Uncoupling Protein 2 Negatively Regulates Mitochondrial Reactive Oxygen Species Generation and Induces Phosphatase-Mediated Anti-Inflammatory Response in Experimental Visceral Leishmaniasis

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Uncoupling Protein 2 Negatively Regulates Mitochondrial Reactive Oxygen Species Generation and Induces Phosphatase-Mediated Anti-Inflammatory Response in Experimental Visceral Leishmaniasis

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To reside and multiply successfully within the host macrophages, Leishmania parasites impair the generation of reactive oxygen species (ROS), which are a major host defense mechanism against any invading pathogen. Mitochondrial uncoupling proteins are associated with mitochondrial ROS generation, which is the major contributor of total cellular ROS generation. In the present study we have demonstrated that Leishmania donovani infection is associated with strong upregulation of uncoupling protein 2 (UCP2), a negative regulator of mitochondrial ROS generation located at the inner membrane of mitochondria. Functional knockdown of macrophage UCP2 by small interfering RNA-mediated silencing was associated with increased mitochondrial ROS generation, lower parasite survival, and induction of marked proinflammatory cytokine response. Induction of proinflammatory cytokine response in UCP2 knocked-down cells was a direct consequence of p38 and ERK1/2 MAPK activation, which resulted from ROS-mediated inhibition of protein tyrosine phosphatases (PTPs). Administration of ROS quencher, N-acetyl-l-cysteine, abrogated PTP inhibition in UCP2 knocked-down infected cells, implying a role of ROS in inactivating PTP. Short hairpin RNA-mediated in vivo silencing of UCP2 resulted in decreased Src homology 2 domain-containing tyrosine phosphatase 1 and PTP-1B activity and host-protective proinflammatory cytokine response resulting in effective parasite clearance. To our knowledge, this study, for the first time, reveals the induction of host UCP2 expression during Leishmania infection to downregulate mitochondrial ROS generation, thereby possibly preventing ROS-mediated PTP inactivation to suppress macrophage defense mechanisms. The Journal of Immunology, 2011, 187: 000–000.

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, a disease caused by the protozoan parasite Leishmania donovani. It is the second largest parasitic killer in the world, responsible for an estimated 60,000 deaths from the disease each year out of half a million infections worldwide. In the mammalian hosts, Leishmania survives and multiplies in macrophages, which are the first line of defense against any invading pathogen. To establish a successful infection, Leishmania parasites must counter the immune responses evoked by macrophages. One of the primary microbicidal molecules in macrophages recognized for its efficacy against Leishmania is reactive oxygen species (ROS), the generation of which is inhibited following infection (1, 2). A few studies suggested the involvement of surface molecules lipophosphoglycan and gp63 of Leishmania (3) and abnormal protein kinase C (PKC) activity in suppressing the ROS generation (2) by the host macrophages. However, no report addressed the possible involvement of host mitochondria, a major ROS generation site, in this regard.

Uncoupling proteins (UCPs) belong to the family of transporters present in the inner membrane of mitochondria and are the major regulator of ROS generation in the macrophage (4, 5). Several studies have emphasized the role of mitochondrial inner membrane UCPs in regulating the mitochondrial ROS generation in diverse cellular contexts and disease conditions. UCP1 was the first member to be identified, and it is exclusively expressed in the brown adipocytes to produce heat by uncoupling oxidative phosphorylation (6). In contrast, UCP2, a negative regulator of mitochondrial ROS generation, was observed to be abundantly expressed in components of the immune system such as spleen, lung, and isolated macrophages (7). Recently, putative involvement in resistance to intracellular pathogens was reported for UCP2. Macrophages from Ucp2−/− mice generated more ROS than did wild-type mice in response to Toxoplasma gondii, and they had a higher toxoplasmacidal activity in vitro (8). Recent data suggested that in Listeria monocytogenes infection, UCP2 modulates innate immunity via the modulation of ROS and cytokine production (9). Moreover, overexpression of UCP2 in RAW 266.7 macrophages has reinforced the belief that UCP2 plays a role in limiting intracellular ROS production (10). The importance of ROS in phagocytic cells is well documented. Therefore, by acting as a negative regulator of ROS production, especially in monocytes/macrophages, UCP2 could be involved in the innate response faster than did wild-type mice in response to Toxoplasma gondii, and they had a higher toxoplasmacidal activity in vitro (8). Recent data suggested that in Listeria monocytogenes infection, UCP2 modulates innate immunity via the modulation of ROS and cytokine production (9). Moreover, overexpression of UCP2 in RAW 266.7 macrophages has reinforced the belief that UCP2 plays a role in limiting intracellular ROS production (10). The importance of ROS in phagocytic cells is well documented. Therefore, by acting as a negative regulator of ROS production, especially in monocytes/macrophages, UCP2 could be involved in the innate response.

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Abbreviations used in this article: ANT, adenine nucleotide translocase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DHR123, dihydrorhodamine 123; H2DCFDA, 2′,7′-dihydrodichlorofluorescein diacetate; NAC, N-acetyl-l-cysteine; PKC, protein kinase C; pNPP, p-nitrophosphoryl phosphate; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; SHP-1, Src homology 2 domain-containing tyrosine phosphatase 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; UCP, uncoupling protein; VL, visceral leishmaniasis.

