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Myeloid Cell IL-10 Production in Response to Leishmania Involves Inactivation of Glycogen Synthase Kinase-3β Downstream of Phosphatidylinositol-3 Kinase

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Leishmania disease expression has been linked to IL-10. In this study, we investigated the regulation of IL-10 production by macrophages infected with Leishmania donovani. Infection of either murine or human macrophages brought about selective phosphorylation of Akt-2 in a PI3K-dependent manner. These events were linked to phosphorylation and inactivation of glycogen synthase kinase-3β (GSK-3β) at serine 9, as the latter was abrogated by inhibition of either PI3K or Akt. One of the transcription factors that is negatively regulated by GSK-3β is CREB, which itself positively regulates IL-10 expression. Infection of macrophages with leishmania induced phosphorylation of CREB at serine 133, and this was associated with enhanced CREB DNA binding activity and induction of IL-10. Similar to phosphorylation of GSK-3β, both phosphorylation of CREB at serine 133 and CREB DNA binding activity were abrogated in cells treated with inhibitors of either PI3K or Akt prior to infection. Furthermore, disruption of this pathway either by inhibition of Akt or by overexpression of GSK-3β markedly attenuated IL-10 production in response to leishmania. Thus, GSK-3β negatively regulates myeloid cell IL-10 production in response to leishmania. Switching off GSK-3β promotes disease pathogenesis. The Journal of Immunology, 2012, 188: 367–378.

Protozoa of the genus Leishmania are the causative agents of a spectrum of diseases known as the leishmaniases. These infections are transmitted by sandflies and they represent a major health problem of global importance. According to the latest World Health Organization report, 14 million people in 88 countries have clinical evidence of leishmaniasis. Additionally, 350 million people live at risk for infection and 2 million new cases occur each year. Moreover, the incidence of the leishmaniases has been on the rise because of the AIDS epidemic, increased international travel, lack of effective vaccines, difficulty in controlling vectors, and the development of resistance to chemotherapy (http://www.who.int/health-topics/leishmaniasis.htm). Depending mostly on the species of leishmania involved, the leishmaniases range from relatively limited cutaneous disease to a progressive and fatal form of visceral disease involving the liver, spleen, and bone marrow. Leishmania donovani is the major causative agent of human visceral leishmaniases.

Mononuclear phagocytes are the primary target cells for leishmania infection, and L. donovani, similar to other leishmania species, cycles between two distinct developmental stages. Infection is initiated when motile infectious promastigotes are inoculated into a mammalian host when an infected sandfly takes a blood meal. Subsequently, promastigotes are taken up by granulocytes (1, 2) and then monocytes and macrophages where they convert into nonmotile amastigotes inside phagocytic vacuoles. Here, they survive and replicate quite readily, despite the ostensibly hostile environment of the phagolysosome. Mononuclear phagocytes are important components of the innate immune system possessing dual responsibilities for the destruction of invading pathogens and, along with dendritic cells, engaging the adaptive immune system in Ag-specific responses with memory. In light of this, it is not surprising that leishmania have evolved strategies to evade or subvert the host-protective properties of mononuclear phagocytes (3–5). Notably, leishmania present significant challenges to the host immune response with their capacity to downregulate the expression of proinflammatory cytokines while promoting the production of anti-inflammatory cytokines (6–10). It has been clearly established that a Th1 cytokine response (IL-12, IFN-γ, TNF) is protective against leishmania infection and, conversely, a Th2 cytokine profile including IL-10 and IL-4 promotes disease progression (11). These latter cytokines along with others have also been associated with macrophage deactivation (12, 13). For example, inhibition of macrophage production of the antimicrobial gas NO has been associated with IL-10 and TGF-β (14), both of which are produced by leishmania-infected macrophages (15). IL-10 in particular has been identified as a critical factor that promotes disease expression after L. donovani infection, including the finding of unrestrained parasite replication in IL-10 transgenic mice (16).

Despite the importance of IL-10 for the biology of leishmania infection, the mechanisms that regulate its production by macrophages in response to leishmania infection are not fully understood.

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Abbreviations used in this article: Akt, Akt inhibitor VIII; CBP, CREB-binding protein; GSK-3β, glycogen synthase kinase-3β; hMDM, human monocyte-derived macrophage; PH, pleckstrin homology.
A limited number of studies have addressed this question and some, but not all, of these have linked activation of MAPKs during leishmania infection to induction of IL-10. For example, ERK1/2 activation was shown to be brought about by lesion-derived Leishmania amazonensis amastigotes that were coated with host IgG (17), and activation of ERK was necessary, but not sufficient, for full IL-10 induction. It was also observed that in addition to ERK, an inflammatory stimulus such as hyaluronic acid from the extracellular matrix was required for maximal induction of IL-10. These findings suggested that disease exacerbation via IL-10, brought about by immune complexes, would likely occur at later stages of infection, when parasite-specific IgG was generated. The results also predicted that reinfection of macrophages by IgG-opsonized amastigotes could contribute to the induction of IL-10.

