for the reduced production of IL-12 seen when DCs are coincubated with both *Giardia* extracts and LPS, DCs were pretreated with either IL-10 receptor antibodies (anti-IL-10R antibody) or control immunoglobulin G before stimulation with LPS and/or *Giardia* extract. Figure 6 shows that the amounts of IL-10 in supernatants of DCs stimulated with LPS alone or with *Giardia* extract increased with anti-IL-10R antibody treatment as expected, since these antibodies blocked the consumption of secreted IL-10. Even in the presence of anti-IL-10R antibody, however, *Giardia* extract alone did not induce any detectable IL-10. The addition of anti-IL-10R also led to a 100% increase in IL-12 production from DCs activated by LPS alone and a 500% increase in IL-12 from DCs activated by LPS and *Giardia* together (Fig. 6B). In the absence of anti-IL-10R antibodies, *Giardia* extract reduced IL-12 production in LPS-activated DCs by 81%. The addition of anti-IL-10R to DCs stimulated with LPS and *Giardia* led to a 70% reduction in IL-12 ($P < 0.05$, $n = 4$). However, the addition of anti-IL-10R to DCs stimulated with *Giardia* extract alone did not lead to any IL-12 production. Thus, IL-10 may contribute slightly to the reduced production of IL-12 but is unlikely to be the primary mechanism by which *Giardia* extracts inhibit IL-12 production by DCs.

PI3K is a potent suppressor of IL-12 production by TLR-activated DCs and monocytes (18, 21). To determine whether *Giardia* suppression of IL-12 is mediated by PI3K, DCs were pretreated with the PI3K inhibitor, wortmannin, or the vehicle (DMSO) before DC stimulation. Although wortmannin had a minimal effect on IL-10 production by DCs stimulated with LPS and/or *Giardia* extract (Fig. 7A and C), there was a dramatic increase in IL-12 production by DCs stimulated with LPS and *Giardia* together (Fig. 7B and D). In six independent experiments, while *Giardia* extract reduced IL-12 production by an average of 83% in the absence of wortmannin, in its presence *Giardia* could only reduce 46% of the IL-12 ($P < 0.001$, $n = 12$). Wortmannin treatment of DCs stimulated with *Giardia* alone had no effect on IL-10 production (Fig. 7C). In contrast, in two of four experiments wortmannin treatment of DCs stimulated with *Giardia* extract alone resulted in modest IL-12 production (Fig. 7D). This suggests that PI3K activity is necessary for inhibition of IL-12 production by the parasite.

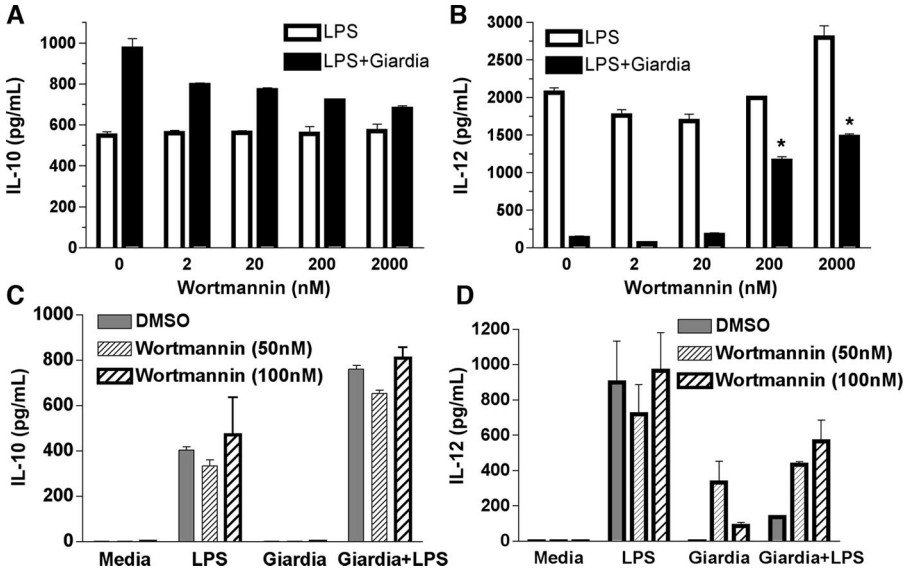

FIG. 7. Effect of PI3K signaling on DC cytokine production. (A to D) DCs were pretreated for 30 min with various concentrations of wortmannin prior to addition of 10 ng of LPS/ml with or without 1 mg of *Giardia* extract/ml. DCs pretreated with wortmannin were incubated with medium alone, with 10 ng of LPS/ml, with 1 mg of *Giardia* extract/ml, or both (C and D). The levels of IL-10 (A and C) and IL-12p70 (B and D) were measured in the supernatants of overnight cultures. Cytokine data are presented as means ± the SEM of duplicate cultures and are representative of four independent experiments. *, $P < 0.05$.

DISCUSSION

Host dendritic cells recognize pathogens and initiate the development of adaptive responses that can lead to protection against infection as well as immune-mediated pathology. The DC response to *Giardia* was unlike most responses to pathogens known today. We have shown that *Giardia* extract induces the maturation of bone marrow-derived DCs, with upregulation of CD40 and partial upregulation of CD80 and CD86. In addition, we show that neither IL-10 nor IL-12 were secreted by *Giardia* activated DCs and only small quantities of IL-6 and TNF-α were produced. Interestingly, IL-6 and TNF-α are the only two cytokines among several tested thus far that have been shown to be necessary for effective *Giardia* control in mice (5, 51, 52). We also found that parasite extracts altered responses to multiple TLR agonists, generally reducing induction of CD80, CD86, and MHC-II on the DC surface and reducing the production of IL-12 while enhancing the production of IL-10. Finally, we showed that parasite extracts continued to inhibit the production of IL-12 by DCs in the presence of anti-IL-10R antibodies, but only minimally after inhibition of PI3K with wortmannin.