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response against \textit{L. donovani}. Many recent studies have revealed that the production of ROS is tightly regulated, engendering the concept that at lower levels than those generated for a microbicidal function, ROS may also function in propagating a signalizing response (11). For example, the reversible oxidation of target proteins in a cell may regulate the function of those proteins in response to various agonists and thus elicit a cellular response to stimulation. In this context, attention has been drawn to the protein tyrosine phosphatases (PTPs), which together with the protein tyrosine kinases are responsible for maintaining a normal tyrosine phosphorylation status. Work from several laboratories demonstrated that PTPs are important targets of ROS, which oxidize the active site Cys of PTP to abrogate its nucleophilic properties, thereby inhibiting PTP activity (12). Moreover, disease progression in VL is exacerbated by a strong parasite-induced macrophage PTP activation, which leads to the negative regulation of host cell functions (13). Although macrophages are an early target of \textit{L. donovani}, where it suppresses the macrophage defense mechanisms by actively inducing PTP activity, and UCP2 modulates the activity of macrophages by regulating the generation of ROS, no direct relationship between these two has yet been documented.

In the present investigation, we set out to explore the mechanisms of establishment of infection by \textit{Leishmania} in macrophages in relationship to mitochondrial ROS regulation by UCP2. The importance of UCP2 and mitochondrial ROS production in host–pathogen interaction have been validated further by silencing UCP2, both in vivo and in vitro situations, and its effects on perturbation of host- and parasite-conducive immune responses in disease progression were studied with emphasis on PTP, MAPK, and pro- and anti-inflammatory cytokines as prime players of immune response in \textit{L. donovani} infection.

Materials and Methods

Reagents

All Abs were from Santa Cruz Biotechnology and Cell Signaling Technology. All other chemicals were from Sigma-Aldrich, unless indicated otherwise.

Animals and parasite

\textit{L. donovani} strain AG83 (MHOM/IN/1983/AG83), isolated from an Indian patient with kala-azar (14), was maintained in inbred BALB/c mice by i.v. passage every 6 wk. \textit{L. donovani} promastigotes were obtained by allowing isolated splenic amastigotes to transform in parasite growth medium for 72 h at 22˚C. The growth medium consisted of medium 199 (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS.

Cell culture, in vitro and in vivo infection

A RAW 264.7 murine macrophage cell line was kept in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM l-glutamine at 37˚C and 5% CO₂. Splenocytes were isolated and cultured as described earlier (15). Macrophages were infected with stationary phase \textit{L. donovani} promastigotes at a 10:1 parasite/macrophage ratio. Infection was allowed to proceed for 4 h, noninternalized parasites were removed by washing the plates with PBS, and cells were cultured for different time periods. Cells were fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. For in vivo infection, female BALB/c mice (∼20 g) were injected via the tail vein with \textit{L. donovani} promastigotes. Infection was assessed by removing spleen from infected mice up to 6 wk. Parasite burdens were determined from Giemsa-stained impression smears (17). Spleen parasite burdens, expressed as Leishman–Donovan units, were calculated as the number of amastigotes/1000 nucleated cells × spleen weight (in grams) (18).

FACS analysis

Intracellular ROS generation was measured using the oxidant-sensitive green fluorescent dye 2’,7’-dihydrodichlorofluorescein diacetate (H₂DCF-DA) (Molecular Probes). Measurement of fluorescence in cells was made by counting at least 10,000 events per test using a FACSCalibur flow cytometer (BD Biosciences) with a FITC filter, and the cells were gated out based on their fluorescent property. The production of mitochondrial ROS was analyzed by labeling cells with 10 μM dihydrorhodamine 123 (DHR123) (Molecular Probes) for 15 min according to Wang et al. (19). Samples were examined by FACSCalibur and the results were analyzed using CellQuest software (BD Biosciences).

Mitochondrial isolation

For the isolation of mitochondria, a mitochondrial isolation kit for cultured cells (Qiagen) was used as instructed.

NBT reduction assay

ROS production was measured in isolated macrophages by measuring their ability to reduce NBT. Macrophages were treated with NBT (100 μM, 20 mg/ml; Sigma-Aldrich) dissolved in PBS containing 5% glucose and incubated at 37˚C. Supernatants were discarded and cells were washed several times with 70% methanol and allowed to dry. Formazan formed was solubilized by adding 100 μl/well KOH (2 M), followed by 100 μl/well DMSO. Absorbance was measured at OD 630 nm.

Aconitase/fumarase activity ratio

Mitochondrial ROS production was estimated by analysis of aconitase and fumarase activities. Macrophages were resuspended in isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris, 0.2% BSA [pH 7.4]) with protease inhibitors (1 μg/ml pepstatin, 4 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 μg/ml bestatin) and homogenized in a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation for 10 min at 750 × g at 4˚C. The supernatant was centrifuged for 20 min at 12,000 × g at 4˚C and mitochondria were resuspended in isolation buffer and then lysed in 0.2% Triton X-100. Enzymatic activities were measured according to Criscuolo et al. (20), by following the absorbance increase at 240 nm for 20 min in appropriate medium (30 mM sodium isocitrate, 50 mM Tris-HCl, 0.6 mM MnCl₂ at pH 7.4 for aconitase, and 50 mM sodium i-malate, 50 mM sodium phosphate at pH 7.4 for fumarase). The aconitase/fumarase ratio was expressed as the ratio of respective rates of absorbance increase.

Immunoblot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM PMSF) and the protein concentrations in cleared supernatants were measured using a Bio-Rad protein assay. The supernatants containing an equal amount of protein (30 μg) from each sample were resolved by 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore). 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 a dilution recommended by the suppliers. Membranes were washed three times with wash buffer and then incubated with HRP-conjugated secondary Ab and detected by the ECL detection system (Amersham Biosciences), according to the manufacturer’s instructions.