In another study, it was suggested that the strength of CD40 signaling may influence the specific MAPK pathway that is activated, thereby influencing the profile of cytokines produced by Leishmania major-infected macrophages (18). In that study, it was found that weak CD40 signaling induced ERK1/2-dependent IL-10 production, whereas more robust CD40 signaling induces p38 MAPK-dependent IL-12 production (18). It has also recently been shown that L. donovani can modulate signaling through the TLR2-mediated MAPK pathway by suppressing p38 MAPK phosphorylation and activating ERK1/2 phosphorylation, resulting in suppression of IL-12p40 production and induction of IL-10 (19).

**FIGURE 1.** Leishmania-induced phosphorylation of Akt is dependent on PI3K. A, hMDM were incubated with promastigotes of L. donovani at a parasite/cell ratio of 10:1. At the indicated time points, uninfected and infected cells were washed with HBBS and cell lysates were collected. Whole-cell lysates were analyzed by immunoblot with Abs specific for phospho-Akt (serine 473) followed by ECL detection. B, The same membrane, stripped and reprobed with anti-Akt. D, The dependence of Akt phosphorylation on PI3K was analyzed when leishmania infection was performed for 4 h in cells pretreated with Akti. LPS-treated cells (100 ng/ml for 30 min) were used as positive controls and for comparison. G, RAW cells were left untreated or infected with promastigotes of L. donovani at a parasite/cell ratio of 10:1 for 6 h. Cells were then lysed and analyzed for the level of Akt phosphorylation on serine 473. The dependence of phosphorylation of Akt on PI3K signaling during leishmania interaction was determined when infection was performed in the presence of PI3K inhibitor wortmannin (Wort). Total Akt was used as a protein loading control (E, H). Again, cells treated with LPS (100 ng/ml for 30 min) were used as positive control. Densitometric analysis of phospho-Akt bands was obtained using ImageJ software and results were normalized to basal level (C, F, I). The data shown are mean ± SD of four independent experiments. Ld, L. donovani.
In contrast to the studies cited above, *L. donovani* was reported to be null with respect to inducing the phosphorylation of p38 MAPK, JNK, and ERK1/2 in macrophages (20). Likewise, *L. donovani* infection did not lead to degradation of IkBα (20). Consistent with these findings, we previously reported attenuation of LPS-induced phosphorylation of ERK1/2 in *L. donovani*-infected mouse macrophages (21).

Taken together, the findings from the various studies summarized above indicate that the regulation of IL-10 production by macrophages in response to leishmania is complex. Notably, none of these studies has addressed the potentially important role for PI3K in regulating the macrophage IL-10 response to leishmania. PI3Ks are a family of lipid kinases that phosphorylate the hydroxyl group of the inositol ring of phosphoinositides at the 3′ position. The resulting 3′-phosphoinositides produce regulate a multitude of cellular events such as mitogenic responses, differentiation, apoptosis, cytoskeletal organization, membrane traffic along the exocytic and endocytic pathways, and cytokine production (22). With regard to the latter, it was recently shown that infection of macrophages with *L. amazonensis* activated PI3K (23) and this was linked to inhibition of IL-12 production (24). This observation coupled with other recent findings suggested to us the possibility that PI3K may also regulate the IL-10 response to leishmania. Thus, PI3K is known to mediate the recruitment and activation of the serine-threonine kinase Akt (25, 26). One of the targets of Akt is glycogen synthase kinase-3β (GSK-3β), which undergoes an inactivating phosphorylation at serine 9 (27). Normally, GSK-3β tonically restricts the activity the CREB (28), which itself is inactivated at the expense of proinflammatory cytokines (31). Evidence that activated Akt positively regulates IL-10 production has been reported by others as well (32). Thus, a model has emerged in which activated Akt functions as a switch to regulate macrophage IL-10 production. Under basal conditions, the GSK-3β switch is turned on and IL-10 production is tonically constrained. Conversely, when GSK-3β is inhibited, this restraint on IL-10 production is removed. Based on these findings, we formulated the hypothesis that leishmania may use the PI3K/Akt pathway to switch off GSK-3β, thereby removing tonic control of CREB and IL-10 production. Below, we present evidence in support of this hypothesis.

**Materials and Methods**

**Chemicals and reagents**

*Leishmania* culture medium-M199, HBSS, PMA, LiCl, Akt inhibitor VIII (Akt; A6730), Escherichia coli LPS 0111:B4, protease inhibitors, and FITC anti-mouse Ab were obtained from Sigma-Aldrich (St. Louis, MO). Wortmannin was obtained from Calbiochem. PBS, RPMI 1640, 1 M HEPES buffer, penicillin G and streptomycin solution, recombinant human GM-CSF, and human monocyte enrichment kit no. 19059 were obtained from StemCell Technologies (Vancouver, BC, Canada). FBS was obtained from Hyclone Laboratories (Logan, UT). Human monocytes were purified using monocyte enrichment kit no. 19059 from StemCell Technologies according to the manufacturer’s instructions. Purity of CD14-positive cells was determined by flow cytometry. For differentiation, cells were cultured in complete RPMI 1640 supplemented with 10 ng/ml recombinant human GM-CSF. After 6 d, culture cells were dislodged using trypsin and plated for experiments.

