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Prostaglandin E₂ Negatively Regulates the Production of Inflammatory Cytokines/Chemokines and IL-17 in Visceral Leishmaniasis

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Persistence of intracellular infection depends on the exploitation of factors that negatively regulate the host immune response. In this study, we elucidated the role of macrophage PGE₂, an immunoregulatory lipid, in successful survival of Leishmania donovani, causative agent of the fatal visceral leishmaniasis. PGE₂ production was induced during infection and resulted in increased CAMP level in peritoneal macrophages through G protein–coupled E-series prostanoid (EP) receptors. Among four different EPs (EP1–4), infection upregulated the expression of only EP2, and individual administration of either EP2-specific agonist, butaprost, or 8-Br-cAMP, a cell-permeable cAMP analog, promoted parasite survival. Inhibition of cAMP also induced generation of reactive oxygen species, an antileishmanial effector molecule. Negative modulation of PGE₂ signaling reduced infection-induced anti-inflammatory cytokine polarization and enhanced inflammatory chemokines, CCL3 and CCL5. Effect of PGE₂ on cytokine and inflammatory/anti-inflammatory balance, PGE₂ inhibited antileishmanial IL-17 cytokine production in splenocyte culture. Augmented PGE₂ production was also found in splenocytes of infected mice, and administration of EP2 antagonist in mice resulted in reduced liver and spleen parasite burden along with host-favorable T cell response. These results suggest that Leishmania facilitates an immunosuppressive environment in macrophages by PGE₂-driven, EP2-mediated cAMP signaling that is differentially regulated by PKA and EPAC. The Journal of Immunology, 2014, 193: 2330–2339.

Prostaglandins, derivatives of arachidonic acid, are among the best studied immunomodulatory lipid mediators (1). The final formulation to PGE₂ is catalyzed by the rate-limiting enzyme cyclooxygenase (COX), which exists as two different isoforms, constitutively expressed COX-1 and inducible COX-2 (2). PGE₂ has potent immunosuppressive properties including inhibition of macrophage activation, oxygen radical generation, leukocyte chemotaxis, generation of myriad proinflammatory cytokines, and modulation of chemokines (3–7), thus resulting in downregulation of inflammation and immunofunctioning. It is known to exert its action by binding to distinct cell-membrane–associated heterotrimetric guanosine triphosphate binding protein (G protein)–coupled E-series prostanoid (EP) receptors termed EP1, EP2, EP3, and EP4 (8). These G protein–coupled receptors are linked to different transduction pathways after associating with different isoforms of G protein that may produce opposite effects, such as activation or inhibition of cellular responses (9). cAMP, the most potent intracellular second messenger of PGE₂, plays a crucial role in PGE₂-dependent modulation of functional activity of macrophages (10). Several reports indicated that PGE₂ mediated EP3-induced inhibition of cAMP production via downregulation of adenylate cyclase, whereas EP2 and EP4 are known to stimulate adenylate cyclase (11) and generate intracellular cAMP ([cAMP]i) (12). Initially, the effects of cAMP could only be attributed to activation of its downstream protein kinase A (PKA) and cAMP-gated ion channels, but recently the contribution of the alternative cAMP target, exchange protein directly activated by cAMP (EPAC), has started gaining attention (13). Although the suppressive effects of cAMP on the production of inflammatory mediators were initially reported to be mediated by PKA rather than EPAC, it is likely that the roles of these two cAMP effectors vary according to the diversity of stimulator and cell type (14). Leishmania donovani, the causative agent of deadly visceral leishmaniasis, is an intramacrophage parasite. The inability of macrophages to kill the parasite is a result of the parasite’s long-reported capacity in skewing the host immune response toward a disease-promoting Th2 phenotype that consequently suppresses host-protective Th1 phenotype (15, 16). To execute this, Leishmania needs specific molecules that are secreted by Leishmania itself or it may activate macrophages to produce immunosuppressive molecules that render the macrophage defense inactive. PGE₂ could be one such immunosuppressive molecule that has been found to be stimulated upon L. donovani infection (17). PGE₂-dependent elevation of cAMP showed increased production of...
of Th2 cytokine, IL-10, in mice (18, 19). Apart from classical Th1 cytokine, Th17 cytokines have recently been reported for their protective role against visceral leishmaniasis (20). A recent study documented that although PGE2 enhances IL-17 production from matured memory T cells, it inhibited the production of IL-17 from naive T cells exposed under Th1 differentiation condition (21). Visceral leishmaniasis has been found to be associated with rapid changes in chemokine expression in both humans and mice, thereby suggesting possible involvement of chemokines in the disease progression (22). L. donovani–infected mice that lack MIP-1α (or CCL3) demonstrated low Ag-specific IFN-γ production during the early phases of infection (23). Treatment with MetRANTES or anti-CCL5 rendered C57BL/6 more susceptible to Leishmania by skewing the immune response toward Th2 phenotype (24). However, little is known about the signaling pathways that lead to altered expression of these chemokines upon L. donovani infection. This study was aimed to underscore the signaling events after the induction of PGE2 in L. donovani–infected macrophages. Both in vitro and in vivo studies were carried out to determine how Leishmania could manipulate the host macrophages to induce PGE2–driven cAMP signaling through PKA and EPAC, resulting in inactivation of effector antileishmanial cytokines and chemokines leading to persistent survival within its niche.

