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Attenuation of Gamma Interferon-Induced Tyrosine Phosphorylation in Mononuclear Phagocytes Infected with *Leishmania donovani*: Selective Inhibition of Signaling through *Janus* Kinases and Stat1

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The induction of gene transcription in response to gamma interferon is impaired in mononuclear phagocytes infected with *Leishmania donovani*, and the mechanisms involved are not fully understood. The changes in gene expression brought about by gamma interferon are thought to involve transient increases in the activities of cellular protein tyrosine kinases, including the *Janus* kinases Jak1 and Jak2, leading to tyrosine phosphorylation of the transcription factor Stat1. To investigate the mechanisms accounting for the impaired responses to gamma interferon, a model system for examining overall changes in protein tyrosine phosphorylation, activation of Jak1 and Jak2, and phosphorylation of Stat1 was developed in phorbol 12-myristate 13-acetate-differentiated U-937 cells. Analysis of whole-cell lysates by antiphosphotyrosine immunoblotting showed that incubation with gamma interferon brought about specific increases in phosphotyrosine labeling of several proteins. Increased labeling of these proteins occurred to similar extents in control cells and in cells that had been infected with *L. donovani* for 16 h. Jak1, Jak2, and Stat1 were immunoprecipitated from control and interferon-treated cells, and tyrosine phosphorylation of these proteins, detected by antiphosphotyrosine immunoblotting, was used to measure their activation. Tyrosine phosphorylation of Jak1, Jak2, and Stat1 increased markedly, in a dose-dependent manner, in U-937 cells incubated with gamma interferon. In contrast, in cells infected with *L. donovani*, tyrosine phosphorylation of Jak1, Jak2, and Stat1 was markedly impaired. This effect was dependent upon the duration of exposure to *L. donovani* and was maximal and complete at 16 h. Results similar to those observed with U-937 cells were also obtained with human peripheral blood monocytes. These findings indicate that infection of human mononuclear phagocytes with *L. donovani* leads to impaired gamma interferon-mediated tyrosine phosphorylation and selective effects on the Jak-Stat1 pathway. Unresponsiveness to gamma interferon for activation of this pathway may explain impaired transcriptional responses in leishmania-infected cells.

Protozoan parasites of the genus *Leishmania* are obligate intracellular parasites of monocytes and macrophages that cause substantial human morbidity and mortality in many parts of the world. An important question in mononuclear phagocyte cell biology and immunology is how leishmanias and other intracellular pathogens have adapted to resist the antimicrobial properties of these cells. Mononuclear phagocytes must be activated to kill intracellular microbes, and while a broad range of agonists are able to bring about various degrees of cell activation, gamma interferon is the most thoroughly characterized agent with respect to the induction of microbial killing (2, 3, 10, 11).

Infection of mononuclear phagocytes with leishmanias and other intracellular pathogens has been shown to result in impaired cellular responsiveness to gamma interferon, and this state of cell deactivation may contribute to microbial persistence (7, 16; for a review, see reference 14). While the exact basis for this deactivated state remains to be fully explored and is likely to be multifactorial, mononuclear phagocytes infected with *Leishmania donovani* and *Leishmania braziliensis* have been shown to produce such autoinhibitory factors as prostanoids (15) and transforming growth factor β (1) that attenuate cellular responses to gamma interferon. In addition, macrophages infected with *L. donovani* have also been shown to have defects in stimulus-response coupling, leading to impaired functional responses (reviewed in reference 14).

It has recently become clear that intracellular signaling in response to gamma interferon involves two cytoplasmic tyrosine kinases that are members of the *Janus* (or Jak) family of nonreceptor protein tyrosine kinases (5, 17). Treatment of cells with gamma interferon results in the tyrosine phosphorylation and activation of Jak1 and Jak2. Activated Jaks are believed to catalyze the phosphorylation on tyrosine of a 91,000-M_r DNA-binding protein known as signal transducer and activator of transcription 1 (Stat1). Phosphorylated Stat1 dimerizes and translocates to the nucleus, where it binds to the gamma interferon activation sequence in gamma interferon-inducible genes, leading to activation of gene transcription. To examine further the basis for unresponsiveness of leishmania-infected macrophages to gamma interferon, the activation of Jak1 and Jak2 leading to the phosphorylation of Stat1 was studied. The results show that gamma interferon-induced tyrosine phosphorylation was attenuated and that Jak1 and Jak2 activation and phosphorylation of Stat1 were selectively impaired in both