**Infection of RAW and THP-1 cells and human macrophages**

Exponentially growing RAW cells or differentiated human macrophages were infected with stationary phase *L. donovani* promastigotes at a parasite/cell ratio of 10:1. At the end of infection period, noninternalized promastigotes were removed by washing with HBSS. The level of infection was determined by staining with Diff-Quik (Dade Behring, Deerfield, IL). For infection studies using THP-1 cells, PMA was used for differentiation.

**EMSA**

For this assay, nuclear and cytoplasmic fractions were prepared based on a protocol from the Skirball Institute of Biomolecular Medicine, New York University Medical Center (33), with minor modifications as published (34). Two to 5 μg nuclear extract and a biotinylated, commercial oligonucleotide encoding the CRE motif 5′-GACCGTGACGTCACACAAAGC-3′ (EMSA gel-shift kit no. YA 1288 P; Panomics) were used for EMSA, based on instructions provided by the manufacturer. EMSA was performed according to our previously published protocol (34).

**Immunoprecipitation and Western blotting**

Control and treated cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, pepstatin, 1 mM Na3VO4, and 1 mM NaF) on ice for 10 min, cleared by centrifugation (12,000 g for 10 min at 4˚C), and immunoprecipitated with an Ab specific for pAkt (serine 473) at 4˚C overnight. After incubation with Ab, protein G-Sepharose was added for 1 h for recovery of immune complexes, washed with lysis buffer, and resuspended in SDS sample buffer for SDS-PAGE (8.5%) and Western blotting analysis. In other experiments, total

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** *Leishmania* selectively induce phosphorylation ofAkt-2. hMDM were incubated with promastigotes of *L. donovani* (Ld) at a parasite/cell ratio of 10:1 for 4 h. Cells lysates were then prepared and incubated with Abs against pAkt (serine 473) for immunoprecipitation. Immunoprecipitates were analyzed by immunoblotting using the Abs indicated: *upper panel*, anti-pAkt (serine 473); *middle panel*, anti–Akt-1; *lower panel*, anti-Akt-2. LPS-treated cells (100 ng/ml for 30 min) were used as positive control and for comparison. The data shown are from one of three independent experiments with similar results.
lysates (20–50 μg proteins) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the relevant Abs, followed by incubation with HRP-conjugated secondary Abs and detection by ECL. Immunoreactive bands were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/index.html).

**Transient transfection**

RAW 264.7 cells and human macrophages were transfected using the Nucleofector (Amaxa Biosystems) according to the manufacturer’s instructions. In brief, 2 × 10⁶ cells were resuspended in 100 μl buffer (Ingenio electroporation solution [Mirus Bio, Madison, WI] for RAW 264.7 cells, and a human monocyte Nucleofector kit for human macrophages [Amaxa Biosystems]) at room temperature and mixed with 2 μg GSK-3β plasmid (a gift from Dr. James Woodgett, Ontario Cancer Institute, Toronto, ON, Canada). Empty vectors as well as 1 μg pmaxGFP were used as controls. Cells were transfected using the program D-032 (for RAW 264.7 cells) or Y-010 (for human macrophages). RAW cells were used 24 h following transfection, whereas human macrophages were used 48 h after transfection.

**RT-PCR**

Total RNA isolation was performed using RNA isolation kits from Promega (Madison, WI) and used for reverse transcriptase reactions. First strand DNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada), and semiquantitative PCR was performed using GoTag Green Master Mix (Promega). Cycling conditions were adapted to each set of primers, but in most cases were as follows: 94°C for 5 min, followed by 33 cycles at 94°C for 30 s, 60°C for 30 s, and extension at 72°C for 30 s. GAPDH was used as a housekeeping gene to assess the relative abundance.

Sense and antisense amplification primers for murine and human IL-10, as well as GAPDH as an internal control, were as follows: murine: IL-10 sense, 5’-AGAAGCATGGCCCAAGAAATCA-3’, antisense, 5’-GCCTTGTAAGACACTTGGG-3’; GAPDH sense, 5’-GACCACAGTTCAGGCACTC-3’, antisense, 5’-GACGGACACATGGGCGTAG-3’; human: IL-10 sense, 5’-ACGGCCGTCTGATCATT-3’, antisense, 5’-TTG GAGCTTATTAAAGG-3’; GAPDH sense, 5’-CGAGATCCCTCCAAAATCAA-3’, antisense, 5’-TGTGGTCATGAGTCCTTCCA-3’; human IL-12p40: sense, 5’-TGGTTTCCATCGT TTGCTG-3’, antisense, 5’-ACAGTGAGTTCACGT TTTCT-3’.

**IL-10 and IL-12 ELISA**

IL-10 and IL-12 measurements were made in 96-well Immulon plates coated overnight with anti–IL-10 mAb or IL-12p40 mAb (BD Pharmingen). After blocking the wells, culture supernatant samples were added and incubated for 2 h. After thorough washing, bound cytokines were detected with biotinylated anti–IL-10 or biotinylated anti–IL-12 and streptavidin-HRP (both from BD Pharmingen). IL-10 and IL-12 produced per million cells were calculated based on viable cell counts, as determined by trypan blue exclusion, at the time that cytokine-containing supernatants were harvested.