Materials and Methods

Reagents

PGE2, AH6809, butaprost free acid, NS-398, and H-89 were purchased from Cayman Chemicals (Ann Arbor, MI), Forskolin, brefeldin A, 2′,5′-dideoxy adenosine (DDA), and LPS (E. coli serotype 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO). rTG–β and IL-6 were purchased from Affymetrix eBioscience (San Diego, CA). CD4 and CD62L were kindly provided by Dr. S. Roy (Indian Institute of Chemical Biology, Kolkata, India); PE-tagged EP2 and EP4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells and parasites

L. donovani (MHOM/IN/1983/AG83) parasites were cultured as promastigotes in medium M199 (Invitrogen, Carlsbad, CA) with Hanks’ salt containing HEPES (13 mM), l-glutamine (20 mM), 10% heat-inactivated FBS along with 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), and were passaged weekly. Peritoneal macrophages from BALB/c mice were isolated as described earlier (25) and cultured at 37°C with 5% CO2 in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. For in vitro infection, cells were infected with L. donovani promastigotes at a parasite/cell ratio of 10:1 and incubated for the specified periods. No cytotoxic or cytostatic effect was observed for all the reagents at the administered doses (data not shown), as studied by examination of cellular morphology and cell viability by MTT assay kit (Roche Applied Science, Indianapolis, IN).

Mice and infections

For in vivo experiments, female BALB/c mice were injected with 105 stationary-phase promastigotes collected from cultures that had not been passaged more than three times via the tail vein. For inhibition of COX-2 and EP2 in vivo, mice were injected i.p. with 10 mg/kg/d of the selective COX-2 inhibitor NS-398 and EP2 antagonist AH6809 twice weekly for 6 wk starting at 1 wk postinfection. Control animals received PBS only. Infection was assessed by removing liver and spleen from infected mice at different time periods, and parasite burdens were determined by Giemsa-stained impression smears. Data are represented as Leishman–Donovan units (26). For in vivo cytokine production, spleenocytes were isolated from mice and cultured as described earlier (27). All the animal care and experimental procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol has been approved by the Committee on the Ethics of Animal Experiments of Indian Institute of Chemical Biology (Permit No. 147-1999).

PGE2 assay

PGE2 was assayed using the PGE metabolite ELISA kit from Cayman Chemicals (Ann Arbor, MI), according to the manufacturer’s instructions.

cAMP assay

[cAMP]i was measured by using a cAMP assay kit from Abcam (Cambridge, MA), according to the manufacturer’s protocol.

Analysis of gene expression by semiquantitative and quantitative PCR

Total RNA was isolated from peritoneal macrophages using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA (1 μg) was used as a template for cDNA synthesis using the superscript-first strand synthesis system for the RT-PCR kit (Invitrogen, Carlsbad, CA). The cDNA was PCR amplified using the following oligonucleotides: COX-2, 5′-ACACTTACATCCT-GCA TTC-3′ and 5′-GAAGGGACACCTTTCCACAT-3′; COX-1, 5′-CC-TGGTACGCAATGTC-3′ and 5′-ACCTTGTTGCTGAGCCG-3′; EP1, 5′-TAACTGAGCTACGGAGATG-3′ and 5′-GCGTCAGGATTGG-ACACATA-3′; EP2, 5′-CCACGATGCCTCCTGGTTAT-3′ and 5′-CAGCCCCCTTACACTTCTCCAAATGA-3′; EP3, 5′-TGACCTTTGCTG-CAACCTG-3′ and 5′-GACCCAGGGAAACAGGTACT-3′; EP4, 5′-CCATCTACGCACTTCTCCTG-3′ and 5′-TGGTGCTCCTACCTACTACCT-3′; and β-actin, 5′-TGGAACTCTTCTGGATCCATG-3′ and 5′-TAAAAGCGAGCTCAAGACGGC-3′. The following amplification was performed in a thermal cycler: 30 cycles of 5 min at 94°C, 15 s at 94°C, 30 s at 58°C, 1 min at 72°C, and 5 min at 72°C. PCR products were then separated on 1.5% agarose gels. Quantitative real-time PCR (ABI 7500 Fast real time PCR system; Applied Biosystems, Foster City, CA) was performed using SyberGreen. The following amplification conditions were maintained throughout the process: 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantitation was performed by the comparative ΔΔ cycle threshold method, and data were normalized to β-actin mRNA level and expressed as a fold change compared with controls.

Cytokine and chemokine analysis by ELISA

Various cytokine and chemokine levels in the cell-free supernatant were assayed using a sandwich ELISA kit (QuantiKine M; R&D Systems, Minneapolis, MN) as described earlier (27).

Assessment of intracellular infection

Peritoneal macrophages were plated overnight in tissue culture plates containing 12 mm2 coverslips at a density of 2 × 105 cells/dish in DMEM medium. Cells were incubated with metacyclic promastigotes (cell/parasite 1:10 ratio) for various time periods and were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 10% FCS. Cells were stained with propidium iodide (PI) (1 μg/ml) in PBS along with 10 μg/ml RNase A for 5 min. Samples were observed under a Olympus IX81 microscope equipped with a FL1000 confocal system using 100× oil immersion Plan Apo (NA 1.45) objectives. Images obtained through section scanning were analyzed by Olympus Fluoview (version 3.1a, Tokyo, Japan) and analyzed using Adobe Photoshop software.