RT-PCR

Total RNA was isolated from RAW 264.7 cells using the RNeasy Mini kit (Qiagen) and treated with DNAse 1, as recommended by the manufacturer. RNA (1 μg) was used as a template for cDNA synthesis using the SuperScript first-strand synthesis system for the RT-PCR kit (Invitrogen). Semi-quantitative RT-PCR was used to detect the mRNA abundance of adenosine nucleotide translocase (ANT1) (forward, 5'-AAAATATTTGGAATACC-CAGCTTCAACA-3'; reverse, 5'-TTTGGTGGTCTAACCA-3'), Aconitase/Fumarase (forward, 5’-ATGTGGTGTGTACCCGCA-3'; reverse, 5’-TCAGAAGATTCCCTCCCAAG-3'), and UCP2 (forward, 5'-TGTCAAGGCAGTTCTCACCC-3'; reverse, 5’-TTCGACATACTGGCAGAGGG-3'). The mRNA was normalized to porin mRNA levels...
Small interfering RNA transfection

Small interfering RNA (siRNA) transfections were carried out using commercial siRNA constructs (Santa Cruz Biotechnology) according to the manufacturer’s protocol. Scrambled siRNA was used as control.

shRNA construct

The UCP2-specific shRNA cassette, driven by the promoter of the small nuclear RNA U6, was generated by PCR-mediated amplification of positions 936–957 of the UCP2 gene (GenBank accession no. NM_003355), and the selection of shRNA target sequences was based on published guidelines (21). The shRNA construct was cloned into a third generation, self-inactivating lentiviral vector pCRI.LV, and virus was produced and concentrated as described earlier (22). Vector-carrying, GFP-specific shRNA was used as control (23). Virus titer was measured at 1 × 10^6 infectious units/ml. For in vivo knockdown of UCP2, 50 µl 1000× vector pCRI.LV was injected into spleen tissue in anesthetized BALB/c mice prior to 3 d infection.

Phosphatase assays

As previously described (15), macrophages were lysed in PTP lysis buffer (50 mM HEPES [pH 7.4] containing 0.5% Triton X-100, 10% glycerol, 1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 µg/ml pepstatin A) and kept on ice for 45 min. Lysates were cleared by centrifugation and protein content was determined by a Bio-Rad protein assay. Lysates were then incubated with 10× PTP reaction buffer (50 mM HEPES [pH 7.5], 0.1% 2-ME, 10 mM p-nitrophenyl phosphate [pNPP]) for 30 min. OD was read at 405 nm. In a separate set of experiments, PTP activity was further determined by the capacity of protein lysates to dephosphorylate a monophosphorylated phosphotyrosine peptide substrate (TRDipYETDYRKR) for 10 min at 37°C. Free inorganic phosphate was detected with malachite green (Sigma-Aldrich), and activity was calculated by subtracting the amount of non-specific hydrolysis by its respective control. Protein extract (10 µg) was used in all experiments.

Statistical analysis

Data shown are representative of at least three independent experiments and are expressed as means ± SD. A Student t-test was employed to assess the statistical significance of differences among pair of data sets with a p value <0.05 considered to be significant.

Results

Decreased cellular and mitochondrial ROS generation in L. donovani-infected macrophages

Successful survival of Leishmania within the host macrophages depends on the impairment of host immune responses. One of the major host defense components against any invading pathogen is the generation of ROS. Inhibition of ROS generation is a crucial adaptive strategy through which Leishmania can survive within the hostile environment of macrophages. We first checked whether Leishmania internalization is associated with neutralization of ROS generation. Because attachment of L. donovani leads to increased ROS generation (24), one set of RAW macrophages was pretreated with cytochalasin D, which prevents the uptake but not the attachment of the parasite (24). Efficacy of cytochalasin D to prevent the parasite internalization within the macrophages was checked and it was observed that the uptake of L. donovani promastigotes was significantly abrogated in the presence of increasing doses of cytochalasin D and found to be maximum at a concentration of 2 µM (82% inhibition of parasite internalization) (Fig. 1A). Next, to measure the level of intracellular ROS, the cytochalasin D-treated or untreated macrophages were infected with L. donovani promastigotes for the indicated time periods, washed in PBS, incubated for 30 min at 37°C in DMEM containing the green fluorescent dye H2DCFDA, and then the fluorescence levels of 50,000 cells were counted. A gate (P2) was established that delineated approximately the upper 5% of fluorescent cells. The percentages of gated cells were 34.5 ± 3.1 and 39.4 ± 4.3% higher in cytochalasin D-treated infected macrophages compared with untreated infected macrophages at 1 and 2 h, respectively, implying significant ROS generation in non-internalized cells (Fig. 1B). In contrast, the percentages of gated cells in untreated infected cells were comparable to those in infected cells, implying that L. donovani infection failed to induce any ROS generation in macrophages. The cellular ROS generation in macrophages did not alter following cytochalasin D treatment (H2DCFDA-positive cells were only 5.6 and 6.4% at 1 and 2 h, respectively, whereas 4.9% H2DCFDA-positive cells were found in untreated controls) (Fig. 1B). This finding was further confirmed by the NBT reduction assay, which is one of the most established methods to detect intracellular O$_2^-$ generation by the reduction of NBT to formazan (25). Similar to FACS analysis, there was no induction of ROS generation in L. donovani-infected macrophages, whereas significantly enhanced levels were observed in cytochalasin D-treated cells at 1 and 2 h postinfection, as evident by 2.1- and 1.9-fold increase in OD values (p < 0.001) (Fig. 1C).