**FIGURE 3.** Leishmania infection-induced phosphorylation of GSK-3β is dependent on PI3K. hMDM (A, B) and RAW cells (D, E) were either left untreated or infected with L. donovani promastigotes (4 h for hMDM and 6 h for RAW cells) at a parasite/cell ratio of 10:1. Cells were then washed with HBBS, and whole-cell lysates were prepared and analyzed by immunoblotting with Abs specific for GSK-3β phosphorylated at serine 9, followed by ECL detection. The same membranes were stripped and reprobed with anti-GSK-3α and GSK-3β (B, E). The dependence of phosphorylation of GSK-3β on PI3K signaling during leishmania interaction was determined when infection was performed in the presence of Akt inhibitor (hMDM) or PI3K inhibitor wortmannin (RAW cells). Cells treated with LPS (100 ng/ml for 30 min) were used as positive control and for comparison. Densitometric analysis of phospho-GSK-3β bands was obtained using ImageJ software, and results were normalized to basal level (C, F). The data shown are means ± SD of three independent experiments. Ld, L. donovani.
**Flow cytometry**

Intracellular staining was performed after cell fixation with 1% paraformaldehyde in PBS for at least 1 h. Cells were then washed and permeabilized with FACS buffer (PBS, 1% BSA) containing 0.1% Triton X-100 for 30 min. One million cells were stained with appropriate primary Ab for 1 h in FACS buffer. Cells were washed once and incubated with FITC-conjugated secondary Ab for 45 min. After incubation, cells were washed, resuspended in PBS, and fluorescence was analyzed according to standard procedures on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with an argon ion laser (488 nm) and data were acquired using CellQuest software.

**Statistical analysis**

Data in the histograms are presented as mean ± SD for the indicated number of independently performed experiments. Statistical analysis was done using a Student t test.

**Results**

*Leishmania infection of human myeloid cells induces phosphorylation of Akt at serine 473*

It had previously been reported that infection of murine macrophages with either *L. major*, *Leishmania pifanoi*, or *L. amazonensis* activated signaling through PI3K to confer macrophage resistance to apoptosis (23). To examine whether activation of PI3K also occurred in human myeloid cells infected with *L. donovani*, we monitored the phosphorylation state of the serine/threonine kinase Akt, a key physiologic mediator of the PI3K pathway, in human monocyte-derived macrophages (hMDM). As shown in Fig. 1A, infection of hMDM with *L. donovani* brought about a time-dependent increase in Akt phosphorylation at serine 473, a marker of Akt activation (35), with maximum increase observed at 4 h. Likewise, infection of murine macrophages (RAW cells) with *L. donovani* also showed an increase in phosphorylation of Akt at serine 473 (Fig. 1G). Note that leishmania induced a maximum level of Akt phosphorylation at 6 h in RAW cells (data not shown). The magnitude of leishmania-induced phosphorylation of Akt in both RAW cells and hMDM was comparable to responses seen to LPS (Fig. 1D, 1G). Furthermore, Akt phosphorylation induced by either leishmania infection or LPS was completely abrogated in cells pretreated with either Akti (Fig. 1D) or the PI3K inhibitor wortmannin (Fig. 1G). Inhibition of activation by Akti is pleckstrin homology (PH) domain-dependent. Akti appears to block binding of PIP3 (produced by PI3K) to the PH domain of Akt-1 and Akt-2, resulting in inhibition of translocation of Akt to plasma membrane and its subsequent activation (36). Thus, Akti, similar to wortmannin, is an inhibitor of the PI3K pathway. It is known that stationary phase promastigotes may contain significant numbers of dead parasites. To ensure that our results were not affected by dead parasites, Ficoll gradients (37) were used to eliminate nonviable leishmania. Raw cells were infected with purified *L. donovani* or stationary phase *L. donovani* for 6 h. The magnitude of Akt phosphorylation in response to purified *L. donovani* was comparable to stationary

**FIGURE 4.** Leishmania infection induces CREB phosphorylation at serine 133. hMDM (A, B) and RAW cells (D, E) were either left untreated or infected with *L. donovani* promastigotes (4 h for hMDM and 6 h for RAW cells) at a parasite/cell ratio of 10:1. Cells were then washed with HBBS, and whole-cell lysates were prepared and analyzed by immunoblotting with Abs specific for CREB phosphorylated at serine 133, followed by ECL detection. The same membranes were stripped and reprobed with anti-CREB (B, E). The requirement for PI3K activity for phosphorylation of CREB was determined when infection was performed in the presence of Akt inhibitor (hMDM) or PI3K inhibitor wortmannin (RAW cells). Cells treated with LPS (100 ng/ml for 30 min) were used as positive control and for comparison. Densitometric analyses of bands for phosphorylation of CREB at serine 133 were obtained using ImageJ software, and results were normalized to basal level (C, F). The data shown are means ± SD of four independent experiments. Ld, *L. donovani*.
phase nonpurified \textit{L. donovani}, indicating that nonviable leishmanias were not responsible for the responses observed (data not shown).