Immunoblotting

Cells were lysed in buffer (Cell Signaling Technology, Danvers, MA), and the protein concentrations in the cleared supernatants were estimated using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The cell lysates were resolved by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Millipore, Billerica, MA). The membranes were blocked with 5% BSA in wash buffer (TBS/0.1% Tween 20) for 1 h at room temperature and probed with primary Ab overnight at dilution recommended by the suppliers. Membranes were washed three times with wash buffer and then incubated with alkaline phosphatase–conjugated secondary Ab and detected by detection of BCIP chromogenic substrate according to the manufacturer’s instruction.

Estimation of reactive oxygen species production

Intracellular reactive oxygen species (ROS) generation was quantified using the oxidant-sensitive green fluorescent dye 2′,7′-dihydrodichlorofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR). Measurement of fluorescence in cells was made by counting at least 10,000 events/test using a FACSCalibur flow cytometer (BD Biosciences, Oxford, U.K.), with an FITC filter, and the cells were gated based on their fluorescence property. Results were analyzed using CellQuest software (BD Biosciences).
Quantification of naive T cell and analysis of Th17 cytokines

To quantify the naive T cell population, we gated CD4+ T cells from the whole splenocyte population; then this gated population was further analyzed for naive T cells. In brief, the splenocytes were washed and FcRs were blocked with anti-Fc IgG for 30 min. The cells were stained for surface markers with monoclonal PE-conjugated Ab directed against mouse CD4 and PerCP-conjugated Ab against CD62L for 30 min in the dark on ice. After staining, cells were centrifuged and resuspended in PBS. Events were acquired on a FACS Canto (BD Biosciences) and then subsequently analyzed using FACSDiva software (BD Biosciences). For Th17 cytokine analysis, petri dishes were first coated with anti-mouse CD3 (5 μg/ml) and then cultured in these coated petri dishes for 3 d in the presence of anti-mouse CD28 (5 μg/ml) with or without rTGF-β (10 ng/ml) and recombinant mouse IL-6 (10 ng/ml). After 3 d, the splenocytes were harvested for RNA isolation and supernatants were harvested for ELISA.

Densitometric analysis

Densitometric analyses for the bands were carried out using QUANTITY ONE software (Bio-Rad, Hercules, CA). Band intensities were quantified and the values were normalized to endogenous control β-actin and expressed as arbitrary units. The ratios of ODs of particular bands/endogenous control are indicated as bar graphs adjacent to corresponding figures.

Statistical analysis

All experiments were performed at least three times. Cell cultures were set in triplicate and the results are expressed as the mean ± SD. Student t test was used to evaluate the statistical significances of differences among paired data sets, and a *p* value <0.05 was considered to be significant.

Results

COX-2–induced macrophage PGE2 production during infection is necessary for parasite survival

Successive invasion and survival of *Leishmania* within the host requires neutralization of both the early and the delayed host responses (28). To determine whether PGE2 has a role in this process, we first measured the level of PGE2 production in *L. donovani*–infected peritoneal macrophages. A time-dependent increase in PGE2 production was observed with a maximum level (3.6-fold; *p* = 0.001) at 24 h postinfection over uninfected cells (Fig. 1A). Because COX-1 and COX-2 are the two major enzymes involved in PGE2 biosynthesis, we measured the expression of these two enzymes during *L. donovani* infection both at the mRNA and the protein levels. COX-2 transcript was found to increase in infected macrophages with a maximum expression (5.6-fold compared with control) at 6 h postinfection (Fig. 1B), whereas expression at the protein level showed a maximum (4.9-fold) at 12 h postinfection (Fig. 1C). In contrast, the expression of COX-1 remained unchanged both at mRNA and at protein levels during infection (Fig. 1B, 1C), suggesting that infection-induced PGE2 is primarily mediated by COX-2. Pretreatment with NS-398, a selective COX-2 inhibitor, showed a dose-dependent reduction in PGE2 production in *Leishmania*-infected cells with maximum inhibition (80.7%; *p* = 0.0004) at a concentration of 1 μM (Fig. 1D). To further examine the role of PGE2, we coadministered macrophages with varying doses of either PGE2 or NS-398 along with *L. donovani* promastigotes (cell/parasite ratio, 1:10) for various time periods. Levels of PGE2 production were measured by enzyme immunoassay in the supernatant (A), whereas expressions of COX-1 and COX-2 were evaluated at the mRNA (B) and protein (C) level by RT-PCR and Western blotting, respectively. Peritoneal macrophages were preincubated with increasing concentrations of NS-398 (0.01, 0.1, and 1 μM) for 30 min. One set of macrophages was then infected with *L. donovani* promastigotes, whereas another set was left uninfected and PGE2 levels were determined in the cell supernatant at 24 h postinfection (D). Macrophages were preincubated with increasing concentrations (0.1 and 1 μM) of either PGE2 for 5 min or NS-398 for 30 min, or both, followed by infection (24 h). The number of parasites per 100 macrophages was determined by PI staining (E). Representative confocal microscopic images of the above experiment (original magnification ×100). Phase-contrast images of each field are shown separately (F). Results are representative of three individual experiments, and the error bars represent mean ± SD. **p < 0.01, ***p < 0.001 versus control, Student t test.
with *L. donovani*, and parasite survival was determined by PI staining. Microscopic evaluation and quantification revealed that administration of PGE$_2$ (1 µM) resulted in an increase of intracellular parasite numbers by 1.7-fold (*p* = 0.003), whereas treatment with NS-398 (1 µM) significantly reduced (73.1%; *p* = 0.0006) the parasite load (Fig. 1E, 1F). PGE$_2$-mediated increase of intramacrophage parasite number was also inhibited by NS-398 pretreatment (47.3%, *p* = 0.001; Fig. 1E, 1F). All of these experiments collectively suggested that *Leishmania* infection induced COX-2–mediated PGE$_2$ production, which has a role in intramacrophage parasite survival.