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differentiated U-937 cells and human monocytes infected with *Leishmania donovani.*

MATERIALS AND METHODS

Reagents and chemicals. RPMI 1640 and Hanks' balanced salt solution (HBSS) were obtained from the Terry Fox Laboratory of the British Columbia Cancer Agency of Vancouver, British Columbia, Canada. Phorbol 12-myristate 13 acetate (PMA) and protease inhibitors phenylmethylsulfonyl fluoride, pepstatin A, aprotinin, and leupeptin were from Sigma Chemical Co., St. Louis, Mo. Microcystin-LR was from Calbiochem, San Diego, Calif. Human AB+ serum was obtained from the Canadian Red Cross, Vancouver, British Columbia, Canada. Affinity-purified antiphosphotyrosine monoclonal antibody 4G10 and rabbit polyclonal antibodies to Jak1 and Jak2 were from Upstate Biotechnology Inc., Lake Placid, N.Y. Rabbit polyclonal antibodies to Stat1 were kindly provided by Andrew Larner, The Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md. Recombinant human gamma interferon was a generous gift of Genentech Inc., South San Francisco, Calif.

L. donovani. Amastigotes of the Sudan strain 2S of *L. donovani* were maintained by serial intracardiac inoculation of the amastigotes into female Syrian hamsters. The amastigotes were isolated from the spleens of hamsters infected 4 to 6 weeks earlier as previously described (12).

Culture and differentiation of U-937 cells. The promonocytic cell line U-937 was obtained from the American Type Culture Collection (CRL 1593) and cultured in suspension in stationary, 75-cm^2 cell culture flasks in RPMI 1640 supplemented with low endotoxin, 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah), and antibiotics as described for human monocytes (see below). Prior to infection with *L. donovani* U-937 cells were cultured for 18 h in the presence of PMA (10 ng/ml). This treatment rendered the cells adherent and capable of phagocytosis of amastigotes.

Isolation and culture of human monocytes. Peripheral blood mononuclear cells were isolated as described previously (9) and were suspended in RPMI 1640 supplemented with 10% (vol/vol) human AB+ serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were seeded into 150-cm^2 cell culture flasks at a density of 1.5×10^8 cells per flask. Monocytes were allowed to adhere for 1 h at 37°C in a humidified atmosphere of 5% CO_2–95% air. Nonadherent cells were removed by three vigorous washings with divalent cation-free HBSS. Adherent monolayers were replenished with supplemented RPMI 1640 without serum and were allowed to equilibrate for 1 to 3 h before infection.

Infection of U-937 cells and human monocytes. Differentiated U-937 cells (5×10^6 cells per 75-cm^2 cell culture flask) were infected with freshly isolated amastigotes of *L. donovani* (10^8 parasites per flask). After the desired period of incubation at 37°C in a humidified atmosphere of 5% CO_2–95% air, noningested amastigotes were removed by washing with HBSS. To determine the rate of infection, cytospin preparations were prepared from dislodged cells, which were stained with Diff-Quik. For the infection of monocytes, monolayers were infected with amastigotes at a parasite-to-monocyte ratio of approximately 20:1. The rate of infection in monocytes was monitored as described for U-937 cells.