In this context, the role of mitochondria, a major cellular organelle involved in ROS generation, could be pivotal, bolstering oxidative stress by contributing to ROS formation. To determine mitochondrial ROS levels, cells were loaded with DHR123. In cytochalasin D-treated macrophages there was significant mitochondrial ROS generation at 1 and 2 h postinfection (34.5 ± 2.8 and 56.1 ± 4.7% DHR123-positive cells, respectively), whereas in untreated macrophages L. donovani infection failed to induce mitochondrial ROS generation (Fig. 1D). Similar to cellular ROS generation, mitochondrial ROS generation in macrophages was almost unaltered following cytochalasin D treatment (DHR123-positive cells were only 7.4 and 6.1% in cytochalasin D-treated macrophages at 1 and 2 h, respectively, whereas 5.1% DHR123-positive cells were found in untreated controls) (Fig. 1D). The above data were further reconfirmed by a fumarase/aconitase ratio assay. Mitochondrial aconitase activity is a functional indicator of mitochondrial ROS levels (26) because the Fe-S center of aconitase is oxidized by superoxide, reducing enzyme activity. However, mitochondrial ROS does not alter fumarase activity. Therefore, we checked the activity ratio of mitochondrial aconitase to fumarase as a functional indicator of ROS production in infected and cytochalasin D-treated cells. (Fig. 1E). Lower aconitase/fumarase ratios signify higher mitochondrial ROS production. In the case of attached parasites, there were 34.2 and 31.1% reductions in aconitase/fumarase ratios over basal levels at 1 and 2 h postinfection, respectively (p < 0.01), whereas the internalization of the parasites failed to induce any mitochondrial ROS generation (2.1 and 4.8% reduction over basal levels at 1 and 2 h postinfection, respectively). Similarly, aconitase activity at 2 h postinfection was significantly lower in cells treated with cytochalasin D (9.2 ± 0.8 U/mg) but was similar in control and L.
FIGURE 1. Effect of *L. donovani* infection on cellular and mitochondrial ROS generation. A, RAW 264.7 cells were pretreated with various doses of cytochalasin D (1–5 μM) for 1 h, followed by infection with *L. donovani* promastigotes (cell/parasite ratio, 1:10) for different time periods as indicated. The number of parasites per 100 macrophages was evaluated by Giemsa staining. B, RAW 264.7 cells were either pretreated with 2 μM cytochalasin D for 1 h or left alone followed by infection with *L. donovani* promastigotes (cell/parasite ratio, 1:10) as described in Materials and Methods. In a separate set, RAW 264.7 cells were treated with 2 μM cytochalasin D for different time periods as indicated. Representative histograms plotting the fluorescence of 50,000 cells treated with 20 μM H2DCFDA to measure cellular ROS generation are shown. The H2DCFDA-positive cells are indicated as the percentage of gated cells. C, Total cellular ROS generation was further determined by the capacity of RAW 264.7 macrophages to reduce NBT. D, To measure mitochondrial ROS generation, macrophages were treated with cytochalasin D followed by infection as indicated above, stained with 10 μM DHR123 for 15 min, and analyzed by FACS. Cytochalasin D-treated uninfected macrophages were used as control. Results are presented as percentage of DHR123-positive cells. E, Mitochondrial ROS production of cytochalasin D-treated or untreated infected macrophages were measured by aconitase activity (inset: the ratio of mitochondrial fumarase/aconitase activities). F, Macrophages were pretreated with CCCP (1 μM) for 1 h or left alone followed by stimulation with LPS (100 ng/ml) plus IFN-γ (100 U/ml) for different time periods (1 and 2 h). The mitochondrial ROS generation was measured by DHR123 probe staining. G, Macrophages were infected with *L. donovani* promastigotes and coincubated with 100 ng/ml LPS plus 100 U/ml IFN-γ with or without 1 μM CCCP for indicated time periods. The number of parasites per 100 macrophages was evaluated by Giemsa staining. Results are representative of three individual experiments, and the error bars represent means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.
Leishmania donovani-infected cells (31.7 ± 3.3 and 31.1 ± 2.9 U/mg, respectively). To further determine the contribution of mitochondrial ROS in total cellular ROS generation, macrophages were stimulated with LPS plus IFN-γ either alone or in combination with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler. A marked elevation of ROS generation was observed in LPS plus IFN-γ-stimulated cells (3.7- and 4.8-fold at 1 and 2 h, respectively, compared with control cells; \( p < 0.0001 \)), which were significantly suppressed in cells coincubated with CCCP (66.2 and 63.5% inhibition at 1 and 2 h, respectively; \( p < 0.0001 \)) (Fig. 1F). Furthermore, inhibitory effects of LPS plus IFN-γ on amastigote multiplication (77.3 and 96.1% parasite killing at 12 and 24 h postinfection, respectively) were markedly reversed in the presence of CCCP (46.2 and 57.3% reduction in parasite killing at 12 and 24 h postinfection, respectively; \( p < 0.001 \)) (Fig. 1G). This observation further strengthens the importance of mitochondrial ROS generation in in vitro killing of parasite. Collectively, these results suggest that Leishmania donovani infection leads to the downregulation of cellular as well as mitochondrial ROS generation, which are essential for parasite survival in phagocytic cells.