**PGE$_2$-induced intramacrophage survival of *L. donovani* is mediated through EP2 receptor**

Four functionally divergent G protein–coupled EP receptors are known to mediate PGE$_2$–dependent intracellular signaling cascade (29). We therefore evaluated the time-course expression of all four EP receptors in *L. donovani*–infected macrophages. Expression analysis of both mRNA and protein levels revealed a time-dependent increase of EP2 receptor in infected cells with maximum expression (5.2- and 4.8-fold of mRNA and protein levels, respectively) compared with control; *p* < 0.001) at 24 h postinfection (Fig. 2A, 2B). On the contrary, infection decreased the expression of both EP1 and EP4. Maximum reduction of EP1 was obtained at 6 h postinfection (60.3% reduction at the mRNA and protein levels, respectively; *p* < 0.001) (Fig. 2A, 2B). EP3 expression remained undetected at all time points, validating earlier studies that EP3 is not expressed in peritoneal macrophages (30). Next, we checked the surface localization of EP2 along with EP4, the expression of which decreased but persisted up to 24 h postinfection. FACS analysis revealed an infection-induced, time-dependent increase of the surface localization of EP2 with maximum expression observed at 36 h postinfection (78.7 ± 6.8% compared with 48 ± 6% in uninfected control; *p* = 0.0001; Fig. 2C). In contrast, infection induced a time-dependent decrease in the surface expression of EP4$^+$ cells as observed up to 48 h (Fig. 2D). To ascertain the involvement of EP2 receptor in intramacrophage survival of *L. donovani* parasites, we treated infected macrophages with increasing doses of either an EP2 antagonist, AH6809, or an agonist, butaprost, and intracellular parasite survival was determined. Significant decrease in intracellular parasites (67.5% reduction as compared with infected cells, *p* = 0.0004) induced by 10 µM AH6809 and reasonable increase in the presence of 10 µM butaprost (1.8-fold; *p* = 0.002; Fig. 2E, 2F) further confirmed that PGE$_2$-induced intramacrophage survival of *L. donovani* might be mediated through EP2.

**EP2 receptor–mediated cAMP production is important for intracellular parasite survival**

EP2 signals through stimulatory G protein $\alpha$ subunit that activates adenylate cyclase and consequently results in [cAMP]$_i$ production (31). An increase in [cAMP]$_i$ is associated with suppression of innate immune functions, including generation of inflammatory mediators and killing of microbes (32). We therefore measured the [cAMP]$_i$ level in peritoneal macrophages at various time points postinfection. Infection induced significant production of cAMP, which started at 2 h postinfection, peaking at 8 h (488 ± 51.5
pmol/ml), and thereafter steadily declined as observed up to 12 h postinfection (Fig. 3A). To ascertain the role of PGE2 in the production of cAMP, we administered increasing concentrations of PGE2 in normal macrophages, and [cAMP]i level was determined. Results revealed a concentration-dependent increase of [cAMP]i, which maximized (5.3-fold over untreated control; p = 0.0002) in the presence of 1 μM PGE2 (Fig. 3B). Infected and PGE2-treated macrophages were preincubated with either NS-398 (COX-2 inhibitor) or AH6809 (EP2 antagonist), to further ascertain whether cAMP generation during infection occurs through COX-2/PGE2/EP2 axis. Pretreatment of macrophages with NS-398 and AH6809 considerably decreased infection-induced cAMP production (69.7 and 57.1% inhibition, respectively, compared with infected macrophages; p < 0.001; Fig. 3C). A similar observation was obtained for PGE2-treated macrophages (Fig. 3C), validating the role of PGE2-induced cAMP in intracellular survival of Leishmania. Because elevation in [cAMP]i level is known to suppress macrophage's microbicidal activity (32), we explored the impact of [cAMP]i on parasite survival. Pretreatment of infected macrophages with 10 μM forskolin (activator of adenylyl cyclase) and 100 μM 8-Br–cAMP (cell-permeable cAMP analog) significantly increased (1.94- and 2.2-fold, respectively; p < 0.01) the intramacrophage survival of parasites (Fig. 3D), validating the role of PGE2-induced cAMP in intracellular survival of Leishmania. Because the effects of cAMP signaling are attributed to its downstream effectors molecules PKA and EPAC, next we tried to explore the role of these molecules on cAMP-mediated parasite survival by using H-89 and brefeldin A, specific inhibitors of PKA and EPAC, respectively. Parasite survival was found to be significantly decreased (54.1% compared with PGE2-treated infected cells; p = 0.006) by 10 μM H-89, but a less prominent decrease was observed (14.4% compared with PGE2-treated infected macrophages; p = 0.2) in brefeldin A pretreated cells. However, PGE2 induces both COX-2 and microsomal PGES-1 via EP2 receptor–mediated signaling. Administration of NS-398 possibly abrogated the feedback mechanism induced by exogenous PGE2, thereby inhibiting cAMP production. Because elevation in [cAMP]i level is known to suppress macrophage's microbicidal activity (32), we explored the impact of [cAMP]i on parasite survival.