\textbf{Leishmania infection induces selective phosphorylation of Akt-2}

To date, three human isoforms of Akt have been identified: Akt-1, Akt-2, and Akt-3. Although the basic activation mechanisms appear to be similar for the three isoforms (38), it was of interest to know whether the Akt response to leishmania was general or specific to one or another isoform. To address this question, initially we used Akt isoform-specific Abs to analyze the expression of the individual isoforms in human macrophages. Akt-1 and -2 were found to be abundant in human macrophages, whereas Akt-3 was barely detectable even after prolonged exposure of blots (data not shown). We then infected hMDM with \textit{L. donovani} for 4 h and, in parallel, cells were also treated with LPS for 30 min for comparison. Cells were then washed with PBS and lysed in ice-cold RIPA buffer containing a mixture of protease and phosphatase inhibitors. Immunoprecipitation was carried out with phosphospecific Abs to phospho-Akt (this reagent recognizes phosphoserine 473 in all the three Akt isoforms) followed by immunoblot analysis using either the same Ab used for immunoprecipitation (pAkt-serine 473) (Fig. 2, top panel) or Abs specific for Akt-1 (Fig. 2, middle panel) or Akt-2 (Fig. 2, bottom panel). This analysis showed that leishmania preferentially induced phosphorylation of Akt-2, whereas LPS was not selective for either Akt-1 or Akt-2.

\textbf{Leishmania infection induces phosphorylation of GSK-3\textbeta at serine 9 in a PI3K-dependent manner}

Activated Akt phosphorylates several downstream targets in the PI3K pathway, including the constitutively active serine/threonine kinase GSK-3\textbeta. Phosphorylation of GSK-3\textbeta at serine 9 results in inhibition of its activity (38), and Akt-mediated phosphorylation of GSK-3\textbeta in human monocytes has been linked to polarization of inflammatory cytokine production favoring IL-10 over IL-12 (31). We therefore examined whether leishmania infection influenced the phosphorylation state of GSK-3\textbeta. As shown in Fig. 3A and 3D, leishmania infection brought about enhanced phosphorylation of GSK-3\textbeta at serine 9 in both RAW cells and in hMDM. In both cases, the magnitude of these responses was comparable to that seen with LPS. Furthermore, leishmania-induced phosphorylation of GSK-3\textbeta at serine 9 was significantly reduced by wortmannin (Fig. 3D) and completely abrogated by Akti (Fig. 3A), indicating that these responses were PI3K-dependent.

\textbf{Leishmania induces phosphorylation of CREB in a PI3K-dependent manner}

One of the transcription factors regulated by GSK-3\textbeta is the phosphoprotein CREB (38). Phosphorylation of CREB at serine 133 is required for recruitment of coactivator CREB-binding protein (CBP), which promotes activation of transcription at CREB promoter regions (39), including the IL-10 promoter (40). The kinase responsible for CREB phosphorylation at serine 133 has not been clearly identified, but it is known that this process is negatively regulated by GSK-3\textbeta (28). Thus, inactivation of GSK-3\textbeta by leishmania should lead to increased abundance of phosphorylation of CREB at serine 133. As shown in Fig. 4, infection of both RAW cells and hMDM resulted in CREB serine 133 phosphorylation. Similar responses were seen to LPS, which was used as a positive control. Notably, leishmania-induced phosphorylation of CREB was shown to be PI3K-dependent, as it was abrogated in cells pretreated with either wortmannin (Fig. 4D) or Akti (Fig. 4A).
Leishmania infection brings about increased CREB DNA binding activity

Next, we examined whether leishmania-induced phosphorylation of CREB resulted in increased CREB/DNA binding activity by EMSA. RAW cells were infected with leishmania either in the presence or absence of PI3K inhibitor wortmannin. Nuclear extracts were then prepared and incubated with a biotinylated CRE probe (5'-GACGGTGCACACAAAGC-3') and processed for EMSA. As shown in Fig. 5A, protein/DNA complexes were detected to indicate that leishmania infection activated CREB binding to the CRE oligonucleotide. Although there appeared to be a low level of constitutive CREB binding to DNA in control cells, this was clearly significantly increased in leishmania-infected cells (Fig. 5A). This interaction was specific, as the signal was completely eliminated by competition with unlabeled probe. Notably, activation of CREB binding appeared to be completely dependent on PI3K, as it was abrogated in leishmania-infected cells treated with wortmannin prior to infection (Fig. 5A).

To confirm that the DNA binding protein activated by leishmania to bind to CRE was bona fide CREB, Ab-based competition experiments were done. As shown in Fig. 5B, when nuclear extracts from leishmania-infected RAW cells were incubated with Ab specific for CREB prior to EMSA, significant inhibition of protein binding to the CRE oligonucleotide was observed as compared with irrelevant, control Ab.