**Effect of Leishmania donovani infection on UCP2 expression**

The mitochondrial ROS generation is regulated by several uncoupling proteins, such as UCP2, UCP3, and ANT (27), located within the inner mitochondrial membrane. We therefore thought it worthwhile to check whether Leishmania infection could modulate the expression of these proteins. Time course analysis (0–4 h) by Western blot demonstrated a considerable increase in UCP2 expression in a time-dependent manner with a maximum of 2.9-fold at 2 h postinfection relative to uninfected control (Fig. 2A), whereas the expression of other uncouplers remained unaltered throughout the course of infection. However, quantification of RTPCR data revealed that at 1 h postinfection there was a strong upregulation of UCP2 mRNA (2.1-fold) in infected cells, which remained unchanged at 2 and 4 h postinfection (1.8- and 2-fold increase, respectively) (Fig. 2B). Perhaps this discrepancy in protein and mRNA level is indicative of the presence of other controlling elements that affect the stability of UCP2 protein. RT-PCR analysis of other uncouplers did not exhibit any change in expression level in the course of infection. Taken together, these findings indicate an increase of UCP2 expression both at protein and mRNA levels during the course of infection.

**Effect of UCP2 silencing on Th1/Th2 cytokine balance, MAPK activation, and parasite survival**

To investigate whether the induction of UCP2 is associated with the inhibition of mitochondrial ROS generation and intracellular Leishmania survival, an siRNA-mediated knockdown system was used. The efficacy of siRNA on UCP2 expression was assessed by Western blotting. UCP2 expression was reduced significantly in cells expressing UCP2-specific siRNA compared with cells expressing control siRNA (Fig. 3A). Silencing of UCP2 resulted in a marked increase in mitochondrial ROS generation with a maximum of 3.8-fold increase at 4 h postinfection (\( p < 0.001 \)), which gradually decreased up to 1.1-fold at 24 h (Fig. 3B). When intracellular survival of Leishmania donovani parasite was measured it was found that although ROS generation was significantly higher at 4 h postinfection in UCP2 knocked-down cells, parasite suppression was only 23.3 ± 3.1% (Fig. 3C). Interestingly, parasite suppression was maximal (77.2 ± 6.1%) at 24 h postinfection in UCP2 knocked-down cells (Fig. 3C), although the level of ROS was only 8.2 ± 1.1% higher than control cells (Fig. 3B). These results suggest that inhibition of UCP2 by siRNA-mediated silencing led to an enhanced mitochondrial ROS production, but the leishmanial activity of the knocked-down cells was not due to the direct effect of ROS (e.g., lipid peroxidation, DNA damage) on parasite killing, as evident from the delay in parasite suppression.

ROS have been demonstrated as one of the key regulators in signal transduction pathways (28), and because the balance of pro- and anti-inflammatory cytokines released from macrophages and other immune cells is an important mediator in the outcome of Leishmania infection (15), we measured the expression of several proinflammatory and anti-inflammatory cytokines by ELISA. Silencing of UCP2 in macrophages resulted in significant reduction of IL-10 and TGF-β expression after Leishmania infection (66.5 and 73.8% reduction in IL-10 and TGF-β, respectively, as compared with infected control; \( p < 0.001 \)) (Fig. 3D). In contrast, UCP2 knocked-down cells showed significantly elevated levels of TNF-α and IL-12 postinfection (5.7- and 3.8-fold for TNF-α and IL-12, respectively; \( p < 0.001 \)) compared with very low levels in infected macrophages (56 and 72 pg/ml for TNF-α and IL-12, respectively) (Fig. 3D). We observed that there was no alteration in the expression profile of pro- and anti-inflammatory cytokines in UCP2 knocked-down cells in the absence of infection. Collectively, these results suggest that induction of UCP2 following infection preferentially turned the immune balance in favor of the parasite, whereas knockdown of UCP2 is associated with enhanced proinflammatory cytokine expression resulting in lower parasite survival within the macrophages.

Pro- and anti-inflammatory cytokine balance in macrophages is dependent on MAPK signal transduction events, and it was previously observed that ERK and p38 pathways are activated in UCP2−/− mice in response to LPS (29). Because of their importance in inflammatory and other immune responses, we thought it worthwhile to examine the effect of UCP2 knockdown on MAPK activation following infection. Activation of MAPKs was assessed by their phosphorylation, and it was observed that in infected macrophages there were very low levels of phosphorylation of

**FIGURE 2.** Expression of uncoupling proteins in RAW 264.7 cells following Leishmania donovani infection. RAW 264.7 cells were infected with Leishmania promastigotes for various time periods (0–4 h) as described in Materials and Methods. The expression of UCP2, ANT1, and UCP3 were evaluated by immunoblot (A) and RT-PCR (B) analysis. Porin was used as an internal control. Band intensities quantified by densitometry are shown as bar graphs on the right-hand side of each panel. Results are representative of three independent experiments and are expressed as means ± SD (\( n = 3 \)).

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p38, ERK, or JNK, whereas knockdown of UCP2 resulted in a gradual increase in the induction of p-ERK1/2 and p-p38, being maximal at 4 and 6 h postinfection, respectively (6.3- and 8.8-fold compared with infected cells treated with control siRNA) (Fig. 3E). We further analyzed the effect of control siRNA on MAPK activation in infected macrophages but did not find any effect on MAPK phosphorylation (Fig. 3F). To evaluate whether activation of p38 and ERK MAPK in UCP2-inhibited infected macrophages was indeed associated with alteration of the proinflammatory/anti-inflammatory cytokine balance, macrophages were treated with pharmacologic inhibitors of the MAPK pathway (SB203580 for p38 and PD98059 for ERK1/2). Preincubation of cells with SB203580 (30 μM) and PD98059 (20 μM) markedly abolished the phosphorylation of MAPK in UCP2 knockdown (E) and control siRNA-treated (F) macrophages were detected by immunoblot analysis following infection with L. donovani promastigotes for various time periods (0–8 h). G and H, Macrophages were transfected (24 h) with UCP2 siRNA as mentioned above and either left alone or followed by treatment with either SB203580 (30 μM) or PD98059 (20 μM) for 1 h. The levels of proinflammatory (G) and anti-inflammatory (H) cytokines were determined by ELISA after 24 h infection. Results are representative of three individual experiments, and the error bars represent means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.