FIGURE 3. Effect of EP2-mediated cAMP production on parasite survival. Intracellular cAMP level was measured in 1 × 10^7 macrophages either infected with L. donovani promastigotes for indicated time periods (A) or treated with increasing doses of PGE2 (0.01, 0.1, 1, and 10 μM) (B). Macrophages were pretreated with either NS-398 (1 μM) or AH6809 (10 μM) for 30 min, infected with L. donovani or treated with PGE2 (1 μM), and [cAMP]i level was measured after 4 h (C). Macrophages were infected with L. donovani promastigotes for 4 h along with increasing doses of the adenylyl cyclase inhibitor, forskolin (1 and 10 μM), or with the cell-permeable cAMP analog, 8-Br–cAMP (10 and 100 μM), and number of amastigotes per 100 macrophages was determined by PI staining after 24 h (D). Macrophages were pretreated with PGE2 (1 μM) (E) or left untreated (F) along with increasing concentrations (0.1, 1, and 10 μM) of either the PKA-specific inhibitor H-89 or EPAC-specific inhibitor brefeldin A, or both (10 μM each). Cells were then infected with L. donovani promastigotes for 4 h, washed, and the number of parasites per macrophage was determined by PI staining after 24 h (E and F). Macrophages were treated with LPS (1 μg/ml) for 30 min and then one set was infected with L. donovani promastigotes in the presence or absence of 100 μM 8-Br-cAMP or DDA. ROS generation was measured after 4 h by H2DCFDA staining followed by flow cytometric analysis. The H2DCFDA+ cells are indicated as the percentage of gated cells (G). Another set of infected macrophages was either left untreated or pretreated with DDA (100 μM) or H-89 (10 μM). ROS generation was measured after 4 h by H2DCFDA staining followed by flow cytometric analysis (H). Results are representative of three individual experiments, and the error bars represent mean ± SD. **p < 0.01, ***p < 0.001 versus control, Student t test.
coadministration of both H-89 and brefeldin A pretreatment resulted in significant reversal (74.2% reduction; \( p = 0.001 \)) of PGE2-mediated increase in intramacrophage parasite survival (Fig. 3E). Similar trend was observed in absence of exogenous PGE2 where administration of 10 \( \mu \)M H-89 caused significant decrease in parasite survival (54.9%; \( p = 0.001 \)) as compared with infected macrophages, whereas a less pronounced effect was observed in the case of 10 \( \mu \)M brefeldin A (12.8%; \( p = 0.3 \); Fig. 3F). When both the inhibitors were added simultaneously, more effective elimination of parasite burden was obtained (72.7% suppression; \( p = 0.0007 \); Fig. 3F). Therefore, both PKA and EPAC may act as the downstream effector molecules of cAMP in PGE2-mediated signaling during Leishmania infection. Production of ROS, a major antileishmanial arsenal (36), was determined vis-à-vis cAMP production in infected cells, to further validate the role of cAMP in parasite survival (Fig. 3G). The percentage of ROS+ cells in LPS (an ROS stimulator)-treated macrophages was found to be 64.4 \( \pm \) 7.1%, which was decreased to 37.7 \( \pm \) 2.9% in LPS + L. donovani coadministered macrophages (Fig. 3F). Leishmania-induced decrease of ROS generation was found to be reversed by cAMP inhibitor DDA (56.1 \( \pm \) 5.9% ROS+ cells compared with 37.7 \( \pm \) 2.9% in LPS-treated infected cells; Fig. 3G). In contrast, administration of 8-Br-cAMP in LPS + L. donovani–treated cells resulted in further decrease of ROS generation (17.9 \( \pm \) 2.3% ROS+ cells compared with 37.7 \( \pm \) 2.9% in LPS-treated infected cells). Macrophages infected with L. donovani alone for 4 h did not induce ROS generation (9.1 \( \pm \) 0.7% ROS+ cells compared with 8.3 \( \pm \) 0.7% in control cells). However, pretreatment of infected macrophages with either DDA or H-89 promoted increase in ROS production (12.7 \( \pm \) 0.9 and 11.6 \( \pm \) 0.9% ROS+ cells, respectively, as compared with 9.1 \( \pm \) 0.7% in infected cells). All of these results collectively demonstrate that EP2-induced, cAMP-dependent signaling is crucial for intramacrophage survival of Leishmania.

**FIGURE 4.** Effect of modulation of PGE2 on cytokine and chemokine levels. Peritoneal macrophages were infected with L. donovani promastigotes either alone or in the presence of various inhibitors like NS-398 (1 \( \mu \)M), AH6809 (10 \( \mu \)M), or DDA (100 \( \mu \)M). Expressions of IL-12, TNF-\( \alpha \), IL-10, and TGF-\( \beta \) were evaluated at protein level by ELISA (A) and at mRNA level by real-time PCR (B). Cells were treated as above, and CCL3 and CCL5 were evaluated at protein (C) and mRNA (D) levels. In another set of experiments, macrophages were infected with L. donovani in the presence or absence of H-89 (10 \( \mu \)M) and brefeldin A (10 \( \mu \)M) for 4 h. Expressions of IL-12, TNF-\( \alpha \), IL-10, and TGF-\( \beta \) were evaluated at protein (E) and mRNA (F) levels, and expressions of CCL3 and CCL5 were evaluated at protein (G) and mRNA (H) levels. Results are representative of three individual experiments, and the error bars represent mean \( \pm \) SD. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) versus infected, Student \( t \) test.
expression (5.5-fold; \( p = 0.0008 \)) compared with infected control (Fig. 4G). In the case of CCL5, the opposite scenario was observed, where H-89 significantly augmented its expression (5.8-fold; \( p = 0.0003 \)), but brefeldin A–administered cells showed comparable levels of expression as infected macrophages (Fig. 4G). Similar expression profile was obtained at the mRNA level of the cytokines and chemokines (Fig. 4B, 4D, 4F, 4H). These results suggest that \( L. donovani \)--induced PGE2 plays an important role in the augmentation of disease-progressing cytokines, which are predominantly regulated by PKA. Moreover, PGE2–mediated decreased expression of chemokines, CCL3 and CCL5, is differentially regulated by PKA and EPAC.