Leishmania infection does not promote a change in the phosphorylation of CREB at serine 129

It has been shown that GSK-3β can negatively regulate CREB DNA binding activity by phosphorylating CREB at serine 129 (40, 41). Thus, it might be predicted that inactivation of GSK-3β by leishmania might result in decreased abundance of phosphorylation of CREB at serine 129. As shown in Fig. 6A and 6C, leishmania infection of hMDM induced CREB 133 phosphorylation without affecting any net change in the signal for CREB 129. Our inability to detect a decrease in signal intensity for phosphorylation of CREB at serine 129 in leishmania-infected cells may be due to the fact that there was little to no signal apparent under basal conditions. When used as a positive control, LPS promoted increased phosphorylation of CREB at both positions (129 and 133), but only the change at position 133 was clearly PI3K-dependent (Fig. 6A, 6C).

Induction of IL-10 production by leishmania is PI3K-dependent

TLR2-induced activation of the PI3K pathway has been shown to promote IL-10 production (31). Moreover, it has been clearly shown that inhibition of GSK-3β downstream of Akt also favors enhanced production of IL-10 (31). It was of interest, therefore, to examine whether leishmania uses the PI3K/Akt pathway to up-regulate expression of IL-10. As shown in Fig. 7A and 7B, infection of hMDM with leishmania significantly enhanced the expression of IL-10, as indicated by increased signals for both IL-10 mRNA and protein. Moreover, these responses to infection were completely blocked in cells pretreated with inhibitors directed at either PI3K or Akt. Taken together, the results of RT-PCR and ELISA assays indicate that leishmania-induced signaling through PI3K and Akt plays a central role in inducing the production of macrophage IL-10. It was also of interest to ask whether activation of PI3K by leishmania and phosphorylation of Akt might influence macrophage IL-12 production. As shown in Fig. 7C and 7D, leishmania did not induce expression of IL-12p40 at either the mRNA or protein levels. Furthermore, despite the fact that inhibition of PI3K reduced IL-10 production, there was no reciprocal enhanced production of IL-12 (Fig. 7C, 7D).

Overexpression of GSK-3β attenuates macrophage IL-10 production in response to leishmania

The findings reported above suggested a model in which inactivation of GSK-3β downstream of PI3K and Akt in leishmania-infected cells resulted in release of CREB from tonic control and activation of IL-10 expression. If this model were correct, we reasoned that overexpression of GSK-3β should reverse this phenotype. This prediction turned out to be correct, as overexpression of GSK-3β in RAW cells for 24 h prior to infection completely abrogated the IL-10 response to leishmania (Fig. 8B). Likewise, and at approximately one half the transfection efficiency when compared with RAW cells (Fig. 8A versus 8D), we also
observed a significant reduction in IL-10 production when GSK-3β was overexpressed in hMDM for 48 h prior to infection with *Leishmania* (Fig. 8E). Notably, induction of IL-10 in response to LPS was relatively insensitive to GSK-3β overexpression, suggesting that LPS can recruit auxiliary pathways to induce this response.

**Phosphorylation of CREB at serine 133 in leishmania-infected macrophages overexpressing GSK-3β**

The results presented thus far are consistent with a model in which leishmania induces inactivation of GSK-3β downstream of PI3K, thereby promoting phosphorylation and activation of CREB at serine 133. Activated CREB can then drive the IL-10 promoter, leading to induction of IL-10 production. If this model were correct, then in addition to blocking IL-10 production, overexpression of GSK-3β should antagonize CREB activation in response to leishmania infection. The findings presented in Fig. 9 demonstrate clearly that leishmania-induced phosphorylation of CREB at serine 133 was abrogated in macrophages overexpressing GSK-3β. These findings provide direct evidence that inactivation of host GSK-3β by leishmania is required to activate CREB, leading to the induction of IL-10.

**Discussion**

Diverse intracellular microbes, including *Leishmania* (5, 42), *Yersinia* (43), *Mycobacteria* (44–46), and the HIV (47) and others, have evolved mechanisms to impair macrophage activation through effects on cell signaling. To ensure their survival, leishmania in particular have the capacity to inhibit numerous macrophage functions and to prevent cell activation (3, 42, 48). The activation states of mononuclear phagocytes are under the influence of the ambient concentrations of both stimulatory and in-
hibitory cytokines in the environment. The production of these cytokines by phagocytic cells are in turn influenced by infection with various pathogens, including leishmania. It has been established that a Th1 immune response is protective against leishmanial infection (11), and Th1 responses are favored by the production of proinflammatory cytokines such as IL-12, IL-18, IL-1, TNF-α, and IL-6. In addition to their ability to infect macrophages either without inducing or minimizing the production of these proinflammatory cytokines (6, 7), leishmania are capable of promoting the production of immunosuppressive cytokines such as TGF-β and IL-10 (8–10, 16, 49). Furthermore, it has been shown that the production of IL-10 in response to leishmania plays an important role in promoting disease progression (9, 10, 16, 50).