Cell incubation, immunoprecipitation, and Western blotting (immunoblotting). Prior to treatment with gamma interferon, the cells were preincubated in serum-free RPMI 1640 for at least 1 h at 37°C in an atmosphere of 5% CO_2–95% air. The cells were then treated with either medium alone or the desired concentration of gamma interferon. After cell treatment, monolayers were washed twice with HBSS and immediately processed for immunoprecipitation. For immunoprecipitation of Jak1 and Jak2, the cells were lysed on ice in modified RIPA buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.15 M NaCl, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM NaF, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 100 mM microcystin, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 2 μg of pepstatin A per ml). For immunoprecipitation of Stat1, the cells were lysed in modified RIPA buffer without sodium deoxycholate. Lysates were centrifuged in a microcentrifuge for 20 min at 4°C. The resulting supernatants were transferred to fresh microcentrifuge tubes and incubated (for 16 to 18 h at 4°C) with a 1:250 dilution of antibodies specific for Jak1, Jak2, or Stat1 or with normal rabbit serum. After incubation with antibodies, protein A-agarose was added for 2 h at 4°C for recovery of immune complexes. The protein A conjugates were washed four times with appropriate lysis buffer. Immune complexes were released by boiling agarose beads in sodium dodecyl sulfate (SDS) sample buffer (8).

Samples were analyzed by SDS–7.5% polyacrylamide gel electrophoresis, and this was followed by electrophoretic transfer to nitrocellulose membranes. The unused sites on the nitrocellulose membranes were blocked with 3% bovine serum albumin in Tris-buffered saline–Tween 20 (TBS-T) (20 mM Tris [pH 7.6] and 0.137 M NaCl with 0.1% Tween 20) for 12 to 16 h at 4°C. The blots were then incubated for 2 h with a 10,000-fold dilution of antiphosphotyrosine monoclonal antibody 4G10 at room temperature. After they were washed in Tris-buffered saline–Tween 20, the blots were incubated for 1 h with horseradish peroxidase conjugates of secondary antibody (Amersham) and washed again as described above. The immunoblots were processed further by incubating the membranes with enhanced chemiluminescence (ECL) substrate, and the immunoblots were developed on ECL hyperfilm (Amersham). To assess the amount of individual proteins immunoprecipitated in each sample, the membranes were stripped after the chemiluminescence detection of the bound antiphosphotyrosine antibodies by incubating them in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8) for 45 min at 50°C. After they were extensively washed and blocked, the membranes were probed with anti-Jak1, anti-Jak2, or anti-Stat1. The membranes were then incubated with horseradish peroxidase conjugates of goat anti-rabbit (Bio-Rad) immunoglobulin G, and the blots were developed on ECL hyperfilm by ECL.

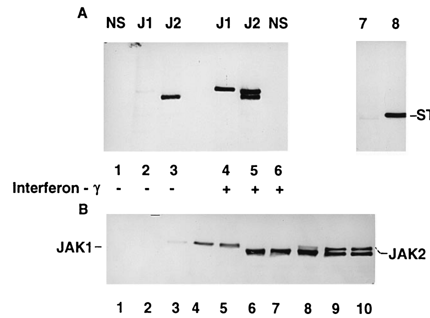

FIG. 1. Gamma interferon-induced tyrosine phosphorylation of Jak1, Jak2, and Stat1 in U-937 cells. (A) Cells were treated either with medium alone (lanes 1 to 3 and 7) or with 100 U of gamma interferon per ml (lanes 4 to 6 and 8) for 10 min at 37°C. Lysates were then prepared and immunoprecipitated with anti-Jak1 (J1), anti-Jak2 (J2), anti-Stat1 (lanes 7 and 8), or normal rabbit serum (NS) as described in Materials and Methods. Immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine antibody and developed by ECL. The arrow indicates the position of a constitutively expressed tyrosine-phosphorylated protein that coprecipitated with Jak2. (B) Concentration dependence of gamma interferon-induced tyrosine phosphorylation of both Jak1 (lanes 1 to 5) and Jak2 (lanes 6 to 10) in U-937 cells. Cells were incubated for 10 min with a range of concentrations of cytokine (0 [lanes 1 and 6], 1 [lanes 2 and 7], 10 [lanes 3 and 8], 100 [lanes 4 and 9], and 1,000 [lanes 5 and 10] U of gamma interferon per ml). Lysates were then immunoprecipitated with either anti-Jak1 or anti-Jak2 as described in Materials and Methods. Immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine antibody and developed by ECL. The data shown are from one of three experiments that yielded similar results.