**FIGURE 3.** Effect of UCP2 silencing on cytokine response, MAPK activation, and parasite survival. A, The specificity of UCP2 siRNA was determined in cell lysates from macrophages expressing either UCP2 targeting or control siRNAs by Western blotting using specific Abs against UCP2. B, To determine the effect of UCP2 inhibition, macrophages were transfected (24 h) with UCP2 siRNA, followed by infection with L. donovani promastigotes for various time periods (0–24 h). B, ROS generation was measured by H2DCFDA probe staining, and (C) number of intracellular parasites were evaluated by Giemsa staining. D, Cytokine levels in UCP2 knockdown macrophages were determined by ELISA after 24 h infection. E and F, The expression and phosphorylation of MAPK in UCP2 knockdown (E) and control siRNA-treated (F) macrophages were detected by immunoblot analysis following infection with L. donovani promastigotes for various time periods (0–8 h). G and H, Macrophages were transfected (24 h) with UCP2 siRNA as mentioned above and either left alone or followed by treatment with either SB203580 (30 μM) or PD98059 (20 μM) for 1 h. The levels of proinflammatory (G) and anti-inflammatory (H) cytokines were determined by ELISA after 24 h infection. Results are representative of three individual experiments, and the error bars represent means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.

**Generation of ROS through knockdown of UCP2 inhibits PTP activity**

Impairment of the MAPK pathway following L. donovani infection is associated with induction of PTP, and kinase–phosphatase balance plays a major role in the disease outcome of leishmaniasis (30). It has previously been shown that modulation of PTPs greatly influences signaling and phagocyte functions (31). We therefore checked whether knockdown of UCP2 has any influence on PTP activity. Macrophage PTP activity was measured by the capacity of total cell lysates to dephosphorylate pNPP as well as a synthetic tyrosine monophosphorylated peptide substrate. L. donovani infection rapidly induced macrophage PTP activity, which was maximal at 2 h (4.6- and 4.3-fold for pNPP and synthetic tyrosine phosphopeptide, respectively) (Fig. 4B). In contrast, in UCP2 knocked-down infected cells the PTP activity was significantly abrogated at oxidation by ROS (12), and therefore to evaluate the role of ROS in inhibition of PTP activity, UCP2 knocked-down macrophages were preincubated (2 h) with N-acetyl-L-cysteine (NAC), an ROS quencher. As depicted in Fig. 4A and 4B, pretreatment of UCP2 knocked-down cells with NAC (5 μM) markedly increased PTP activity following infection and the maximum activity was observed at 2 h postinfection (4.28- and 4.12-fold for pNPP and synthetic tyrosine phosphopeptide, respectively). These data in-
dicated that increased mitochondrial ROS generation in UCP2 knocked-down infected cells is associated with abrogation of macrophage PTP activity.

To gain insight into the cellular mechanism underlying the PTP inactivation associated with increased mitochondrial ROS generation, we sought to identify whether expression of a broad range of macrophage-specific PTPs were altered in UCP2 knocked-down infected cells. As far as individual phosphatases were concerned, immunoblot analysis of various PTPs revealed a strong upregulation of PTP-1B and SHP-1 at 4 h postinfection (2.1- and 2.8-fold for PTP-1B and SHP-1, respectively; $p < 0.001$), whereas the expression levels of other PTPs (TCPTP, SHP-2, PTP-PEST, PTEN) remained comparable in control and infected macrophages (Fig. 4C). However, knockdown of UCP2 did not alter the increased expression of PTP-1B and SHP-1 in infected macrophages. We then checked the specific activities of these two PTPs in UCP2 knocked-down cells. The specific activities of immunoprecipitated SHP-1 and PTP-1B were $66.1 \pm 6.2$ and $73.3 \pm 7.6$ lower in UCP2 knocked-down cells compared with infected macrophages (Fig. 4D). However, pretreatment of UCP2 knockdown cells with NAC resulted in almost comparable levels of SHP-1 and PTP-1B activity as in infected macrophages (Fig. 4D). These results indicated that UCP2 knockdown in infected macrophages resulted in deactivation of PTPs (SHP-1 and PTP-1B), which was mediated by increased ROS generation.