**PGE2 produced during Leishmania infection negatively modulates host Th17 response**

PGE2 has been reported to act on naïve T cells and modulate Th17 cytokine production (21). We have recently demonstrated that along with Th1 cytokines, Th17–inducing cytokine IL-23 and Th17 cytokine IL-17 also play a protective role against visceral leishmaniasis (20). Therefore, our next aim was to determine whether PGE2 has any influence over Th17 cytokine regulation during infection. To this end, splenocytes were isolated from normal healthy mice (6–8 wk old), and FACS analysis using specific marker revealed among 21.6% of the total T cell population, 73.4 ± 7.6% of T cells in the splenocyte population were in naïve state (Fig. 5A). In vitro treatment of splenocytes with TGF-β and IL-6 for 3 d resulted in the differentiation of Th17 cells from naïve T cells (21) and produced significant levels of Th17 cytokines, IL-17 and IL-22 (6.7 ± 0.75 and 1.1 ± 0.15 ng/ml, as compared with 0.2 ± 0.04 and 0.3 ± 0.05 ng/ml in supernatants of control splenocytes; Fig. 5B). In contrast, treatment with either \( L. donovani \) or PGE2 (1 \( \mu \)M) for 3 d during primary polarization phase dramatically reduced TGF-β and IL-6–promoted IL-17 hike by 76.1 and 95.3%, respectively (Fig. 5B). To evaluate whether the suppression of IL-17 by \( L. donovani \) is due to the production of endogenous PGE2, we administered NS-398 to inhibit macrophage PGE2 production. Suppression of PGE2 production significantly reversed \( L. donovani \)–promoted IL-17 suppression and increased IL-17 level by 5.1-fold over infected control during primary differentiation phase (Fig. 5C). To ascertain the role of PGE2–induced signaling in IL-17 production, we administered AH6809 (10 \( \mu \)M) or H-89 (10 \( \mu \)M) or DDA (100 \( \mu \)M) during the primary differentiation phase of naïve T cells. Significant increase in IL-17 production was found in case of treatment with AH6809 (3.9-fold; \( p = 0.0006 \)), DDA (5.5-fold; \( p = 0.0002 \)), and H-89 (5.3-fold; \( p = 0.0003 \)) over infected control (Fig. 5C). However, there was no change in the level of IL-22 by these treatments (Fig. 5C). We next treated the splenocyte culture with positive modulators of PGE2 signaling cascade. The inhibitory effect of PGE2 on IL-17 production was mimicked by treatment with EP2 agonist, butaprost, and cAMP activating agent, forskolin (61.9 and 88.2% reduction, respectively, compared with treated control; \( p < 0.001 \)), demonstrating that EP2–mediated increase in cAMP levels play an important role in PGE2–mediated inhibition of IL-17 production (Fig. 5D). We also evaluated the level of another Th17 cytokine, IL-22, and neither Leishmania infection nor administration of NS-398, butaprost, or forskolin during primary stimulation of splenocytes brought out any marked change in the IL-22 production compared with the level obtained in differentiated cultured supernatant (Fig. 5C, 5D). Taken together, these findings suggest that \( L. donovani \)--induced PGE2 can directly act on Th17 cells during primary stimulation to inhibit IL-17–dependent protective immune response against experimental visceral leishmaniasis.

**Importance of COX2/EP2/cAMP axis during in vivo infection and cytokine/chemokine production**

To determine whether \( L. donovani \)--induced PGE2 signaling is operating in an in vivo situation, we used the experimental BALB/c mouse model of visceral leishmaniasis. Similar to an in vitro scenario, increasing levels of PGE2 were obtained in the culture supernatants of splenocytes isolated from infected mice at 2, 4, and 6 wk postinfection with maximum level obtained at 6 wk postinfection (7.1 ± 0.82 ng/ml; \( p = 0.0005 \); Fig. 6A). To evaluate the role of PGE2 signaling on parasite survival, we then treated infected mice with either NS-398 or AH6809 over a 6-wk period. NS-398 treatment reduced the spleen and liver parasite burden by 58.1 and 54.1% (\( p < 0.01 \)), respectively, whereas 51.2 and 49.1% reduction (\( p < 0.01 \)) were observed in the case of AH6809 treatment (Fig. 6B, 6C). Significant reduction (\( p < 0.001 \)) in mean spleen weight was also observed for NS-398 and AH6809 (72.1 and 64.8%, respectively) compared with infected ones (Fig. 6D).