RESULTS

Gamma interferon induces tyrosine phosphorylation of Jak1, Jak2, and Stat1 in U-937 cells. To examine the Jak-Stat1 signaling pathway in gamma interferon-treated cells, exponentially growing, undifferentiated U-937 cells were incubated for 10 min at 37°C with either medium alone or gamma interferon (100 U/ml). The cells were then washed once with HBSS and lysed in detergent containing RIPA lysis buffer as described in Materials and Methods. Immunoprecipitation with antibodies specific for Jak1 and Jak2 followed by immunoblot analysis with antiphosphotyrosine antibodies showed that gamma interferon induced substantial tyrosine phosphorylation of both Jaks (Fig. 1A). It should also be noted that a second protein with an M_r of approximately 120,000 that appeared to be constitutively phosphorylated on tyrosine was consistently coimmunoprecipitated from U-937 cells with Jak2 (Fig. 1A and B). The identity of this constitutively phosphorylated protein is unknown, but as will be seen below, it was not detected in human monocytes.

Activation of Jak1 and Jak2 in gamma interferon-treated U-937 cells suggested that this would likely be accompanied by tyrosine phosphorylation of DNA-binding protein Stat1. Immunoprecipitation with anti-Stat1 followed by antiphosphotyrosine Western blotting demonstrated that a marked increase in the phosphorylation of a protein with an expected M_r of 91,000 took place (Fig. 1A, lane 8).

To examine the gamma interferon dose-response relationship for tyrosine phosphorylation of Jak1 and Jak2, U-937 cells were incubated for 10 min with a range of concentrations of

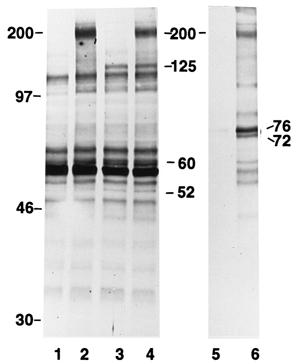

FIG. 2. Effect of leishmania infection on gamma interferon-induced tyrosine phosphorylation in U-937 cells. Differentiated U-937 cells were either left untreated or incubated with leishmania amastigotes at an approximate parasite-to-cell ratio of 20:1. After overnight incubation (16 h), control (lanes 1 and 3) and infected (lanes 2 and 4) cells were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 U of gamma interferon per ml for 10 min at 37°C. Cells were lysed in Laemmli sample buffer, electrophoresed, and transferred to a nitrocellulose membrane. The membrane was probed with antiphosphotyrosine antibody 4G10 and developed by ECL. The autoluminogram of a blot is shown, with the molecular masses (in kilodaltons) of the marker proteins on the left. The relative electrophoretic mobilities of the proteins of interest are shown on the right in kilodaltons. Lanes 5 and 6 show phosphotyrosine-containing proteins in a direct cell lysate of amastigotes. Lanes 5 and 6, respectively, were loaded with 2×10^6 and 10^7 amastigotes. The data shown are from one of two independent experiments that yielded similar results.

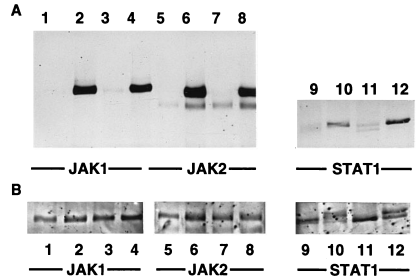

FIG. 3. *Leishmania* infection attenuates gamma interferon-induced tyrosine phosphorylation of Jak1, Jak2, and Stat1 in U-937 cells. Differentiated U-937 cells were incubated with amastigotes of *L. donovani* at an approximate parasite-to-cell ratio of 20:1. Parallel flasks were incubated with fixed *S. aureus* (0.002%). After overnight incubation (16 h), control and infected cells were washed with HBBS and incubated with gamma interferon (100 U/ml) for 10 min at 37°C. Cells were then lysed and immunoprecipitated with the antibodies indicated. Immunoprecipitated proteins were separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, probed with antiphosphotyrosine antibodies, and developed by ECL. (A) Anti-Jak1 (lanes 1 to 4), anti-Jak2 (lanes 5 to 8), and anti-Stat1 (lanes 9 to 12). Lanes 1, 5, and 9 are controls (uninfected and with no gamma interferon. Lanes 2, 6, and 10 represent uninfected cells incubated with gamma interferon. Lanes 3, 7, and 11 are infected cells incubated with gamma interferon. Lanes 4, 8, and 12 represent cells that had ingested *S. aureus* before incubation with gamma interferon 16 h later. (B) The same membrane, stripped and reprobed with anti-Jak1 (lanes 1 to 4), anti-Jak2 (lanes 5 to 8), and anti-Stat1 (lanes 9 to 12) and developed by ECL. The data shown are from one of three experiments that yielded similar results.