Although IL-10 is known to suppress IL-12 production (4, 14), blocking IL-10 through two different approaches failed to restore IL-12 production by DCs stimulated with *Giardia* alone. However, *Giardia* enhanced DC IL-10 production in response to TLR ligands (Fig. 5). Blocking activation of the ERK1/2 pathway with the MEK1/2 inhibitor U0126 did not reverse IL-12 inhibition by *Giardia*, despite the reduced production of IL-10 (Fig. 5). Moreover, blocking IL-10 signaling by adding neutralizing anti-IL-10R monoclonal antibodies did not restore IL-12 production (Fig. 6). Together, these data suggest that the augmented IL-10 plays only a minor role in the IL-12 suppression due to *Giardia*. Thus, an IL-10-independent mechanism of IL-12 suppression by *Giardia* exists.

The general pattern of DC response seen in these studies is most similar to those observed in response to SEA from the helminth parasite *S. mansoni* (10, 24, 49). SEA augmented IL-10 production from LPS-stimulated DCs and inhibited IL-12 production. The addition of anti-IL-10R antibodies also resulted in restoration of some IL-12 production by DCs stimulated with both SEA and LPS, but not DCs stimulated with LPS alone. An IL-10 independent mechanism was also clearly present in these studies as well. The PI3K/Akt pathway has been shown to mediate a negative regulation of TLR-induced IL-12 production, as well as that of other inflammatory cytokines (18, 21). We show that blocking this pathway with a specific inhibitor for PI3K, wortmannin, restores the majority of LPS induced IL-12 that is otherwise inhibited by *Giardia* (Fig. 7). In addition, wortmannin-treated DCs stimulated with *Giardia* extract alone were able to produce IL-12. Thus, PI3K activity plays a major role in the inhibition of DC responses to TLR signals by *Giardia* and may well be the IL-10-independent mechanism noted by Kane et al. (24).

The PI3K/Akt pathway has been proposed as an early mechanism of immune regulation in DCs, as opposed to pathways such as the suppressor of cytokine signaling 1 (SOCS1) and the IL-1R-associated kinase-M (IRAK-M), which also negatively regulate IL-12 production, but only after its initial induction (17). Manipulation of the PI3K/Akt pathway may be a key approach in the management of immune pathology. Even at very high doses (2,000 nM) of wortmannin, the reversal of *Giardia* IL-12 inhibition was not complete (Fig. 7), suggesting that other mechanisms might be involved. Since cyclo-oxygenase activity has been shown to block IL-12 production, we also attempted to reverse the inhibition of LPS responses using indomethcain and aspirin, but these compounds had no effect in this system (data not shown). Several additional transmembrane protein immune regulators are known, such as the IL-33 receptor, suppressor of tumorigenicity 2, and the single immunoglobulin IL-1R-related receptor, both of which are members of the TIR superfamily of transmembrane receptors; the TNF-related apoptosis-inducing ligand receptor; and the DC-specific ICAM-grabbing nonintegrin (DC-SIGN) (28). DC-SIGN is highly expressed on immature DCs and has been shown to serve as recognition receptors for the internalization of several pathogens, including human immunodeficiency virus, by DCs and more recently *S. mansoni* SEA (43). Moreover, ligation of DC-SIGN on the DC surface leads to cytokine modulation in a PI3K- and ERK1/2-dependent manner (9). The *Giardia* trophozoite surface contains abundant amounts of carbohydrates of the GlcNAc family (38, 47), and there are indications of binding of GlcNAc sugars to DC-SIGN (50). The addition of soluble Glc-NAc, however, failed to block the inhibitory activity of *Giardia* extracts.

How DCs direct the development of T-cell responses during *Giardia* infection remains unclear. It has been well established that intestinal $CD4^+$ and not $CD8^+$ T cells are required for effective *Giardia* control (22, 33, 39, 46). However, IL-4-, IL-4Rα-, STAT-6-, and IFN-γ-deficient mice all control infections approximately as well as wild-type mice (40). The parasite's ability to inhibit IL-12 production is consistent with the lack of a requirement for gamma interferon. Although TNF-α has been shown to play a role in controlling infections (51), whether T cells produce this cytokine has not yet been addressed. The present study is the first to address the interaction between DCs and *Giardia*.

Modulation of DC responses to pathogens has been noted in several other infections. Presumably, a reduced response to the pathogen will benefit the microbe by extending the length of infection and allowing greater time for transmission to a new host. Reduced responses might also benefit the host, if immune-mediated pathology is a significant contributor to the disease. Unlike most intestinal pathogens, *Giardia* induces diarrhea without necessarily causing significant infiltration of neutrophils or macrophages (15, 32). The ability of *Giardia* to inhibit the production of IL-12 and enhance the production of IL-10 by DCs in response to other TLR agonists may play significant role in contributing to the maintenance of an anti-inflammatory milieu in the gut. Further studies to investigate the mechanisms of *Giardia* inhibition of DC might therefore provide novel ways to deal with chronic inflammatory conditions such as inflammatory bowel disease.

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