**FIGURE 5.** Effect of PGE2 on the production of Th17 cytokines. Splenocytes were isolated from BALB/c mice and were first gated on the basis of surface expression of CD4; then the gated population was analyzed by FACS for the expression of CD62L (A). These cells (naïve T cells) were isolated and activated with TGF-β and IL-6 for 3 d. IL-17 and IL-22 levels were then measured by ELISA in these cells (2 \( \times 10^6 \)), which were subjected to the following treatments: \( L. donovani \) infection along with PGE2 (1 \( \mu \)M) (B), or \( L. donovani \) infection alone or in presence of various inhibitors like NS-398 (1 \( \mu \)M), AH6809 (10 \( \mu \)M), H-89 (10 \( \mu \)M), or DDA (100 \( \mu \)M) (C), or treatment with butaprost (1 \( \mu \)M) and forskolin (10 \( \mu \)M) (D). Results are representative of three individual experiments, and the error bars represent mean ± SD. ***\( p < 0.001 \), Student \( t \) test.
During the experimental period, all animals remained healthy without any apparent loss in body weight. Next, we examined the proinflammatory and anti-inflammatory cytokine and chemokine profiles in splenocytes from control, infected, and infected mice treated with either NS-398 or AH6809 by ELISA at 2, 4, and 6 wk postinfection. Both NS-398 and AH6809 treatment resulted in significant changes in the level of cytokine and chemokine production as observed at 2, 4, and 6 wk postinfection. Treatment with NS-398 resulted in 76.5 (p = 0.0009) and 48.1% (p = 0.003) reduction of IL-10 and TGF-β (Fig. 6F) along with 3.4- and 7.6-fold (p < 0.01) increase in the production of IL-12 and TNF-α (Fig. 6E) at 4 wk postinfection compared with untreated infected mice. In the case of AH6809 pretreatment, 63.4 and 28.1% reduction of IL-10 and TGF-β were obtained along with 2.6- and 4.9-fold increase in the level of IL-12 and TNF-α at 4 wk postinfection (Fig. 6E, 6F). Splenocyte supernatant analysis of NS-398–treated infected mice showed a pronounced increase in the chemokine level also (3.4- and 4.5-fold for CCL3 and CCL5, respectively, over infected control; p < 0.001; Fig. 6G) at 4 wk postinfection. Treatment with AH6809 demonstrated a similar trend in the levels of CCL3 and CCL5 (1.9- and 2.2-fold increase over infected control; p < 0.01; Fig. 6G) at 4 wk treatment. AH6809 resulted in an appreciable increase in the level of IL-17 (5.5- and 4.1-fold, respectively), with much less pronounced increase in IL-22 level (1.4- and 1.1-fold, respectively) compared with infected control at 4 wk postinfection (Fig. 6H). These results demonstrated that PGE2 signaling induced upon Leishmania infection may modulate the cytokine and chemokine balance in favor of the parasite, which is crucial for its intramacrophage survival.

**Discussion**

Despite the large amount of Ags presented by *L. donovani*, the inability of macrophages to kill the parasite is a result of the parasite’s long-reported capacity to modulate several key signaling pathways of the host (38). *Leishmania* achieves this either by using strategies to inhibit proteins that play a positive role in immune cell activation or by activating molecules known to play key roles in the negative regulation of immune function (39). We hypothesized that the lipid mediator PGE2 could be one of them (17), and this study describes the detailed downstream signaling triggered by PGE2 to aid persistent parasite survival inside the macrophages. Our findings of *L. donovani*–induced increased expression of COX-2, the rate-limiting enzyme for PGE2 biosynthesis, and reduced intracellular parasite survival in the presence of specific COX-2 inhibitor necessitates the need for a deeper analysis of PGE2 signaling. Studies with *Streptococcus pyogenes* showed that inhibition of COX-2 both by genetic ablation and by pharmacological inhibition significantly improved not only the ability of mice to control infection, but also the survival period of infected animals (40), and in the case of *L. major* infection, causative agent of cutaneous leishmaniasis, administration of COX-2 inhibitor markedly decreased the number of metastatic lesions in mice (41). Our findings are in apparent discrepancy with the study by Mauel et al. (42), where they concluded that PGE2 and cAMP increase NO-mediated killing of certain *Leishmania* species in B6 and CBA mice. However, they first activated the macrophages with IFN-γ and TNF-α, which might lead to NO-mediated leishmanicidal activity, whereas we performed all the experiments in resting macrophages. Difference in mouse strain could be another explanation for this anomaly because we have used BALB/c mice in our study. It is known that difference in mouse strain exhibits different outcomes not only in visceral leishmaniasis, but also in different disease models (43–45). PGE2 exerts its effect by binding to a family of G protein–coupled E-prostanoid receptors termed EP1, EP2, EP3, and EP4. Among these, EP2 was found to be markedly induced in *L. donovani*–infected condition. Infection upregulated the expression and sur-