cytokine. The results shown in Fig. 1B demonstrate that phosphorylation of both Jak1 and Jak2 was detectable at 10 U of gamma interferon per ml and was maximal at a concentration of 100 U/ml.

Effect of leishmania infection on gamma interferon-induced tyrosine phosphorylation in U-937 cells. Incubation of exponentially growing U-937 cells with *Leishmania* amastigotes resulted in infection of only 10 to 20% of the cells. In contrast, when U-937 cells were allowed to differentiate in the presence of PMA (10 ng/ml) for 24 h, the cells became adherent, and upon incubation with *Leishmania* amastigotes, infection rates of >90% were achieved. In initial studies, responses of phorbol ester-differentiated U-937 cells to gamma interferon with respect to the activation of Jak1 and Jak2 and tyrosine phosphorylation of Stat1 were observed to be indistinguishable from those observed in nondifferentiated cells. Differentiated U-937 cells were, therefore, considered to be a suitable model system in which to examine the effects of infection with *L. donovani*, and these cells were used in all studies concerned with leishmania infection.

Differentiated U-937 cells, infected and noninfected, were either left untreated or incubated with gamma interferon (100 U/ml) for 10 min at 37°C, and this was followed by lysis in SDS sample buffer. Lysates were analyzed by antiphosphotyrosine Western blotting. Basal (in non-interferon-treated cells) patterns of tyrosine-phosphorylated proteins were different for control and leishmania-infected cells (Fig. 2; compare lanes 1 and 2). For example, a prominent band with an M_r of approximately 200,000 was present with infected cells but not with control cells. In order to ascertain to what extent amastigote-derived proteins may have contributed to the differences observed, the pattern of tyrosine-phosphorylated proteins in an amastigote lysate was examined in parallel. As can be seen in Fig. 2 (lane 6), a prominent protein band with an M_r of 200,000 was also observed with the amastigote preparation. This suggested that the corresponding band in infected cells was likely leishmania derived and not an induced host cell protein. Of interest was that some leishmania-derived phosphotyrosine-containing proteins—for example, the prominent doublet with M_rs of approximately 76,000 and 72,000—actually appeared to be diminished in intensity in infected cells.

Treatment of noninfected control cells with gamma interferon brought about increased tyrosine phosphorylation of at least three proteins with M_rs of approximately 125,000, 60,000, and 52,000 (Fig. 2; compare lanes 1 and 3). Increased labeling of these proteins also occurred to a comparable extent in leishmania-infected cells (Fig. 2; compare lanes 2 and 4). These results indicate that leishmania infection does not globally inhibit gamma interferon-induced changes in tyrosine phosphorylation in U-937 cells.

Gamma interferon-induced tyrosine phosphorylation of Jak1, Jak2, and Stat1 is attenuated in leishmania-infected U-937 cells. Differentiated U-937 cells, infected (for 16 to 18 h) and noninfected, were incubated with gamma interferon and analyzed for tyrosine phosphorylation of Jak1, Jak2, and Stat1 (Fig. 3). As can be seen in Fig. 3, gamma interferon-induced phosphorylation of Jak1 (panel A, lanes 1 to 4), Jak2 (panel A, lanes 5 to 8), and Stat1 (panel A, lanes 9 to 12) appeared to be abrogated in *Leishmania*-infected cells. To control for the nonspecific effects of phagocytosis, an additional group of U-937 cells was allowed to ingest fixed *Staphylococcus aureus*. As can be seen in Fig. 3 (panel A, lanes 4, 8, and 12), these cells were fully responsive to gamma interferon.