As summarized above, a number of studies have addressed the mechanisms that regulate IL-10 production in response to leishmania (17–21), and evidence both for and against roles for ERK and TLR signaling have been reported. Although the rules that govern the production of pro- versus anti-inflammatory cytokines are still being written, recent studies have established that PI3K regulates these responses (30–32). There are likely multiple mechanisms through which PI3K influences cytokine production, and some of these are yet to be discovered. Nevertheless, it is clear that Akt and GSK-3β are centrally involved. For example, it has been shown that GSK-3β in its constitutively active state preferentially favors production of proinflammatory cytokines, including IL-12 (30). Conversely, inactivation of GSK-3β favors the production of excess IL-10 at the expense of proinflammatory cytokines (31). Evidence that activated Akt promotes IL-10 production has also been reported (32). Not only do three kinases all contribute to cytokine regulation, but their actions are linked directly. Thus, activation of PI3K mediates the recruitment and activation of Akt (25, 26), which in turn brings about the phosphorylation and inactivation of the regulator kinase GSK-3β at serine 9 (27). One of the transcription factors that under the control of GSK-3β is the phosphoprotein CREB (28). Under basal conditions in its active state, GSK-3β phosphorylates CREB on serine 129, which is inhibitory (40, 41). In contrast, in the absence of functional GSK-3β, phosphorylation of CREB at serine 133 predominates. This phosphorylation promotes CREB activation and leads to the recruitment of the coactivator CBP to enhance the transcriptional activities of gene promoters that bind the complex, as is the case for IL10 (28). Thus, it has been shown that IL-10 production requires activated CREB, which recruits CBP and the complex to the IL-10 promoter. Recruitment of CBP to CREB occurs at the expense of NF-κB, and hence IL-10 production is favored over that of IL-12 (31). Under normal conditions, constitutively active GSK-3β restrains IL-10 production by maintaining CREB in a relatively inactive state.

In the light of the important roles for PI3K in modulating cytokine production in general and IL-10 in particular (31), we investigated whether leishmania hijacks the PI3K/Akt/GSK-3β pathway and CREB signaling to upregulate the expression of IL-10, leading to an immunosuppressive environment. First, we obtained evidence that leishmania promoted activation of Akt in human macrophages. It is well known that phosphorylation of serine 473 is involved in Akt activation (35). Leishmania-infected cells showed clear induction of Akt phosphorylation in a PI3K-dependent manner (Fig. 1). This result was consistent with the recently published finding that infection of murine macrophages with either L. major, L. pifanoi, or L. amazonensis activated signaling through PI3K/Akt (23). These investigators concluded that leishmanial promastigotes engaged the PI3K pathway to render infected cells resistant to death from apoptosis.

In mammalian cells, three isoforms of Akt, Akt-1/PKBα, Akt-2/ PKBβ, and Akt-3/PKBγ, have been identified, all of which are activated in a PI3K-dependent manner (51). Although Akt has been studied extensively, the specific functions of individual Akt isoforms have been emphasized only recently. Akt-1, Akt-2, and Akt-3 are encoded by three separate genes with >80% sequence homology (52). Akt-1 and Akt-2 are ubiquitously expressed, whereas Akt-3 is expressed in testicular and neuronal tissues. Additionally, all three isoforms share the same structural organization. Thus, all Akt isoforms have been previously considered to possess identical or similar substrate specificities (53). However, recent in vivo studies using knockout mouse models have provided evidence for isotype-specific functions for Akt. For example, Akt-1 knockout mice are growth retarded (54), whereas Akt-2 knockout mice have findings consistent with a diabetic state (55), and Akt-3 knockout mice show reduced brain size (56). These results indicate that individual Akt isoforms have both distinct and overlapping functions. It was of interest, therefore, to investigate whether induction of Akt phosphorylation by leishmania was isoform-specific. Indeed, our results showed that leishmania induced the selective phosphorylation of Akt-2 (Fig. 2). This was in contrast to LPS, which brought about phosphorylation of both Akt-1 and Akt-2. The basis for selective phosphorylation of host Akt-2 in response to leishmania is presently unknown. Although Akt isoforms exhibit extensive sequence homology and possess similar domain structures, the greatest variations are located within the phosphoinositide-binding PH domains. Indeed, the c-Jun NH2-terminal kinase interacting protein 1 has been shown to interact selectively with the PH domain of Akt-1 (57). Thus, it is possible that a leishmania-derived factor or a leishmania-induced host factor may bind selectively to the PH domain of Akt-2 in such a manner as to increase its affinity for PIP3. As to the functional impact of selective activation of Akt-2 in infected cells, it is interesting to speculate that this may influence either glucose transport, glucose homeostasis, or both in such a manner as to provide a more permissive environment for infection.
To understand the importance of activation of the PI3K/Akt-2 pathway by leishmania, including how cytokine production is affected, it was important to identify downstream targets. Given its important role in regulating cytokine production (30, 31), GSK-3β was an attractive candidate. GSK-3 is a ubiquitous serine/threonine kinase expressed in two isoforms (α and β), and it has been implicated in multiple biological processes (58, 59). Unlike most protein kinases, GSK-3 is constitutively active in resting cells and may become switched off or relatively active upon appropriate cell stimulation. For example, stimulation of cells with insulin causes inactivation of GSK-3β through a PI3K-dependent mechanism involving Akt (27).