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**FIGURE 6.** Effect of NS-398 and AH6809 on infection. BALB/c mice were infected with 10^7 *L. donovani* promastigotes, and the splenocytes were isolated after various time periods as indicated. PGE2 production per 2 × 10^6 splenocytes was evaluated by enzyme immunoassay (A). Another set of infected mice was treated i.p. with NS-398 or AH6809 twice weekly at a dose of 10 mg/kg/d for 6 wk starting at 1 wk postinfection. Disease progression was determined by measuring spleen and liver parasite burden as Leishman–Donovan units at various time periods postinfection (B and C) and spleen weight (D) at 6 wk postinfection. Splenocytes (2 × 10^6) were isolated from control, infected, and infected plus NS-398–treated or AH6809–treated mice at 2, 4, and 6 wk postinfection, and cytokine and chemokine levels were determined by ELISA. TNF-α and IL-12 (E), TGF-β and IL-10 (F), CCL3 and CCL5 (G), and IL-17 and IL-22 (H). Results are representative of three individual experiments, and the error bars represent mean ± SD. **p < 0.01, ***p < 0.001 versus infected, Student t test.
face localization of EP2, and the role of EP2 in the suppression of severity of visceral leishmaniasis was further confirmed by in vivo administration of EP2-specific antagonist in the BALB/c mouse model, thus pointing directly to a key contribution of EP2 in the augmentation of Leishmania growth in macrophages. This is in agreement with previous studies that showed that in Streptococcal infection, mice that lack the gene for EP2 were protected from death caused by group A Streptococcus infection (46). However, in the case of L. major, increased expression of EP1 and EP3 was reported during infection (47), which may be because of difference in strain. EP2 is known to couple with Gs protein, leading to increase in [cAMP], level (8). cAMP has been postulated to inhibit microbialized capacity of macrophages (25, 48); we speculated that this pathway might be responsible for generating ultimate downstream effects mediated by PGE2 in parasite survival. Administration of cell-permeable cAMP analog and adenylyl cyclase activator enhanced Leishmania survival, and cAMP-treated macrophages prevented ROS generation, a major contributor in anti-leishmanial mechanism. The role of cAMP in augmenting infection has been shown by others (48), and our study adds further evidence to this notion. The effects of cAMP are mediated by two ubiquitously expressed [cAMP] receptors: the classical PKA and the recently discovered EPAC (49). Significant reduction in parasite number in the presence of PKA inhibitor suggested greater contribution of PKA in mediating downstream effects of cAMP. However, more pronounced effect observed with combined administration of both EPAC and PKA inhibitor indicated that the role of EPAC cannot be completely negated in fostering parasite survival. Modulation of chemokine and inflammatory/anti-inflammatory cytokine profile plays a decisive role during visceral leishmaniasis; therefore, we analyzed the importance of PKA and EPAC in shifting the balance toward anti-inflammatory cytokine and chemokine production during infection. Proinflammatory cytokines TNF-α and IL-12, and anti-inflammatory cytokines TGF-β and IL-10 have gained utmost importance because depletion of TNF-α and IL-12 and augmentation of TGF-β and IL-10 are known to aggravate progression of visceral leishmaniasis (15). Using peritoneal macrophages exposed to various treatment conditions, we inferred that PGE2-mediated induction of PKA causes attenuation of IL-12 and TNF-α production, as well as enhancement of IL-10 and TGF-β. Moreover, in vivo administration of COX-2 inhibitor and EP2 antagonist greatly increased the splenic secretion of TNF-α and IL-12, whereas substantially inhibiting the release of the anti-inflammatory cytokines IL-10 and TGF-β from the splenocytes of infected mice. Chemokines are the key molecules in recruiting immune cells by chemotaxis, which also act in leukocyte activation, inflammatory diseases, and antimicrobial mechanisms (22, 50). Chemokines seem to be implicated in T cell amplification of the inflammatory response, an important step for protective host defense in visceral leishmaniasis (22). Although there are reports of overexpression of CCL3 as the host-protective chemokine (23), there are also reports of CCL3 playing a deleterious role in the outcome of L. donovani infection (51). Moreover, blockade of CCL5 was found to render mice more susceptible to L. major infection (24). In this study, we wanted to determine the role of L. donovani-induced PGE2 in the modulation of CCL3 and CCL5 in peritoneal macrophages, and found suppression of both of these proinflammatory chemokines. We further observed that the attenuation of CCL3 might be occurring through the cAMP/EPAC pathway, whereas that of CCL5 is through the cAMP/EPAC pathway. These results complement a previous study by Jing et al. (5) reporting inhibition of CCL3 by EPAC-mediated glycogen synthase kinase 3 pathway in dendritic cells and by Qian et al. (52) stating PKA as the sole player in suppression of CCL5 in RAW 264.7 cells. It might be possible that L. donovani–induced PGE2 differentially regulates the secretion of CCL3 and CCL5, and EPAC contributes in parasite survival by augmenting the increase of CCL3, which accounts for the previously observed inhibitory effect of EPAC on parasite survival.

We recently reported the importance of Th17 cytokines in the establishment of antileishmanial response (53). We therefore checked whether in addition to inflammatory cytokines, PGE2 also can modulate secretion of the Th17 cytokines in Leishmania infection. Although PGE2 was shown to enhance IL-17 production from CD4+ memory T cells, it has recently been shown that the pathogenic fungus, Cryptococcus neoformans, induces PGE2 production upon infection and uses PGE2- and IRF4-dependent mechanisms to specifically inhibit induction of IL-17 during Th17 differentiation of T cells in the naive condition (21). Our studies are in agreement with this as we showed that in L. donovani infection, the induction of PGE2 downregulates the secretion of IL-17 during the primary differentiation phase induced by TGF-β and IL-6, but not in matured Th17 cells. PGE2 therefore not only modulates inflammatory cytokines and chemokines, but also helps in parasite survival by downregulating the production of antileishmanial IL-17 cytokine. Our study thus provides strong evidence that by inducing COX-2 expression and its product PGE2, L. donovani exploits the EP2/cAMP signaling pathway as a virulence mechanism to frustrate the host defense strategies. Finally, we envisage a circuit in which the exacerbated generation of PGE2 induced by Leishmania parasites results in the attenuation of disease-resolving inflammatory and Th17 cytokines and proinflammatory chemokines, which would in turn augment Leishmania growth.

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Disclosures

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References