The simplest explanation for decreased signal intensity in samples from *Leishmania*-infected cells in the 4G10 antiphosphotyrosine immunoblots in Fig. 3A is impaired gamma interferon-induced signaling. This would result in the diminished activation of Jak1 and Jak2, in the decreased autophosphorylation of these kinases, as well as in the diminished phosphorylation of Stat1. Alternatively, these results could also be explained if infection had in some manner led to reduced levels of expression of these three proteins. As can be seen in Fig. 3B, however, the latter possibility was eliminated when the membranes were stripped and reprobed with antibodies specific for Jak1, Jak2, and Stat1. As can be seen, relative to those in the

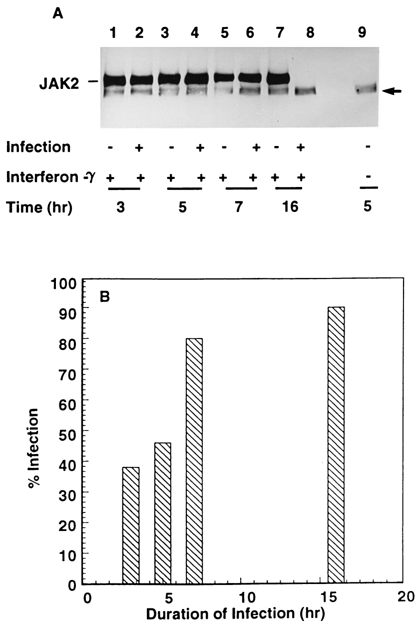

FIG. 4. Kinetic analysis of the effects of leishmania infection on gamma interferon-induced tyrosine phosphorylation of Jak2 in U-937 cells. Differentiated U-937 cells were exposed to amastigotes for the indicated time periods and were subsequently incubated with gamma interferon (100 U/ml) for 10 min at 37°C. Lysates were prepared and immunoprecipitated with anti-Jak2 as described in Materials and Methods. Immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine antibody and developed by ECL. (A) Autoluminogram of blot. (B) Histogram showing the frequency of infected cells as a function of duration of exposure to amastigotes. The data shown are from one experiment.

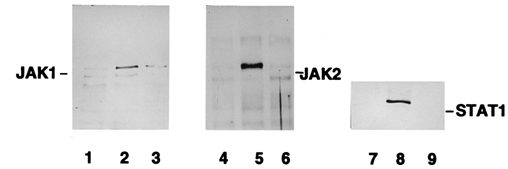

FIG. 5. Leishmania infection attenuates gamma interferon-induced Jak-Stat tyrosine phosphorylation in human monocytes. Monolayers of freshly isolated human monocytes were either left untreated or exposed to amastigotes at an approximate parasite-to-cell ratio of 20:1 (lanes 3, 6, and 9). Sixteen hours later, the cells were washed and incubated with gamma interferon (100 U/ml) for 10 min at 37°C. Cell lysates were then prepared and immunoprecipitated with anti-Jak1 (lanes 1 to 3), anti-Jak2 (lanes 4 to 6), and anti-Stat1 (lanes 7 to 9) as described in Materials and Methods. Immunoprecipitates were separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, probed with antiphosphotyrosine antibodies, and developed by ECL. Lanes 1, 4, and 7 are control cells (uninfected and with no gamma interferon), lanes 2, 5, and 8 are uninfected cells incubated with gamma interferon, and lanes 3, 6, and 9 are infected cells incubated with gamma interferon. The data shown are from one of three independent experiments that yielded similar results.

control cells, the levels of expression of all three proteins in the infected cells were not reduced.

Attenuation of Jak-Stat signaling during leishmania infection is time dependent. To examine whether the effects of leishmania infection were time dependent, cells were incubated with amastigotes for various periods of time. The incubations were then terminated by lysis in RIPA buffer, and the cells were processed for immunoprecipitation and immunoblotting. As can be seen in Fig. 4, gamma interferon-induced activation of the Jak-Stat pathway occurred normally through 7 h of infection (80% of the cells were infected with 7.9 ± 4.4 [mean ± standard deviation] amastigotes per cell). In contrast, by 16 h (90% of the cells infected with 15 ± 8 amastigotes per cell), there was no evidence for activation of the Jak-Stat pathway.