Role of UCP2 in in vivo Leishmania infection

Because our in vitro observations suggested that UCP2 induction decreased ROS generation and proinflammatory cytokine expression, thereby facilitating parasite survival in infected phagocytic cells, we thought it worthwhile to evaluate the role of UCP2 in the disease progression of leishmaniasis in vivo conditions. Immunoblot analysis of the splenocytes of $L. donovani$-infected animals at various time periods postinfection revealed a strong upregulation of UCP2 expression at protein level, similar to the in vitro scenario. $L. donovani$ infection caused a substantial increase in UCP2 protein expression in the spleen cells, with a maximum induction of 4.3-fold at 2 wk postinfection ($p < 0.001$) (Fig. 5A). We then checked whether inhibition of UCP2 could modulate the ROS generation and parasite persistence in vivo, and for that silencing of UCP2 was achieved through administration of lentiviral vector-mediated shRNA. To observe which spleen cells were effectively infected by shRNA lentiviral particles, GFP-encoding control shRNA was injected to the spleen tissue of BALB/c mice and GFP expression in macrophages was evaluated by flow cytometry. Based on surface expression of CD11b, macrophages of splenocytes were gated by anti–CD11b-PE, and GFP-expressing macrophages were found to be $87.7, 85.5,$ and $89.6\%$ at 2, 4, and 6 wk posttransfection, respectively (Fig. 5B). This observation suggested that among the total spleen cell population, macrophages are effectively infected by the shRNA lentiviral particles. The efficacy and specificity of shRNA on UCP2 expression was further evaluated by immunoblot analysis in splenic macrophages (Fig. 5C) as well as splenocytes of infected mice at various time periods postinfection (Fig. 5D, inset). Splenocytes from UCP2 shRNA-treated mice showed 3.35- and 3.58-fold induction of ROS generation at 2 and 4 wk postinfection, respectively ($p < 0.001$), compared with control shRNA-treated infected mice (Fig. 5D). Similarly, UCP2 inhibition in infected mice drastically reduced the spleen parasitic burden at 6 wk postinfection (75.8% reduction in spleen parasite burden compared with control shRNA-treated infected animal).
FIGURE 5. Role of UCP2 in in vivo infection, ROS generation, phosphatase activity, and cytokine balance. A, BALB/c mice were infected with $10^7$ L. donovani stationary-phase promastigotes as described in Materials and Methods. UCP2 expression at various time periods (0–6 wk) in the splenocyte lysates of infected mice were evaluated by immunoblot analysis ($n = 5$). B, Mice were administered 50 µl 1000× vector concentrate of GFP-encoding shRNA construct into spleen tissue, and splenocytes were isolated at various time periods. FACS analyses were performed in splenocytes where macrophage population was gated by anti–CD11b-PE Ab. Transfection specificity of GFP-encoding lentiviral particles was calculated in the double-positive subpopulation of macrophages ($n = 5$). C, To observe the efficacy of in vivo knockdown of UCP2, mice were injected with either GFP-encoding shRNA or UCP2-specific shRNA construct as described above. UCP2 expression was evaluated in isolated splenic macrophages by immunoblot analysis at various time intervals as indicated ($n = 5$). D, To evaluate the effect of knockdown of UCP2 on in vivo ROS generation, anesthetized BALB/c mice were injected with either GFP-encoding shRNA or UCP2-specific shRNA construct 3 d prior to infection. Splenocytes (2 × $10^6$ cells) from different groups of infected mice were isolated at various time periods (2–6 wk), and ROS generation was measured by H$_2$DCFDA probe staining as described in the legend of Fig. 1 ($n = 5$) (inset: UCP2 expression in UCP2-shRNA–treated mice splenocytes). E, Spleen parasite burdens were determined weekly in different groups of infected mice at various time points (1–6 wk) as described in Materials and Methods and are expressed as Leishman–Donovan units (LDU) ± SD for five.
FIGURE 6. *L. donovani* infection strongly upregulated UCP2, a mitochondrial inner membrane protein, which downregulated mitochondrial ROS generation, thereby preventing ROS-mediated PTP inactivation. This in turn led to deactivation of MAPKs, thereby shifting the pro-inflammatory/anti-inflammatory cytokine balance to anti-inflammatory phenotype and facilitating parasite survival.

(Fig. 5E). PTPs play a crucial role in parasite survival, and our in vitro observation revealed a strong induction PTP-1B and SHP-1 following *L. donovani* infection. We therefore assessed whether in vivo UCP2 inhibition could modulate the PTP activation. To this end, the activities of specific phosphatases were assessed in splenocytes of UCP2 knocked-down mice. *L. donovani* infection resulted in increased activity of SHP-1 and PTP-1B in spleen cells with a maximum induction at 3 wk postinfection (5.6-fold for SHP-1 and 4.9-fold for PTP-1B) (Fig. 5F, 5G). In contrast, in vivo silencing of UCP2 resulted in a gradual decrease in SHP-1 and PTP-1B activity, with a maximum inhibition at 3 wk postinfection (70.1 and 62.6% decrease for SHP-1 and PTP-1B, respectively, compared with control shRNA-treated infected animals) (Fig. 5F, 5G). Moreover, in vivo silencing of UCP2 also resulted in increased levels of TNF-α (790 ± 77 compared with 135 ± 12 pg/ml; p < 0.001) (Fig. 5H) with a concomitant decrease in IL-10 synthesis at 4 wk postinfection (220 ± 24 compared with 970 ± 95 pg/ml in infected mice) (Fig. 5I). These results suggest that strong upregulation of UCP2 expression following *L. donovani* infection may be associated with the concomitant induction of PTP activity resulting in anti-inflammatory immune response (Fig. 6), thus helping in the establishment of infection.