Inhibition of GSK-3β most commonly involves direct phosphorylation of an N-terminal serine residue (serine 9) (59) by Akt (60). Recently, it was shown that GSK-3β is involved in regulating TLR-mediated cytokine induction (31). Notably, inhibition of GSK-3β was shown to result in the production of excess IL-10 at the expense of proinflammatory cytokines (31). Consistent with this model, we found that activation of the PI3K/Akt pathway by leishmania brought about phosphorylation of GSK-3β on serine 9 in both murine and human macrophages (Fig. 3).

The identification of GSK-3β as a downstream target for inactivation by PI3K and Akt in leishmania-infected cells was an important finding precisely because inactive GSK-3β has been linked to excess IL-10 production (31). This relates directly to a role for active GSK-3β in maintaining CREB in a relatively inactive state by preventing the formation of complexes containing CBP and CREB at serine 133, thereby constraining CREB DNA binding activity (28). The complex containing CBP and CREB at serine 133 is important to activate transcription at CREB promoter regions (39), including the IL-10 promoter (40). The critical role for phosphorylation of CREB at serine 133 to activate transcription is supported by the finding that mutation of this residue results in complete loss of transcriptional activity (61, 62). Indeed, when we examined CREB, coordinated with inactivation of GSK-3β during leishmania infection, we observed increased phosphorylation of CREB at serine 133 (Fig. 4) and increased CREB DNA binding activity (Fig. 5), both of which were PI3K-dependent. As discussed above, phosphorylation of CREB at serine 133 exposes a site for phosphorylation by GSK-3β at serine 129, which itself is inhibitory. In this context, it was surprising that leishmania-induced phosphorylation of CREB at serine 133 did not result in a parallel increase in phosphorylation of CREB at serine 129 (Fig. 6). As expected, LPS-induced phosphorylation of CREB at serine 133 was accompanied by increased phosphorylation of CREB at serine 129 (Fig. 6), suggesting differences in LPS- and leishmania-induced signaling through PI3K, at least at the level of CREB phosphorylation.

Importantly, our results linked inactivation of GSK-3β directly to activation of CREB by showing that overexpression of GSK-3β prevented serine 133 phosphorylation of CREB during leishmania infection (Fig. 9). Note that in these experiments transfection with GSK-3β plasmid alone modestly increased the signal for phosphorylation of CREB at serine 133. The explanation for this unexpected finding is not clear at this time. However, it is noteworthy that despite this effect of GSK-3β plasmid transfection alone, overexpression of GSK-3β was still able to completely abrogate leishmania-induced phosphorylation of CREB at serine 133.

This and the other findings discussed thus far are consistent with a model in which leishmania infection activated the PI3K/Akt pathway, leading to inactivation of GSK-3β and induction of IL-10 production via enhanced activity of CREB promoter binding complexes. We provided formal proof that this model is correct by showing that either PI3K inhibitors (Fig. 7) or overexpression of GSK-3β (Fig. 8) abrogated the phenotype of excess IL-10 production by leishmania-infected cells. Interestingly, the IL-10 response to LPS was not as sensitive to either PI3K inhibition (Fig. 7) or GSK-3β overexpression (Fig. 8) as was the IL-10 response to leishmania, suggesting that LPS can recruit auxiliary pathways to induce IL-10 production.

Importantly, during the course of our experiments, we monitored IL-12 production in parallel with IL-10 to determine whether we could find evidence that it was regulated in a reciprocal fashion. In fact, inhibition of PI3K did not affect IL-12p40 production in leishmania-infected human (Fig. 7C, 7D) and murine macrophages (data not shown). This is in contrast to a recent report indicating that inhibition of PI3K/Akt signaling resulted in the induction of IL-12 production by murine macrophages infected with L. amazonensis (24). This discrepancy may be due to the differences in leishmania species or to other unidentified factors.

In summary, we have shown in this study that inactivation of GSK-3β downstream of PI3K leads to induction of IL-10 production in leishmania-infected myeloid cells. The mechanism leading to activation of PI3K by leishmania is a focus of current interest and may or may not involve a transmembrane receptor. In this regard, it was recently reported that Akt phosphorylation was induced as early as 15 min after exposure of murine macrophages to either L. major, L. pifanoi, or L. amazonensis (23). This suggests receptor-mediated activation; however, in our model system, activation of Akt in response to L. donovani had a delayed onset at 4 h in hMDM (Fig. 1) and at 6 h in RAW cells (data not shown). It is possible that multiple mechanisms are invoked by leishmania to ensure Akt activation at both early and late stages of infection, thereby maximizing the likelihood of intracellular survival. One possibility to consider, and the one that we favor, is that leishmania secrete a factor (or factors) into the cytosol of infected cells (Fig. 10) that brings about activation of PI3K either directly or indirectly, leading to changes in cell regulation that favor suc-

![FIGURE 10. Induction of myeloid cell IL-10 production in response to leishmania. Model illustrating how leishmania hijacks the PI3K pathway to inactivate GSK-3β, leading to induction of IL-10 production. Potential mechanisms (A) transmembrane receptor-dependent or (B) cytosolic involving a leishmania-secreted factor, contributing to PI3K activation, are shown.](http://www.jimmunol.org/)
cessful infection. Activation of PI3K via a transmembrane receptor (Fig 10) is also a possibility worth investigating.

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Disclosures
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