Leishmania infection attenuates interferon-induced tyrosine phosphorylation of Jak1, Jak2, and Stat1 in human monocytes. The results presented above indicate that infection of differentiated U-937 cells with *L. donovani* induces a state of unresponsiveness to gamma interferon-mediated signaling through Jak1, Jak2, and Stat1. To validate differentiated U-937 cells as a model system in which to study this interaction further, the effects of *L. donovani* infection on gamma interferon signaling were also examined in primary human peripheral blood monocytes. Monocyte monolayers were incubated with freshly isolated amastigotes of *L. donovani* at a parasite-to-cell ratio of approximately 20:1. This resulted in an infection rate of >95% at approximately 9.1 ± 5.3 (mean ± standard deviation) amastigotes per cell. After 16 to 18 h of incubation, infected and control cells were incubated with gamma interferon for 10 min. The cells were then lysed and processed for immunoprecipitation and antiphosphotyrosine Western blotting. The results shown in Fig. 5 demonstrate that—as was the case with differentiated U-937 cells—leishmania infection induces a state of unresponsiveness to gamma interferon for the activation of Jak1 and Jak2 and for the tyrosine phosphorylation of Stat1.

DISCUSSION

Mononuclear phagocytes infected with *L. donovani* have impaired responses to gamma interferon for the induction of gene transcription (7, 14, 16). The aim of the present study was to investigate the possibility that this state of diminished responsiveness may be related to defective gamma interferon-mediated cell signaling. Agonist-mediated changes in the activities of both receptor and nonreceptor tyrosine kinases are known to play central roles in the regulation of cell function (5, 17, 18). In this respect, a direct pathway leading from the gamma interferon receptor to activation of gene transcription is now known to involve two members of the *Janus* family of cytoplasmic tyrosine kinases, Jak1 and Jak2, and in addition, the latent cytoplasmic DNA-binding protein Stat1 (5, 17). Activation of Jak kinases involves their autophosphorylation on tyrosine, and Jak1 and Jak2 appear to be involved in bringing about tyrosine phosphorylation of Stat1 (5). Tyrosine-phosphorylated Stat1 dimerizes through SH2 domain interactions and translocates to the nucleus, where it binds to a specific DNA consensus sequence, referred to as the gamma interferon activation sequence, present within promoter regions of certain gamma interferon-inducible genes (5). Thus, regulation of tyrosine phosphorylation at the whole-cell level and specifically the Jak-Stat signaling pathway are logical targets upon which to focus an investigation of impaired responses to gamma interferon during leishmania infection.

In initial experiments, it was observed that treatment of undifferentiated U-937 cells with gamma interferon resulted in rapid increases in tyrosine phosphorylation of Jak1, Jak2, and Stat1 (Fig. 1A), and this was found to be concentration dependent for gamma interferon (Fig. 1B). However, because these cells ingested *L. donovani* only to a limited extent, it was necessary to differentiate them with PMA, after which they became adherent and were readily infected with *L. donovani*. The Jak-Stat pathway was also observed to be rapidly activated in PMA-differentiated U-937 cells (Fig. 3). Overall, the responses observed in differentiated U-937 cells were indistinguishable from those observed in non-PMA-treated cells. Taken together, these findings indicate that differentiated

U-937 cells are a suitable model system in which to examine the effects of *L. donovani* on gamma interferon-regulated changes in tyrosine phosphorylation at the whole-cell level as well as on the more specific activation of Jak1 and Jak2 leading to the phosphorylation of Stat1. It should be noted that a constitutive phosphotyrosine-containing protein with a slightly lower molecular mass than that of Jak2 was consistently coimmunoprecipitated with Jak2 in U-937 cells (Fig. 2 to 4). The identity of this protein is not known, but it may not be unique to this system. Recently, a constitutively phosphotyrosine-containing protein with an electrophoretic mobility similar to that of Jak2 was also observed to be present in Jak2 immunoprecipitates from HeLa cells (4).