**Discussion**

The capacity of *Leishmania* to survive within the phagolysosomes of macrophages has been shown to involve the suppression ROS generation (1, 2). Previous studies indicated that *Leishmania* parasites avoid triggering the oxidative burst by actively inhibiting PKC-mediated NADPH activation. While mitochondria are potent producers of ROS and are a major contributor of the cellular oxidative burst, the role of mitochondrial ROS has not been studied previously in the disease condition of VL. In our present study, we sought to determine whether *Leishmania* infection could modulate the production of mitochondrial ROS for its survival within the phagolysosomes of the macrophages. Our main finding is that *L. donovani* infection resulted in suppression of mitochondrial ROS generation, which is associated with a strong upregulation of UCP2, a mitochondrial inner membrane protein. Induction of UCP2 is possibly involved in preferential activation of macrophage PTP, thereby preventing the positive p38 and ERK signal transduction, resulting in the increased synthesis of anti-inflammatory cytokines and subsequent survival of parasites. Inducing mitochondrial ROS generation by siRNA-mediated silencing of UCP2, we have demonstrated a definite role for mitochondrial ROS and UCP2 in disease progression of VL. This observation was extended to in vivo situation by shRNA-mediated silencing of UCP2, which resulted in enhanced ROS generation, macrophage PTP deactivation, and induction of proinflammatory cytokine response and subsequent suppression of organ parasite burden of infected mice.

Previous reports have indicated that *Leishmania* parasites are susceptible to ROS-mediated toxicity and avoid the induction of ROS generation by actively inhibiting PKC-mediated signaling (2). Although PKC activation is associated with NADPH-mediated cellular oxidative burst, the production of ROS by mitochondria has not been addressed previously in the disease context of VL. In our study, we have shown that *L. donovani* infection suppresses cellular as well as mitochondrial ROS generation in macrophages. Mitochondrial ROS are the byproduct of electron transport chain (32) and are negatively regulated by several uncoupling proteins present in the inner membrane of mitochondria at different cellular contexts. However, in macrophages, the generation of mitochondrial ROS is regulated by UCP2 (4). Our observation suggested that suppression of mitochondrial ROS generation following *L. donovani* infection is associated with strong upregulation of UCP2 in macrophages. In this context, we have evaluated the possible role of other uncouplers such as ANT1 and UCP3, but could not find any change in the expression level of these proteins.

Early studies with *Ucp2*−/− mice revealed that the deletion of the *Ucp2* gene markedly enhanced the microbicidal activity of the macrophages and this increased activity was associated with an
elevated level of ROS (8, 9). We observed an increase in mitochondrial ROS production in UCP2-siRNA–treated macrophages along with suppression of parasite survival. These findings seem to be in good agreement with the fact that UCP2 induction is correlated with the suppression of mitochondrial ROS generation following infection and that silencing of UCP2 resulted in reduced intramacrophage parasite survival. However, one interesting point in this study was the long interval observed between the surge in mitochondrial ROS generation (4 h postinfection) and effective parasite clearance (24 h postinfection). This observation is indicative of the fact that there may be other cellular processes involved in the elimination of the parasites apart from ROS-mediated direct effects such as DNA damage and lipid peroxidation. Induction of proinflammatory cytokines has been documented to evoke healing responses against L. donovani infection (33), and previous studies have suggested that stimulation of Ucp2−/− macrophages with LPS could modulate the cytokine balance toward the proinflammatory phenotype (9). In our present study, the increased synthesis of proinflammatory cytokines at a later time point (24 h) might be accountable for the delay observed in effective parasite clearance by the UCP2 knocked-down macrophages. However, one interesting observation in this study was that despite silencing UCP2, there was a gradual decrease in mitochondrial ROS production during a time period of 24 h. This may be explained by the activation of the macrophage antioxidant defense mechanism, either by the cell itself or by the parasite following infection, to reduce prolonged ROS-mediated toxicity.

Proinflammatory cytokine gene expression is known to be regulated by an upstream activation of MAPKs in different cellular contexts (34). In the present study, we observed a strong phosphorylation of p38 and ERK1/2 MAPK in UCP2 knocked-down infected macrophages, and pharmacological blockade of p38 and ERK1/2 pathways suggested that the induction of proinflammatory cytokines might be a direct consequence of MAPK activation. MAPK activation is an outcome of the balanced action of protein kinases and protein phosphatases. MAPks have been implicated in several pathways that modulate macrophage functions (35), and ROS have been shown to regulate the activity of several PTPs (12). We sought to evaluate the status of PTP activity in UCP2 knocked-down macrophages. We assessed the induction of leukocyte PTPs following infection and found that the specific activities of two PTPs, SHP-1 and PTP-1B, were significantly lower in UCP2 knocked-down macrophages, which might contribute to the higher phosphorylation of MAPks and consequently to the enhanced proinflammatory cytokine synthesis. Moreover, use of ROS quencher in UCP2 knocked-down infected macrophages suggested a definite role of ROS in the deactivation of PTPs. Disease progression of VL is exacerbated by a strong parasite-induced PTP activation, which leads to the negative regulation of host cell functions. Our study demonstrated that L. donovani infection is associated with strong upregulation of UCP2 in macrophages, resulting in marked induction of PTP activity possibly by inhibition of mitochondrial ROS generation. This was ascertained by the fact that UCP2 silencing resulted in increased ROS generation and inhibition of PTP activity in infected macrophages, thereby promoting positive MAPK signal transduction and induction of proinflammatory cytokine synthesis. These observations were further validated in the vivo situation, which revealed that, in infected mice, shRNA–mediated silencing of UCP2 resulted in inactivation of specific PTPs (SHP1 and PTP-1B) associated with strong induction of proinflammatory cytokine synthesis and significantly suppressed organ parasite burden.

The study has shed light on a novel pathway involving mitochondrial uncoupler favoring parasite persistence and disease progression in VL. Finally, a better understanding of the parasite-induced host inhibitory mechanisms will help in developing intervention strategies not only for nonhealing leishmaniasis but also for other macrophage-associated parasitic diseases.

Disclosures

The authors have no financial conflicts of interest.

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