The overall basal patterns of phosphotyrosine-containing proteins in whole-cell lysates were different for noninfected and infected U-937 cells (Fig. 2). For example, a prominent band with an M_r of approximately 200,000 was present in infected cells but not in control cells. A phosphotyrosine-containing protein with an M_r of 200,000 was also relatively abundant in 4G10 immunoblots of amastigote lysates, indicating that the corresponding band in infected cells was likely leishmania derived and not an induced host cell protein. In contrast, two other prominent phosphotyrosine-containing proteins that were also observed in amastigotes and that had M_r of approximately 72,000 and 76,000 were significantly diminished in intensity in infected cells. These results indicate that leishmania-derived phosphotyrosine-containing proteins are differentially expressed in infected macrophages. There are several possible explanations for this finding. First, this could be explained by the differential synthesis or stabilities of these amastigote proteins within U-937 cells. Alternatively, the activities of leishmania tyrosine kinases, phosphotyrosine phosphatases or both towards these proteins may be differentially regulated in amastigotes and in infected cells. Third, it is also possible that a host cell phosphatase may have greater activity toward the 72,000- and 76,000-M_r leishmania proteins compared with that toward the 200,000-M_r phosphotyrosine-containing protein. Further work will be required to identify these leishmania phosphotyrosine-containing proteins and to define their potential roles in disease pathogenesis.

Analysis of the patterns of phosphotyrosine-containing proteins in U-937 whole-cell lysates indicated that at least three proteins with M_rs of approximately 125,000, 60,000, and 52,000 (Fig. 2) showed increased phosphotyrosine content after incubation with gamma interferon. Notably, increased labeling of these proteins occurred to similar extents in control and leishmania-infected cells. The finding that gamma interferon-induced tyrosine phosphorylation is not globally inhibited in leishmania-infected cells indicates (i) that the gamma interferon receptor is functionally intact under these conditions, which is consistent with previously published findings (16), and (ii) that at least one gamma interferon-regulated signaling pathway leading to changes in phosphotyrosine labeling appears to be unimpaired in these cells. In contrast, more specific analysis of the gamma interferon responsiveness of Jak-Stat signaling components indicated that this pathway is selectively impaired in leishmania-infected cells (Fig. 3 to 5).

Attenuation of Jak-Stat signaling under these conditions appeared to be relatively specific for *L. donovani*, since activation of Jak1 and Jak2 and phosphorylation of Stat1 occurred normally in U-937 cells that had ingested fixed *S. aureus*. The effects of leishmania infection were observed to be time dependent in that signaling through Jak-Stat appeared to be intact through 7 h of infection but was virtually nonexistent by 16 h (Fig. 4). The intensity of infection of U-937 cells at 16 h was approximately twice that observed at 7 h. Thus, the delayed effect of *L. donovani* on the Jak-Stat pathway may reflect dependency on the level of infection, a requirement for synthesis of an inhibitor, or both. Ongoing experiments are examining these possibilities.

The exact basis for the attenuation of gamma interferon-induced activation of the Jak-Stat signaling pathway in leishmania-infected cells remains to be defined. However, a number of potential mechanisms may be considered. For example, enhanced production of such autoinhibitory molecules as interleukin 10, transforming growth factor β, or prostaglandins by infected cells could be involved. Results of initial experiments, however, showing no effects of either transforming growth factor β or interleukin 10 on gamma interferon-induced activation of the Jak-Stat pathway in U-937 cells suggest that these molecules may not be relevant (10a). Consistent with this observation, the responsiveness of the Jak-Stat pathway to gamma interferon in astrocytes was also observed to be unaffected by transforming growth factor β (13).

Activation of Jak-Stat signaling by specific ligands is believed to involve receptor dimerization or oligomerization as well as binding of Jaks to receptor membrane proximal domains (5). While it is possible that these interactions could be disrupted by leishmania infection, leading to impaired signaling, it seems unlikely that such mechanisms would have delayed kinetics. An alternative mechanism to explain delayed attenuation of Jak-Stat activation during leishmania infection could involve induction of cellular phosphotyrosine phosphatases. One potential candidate is a hematopoietic cell phosphatase (known otherwise as PTP1C, SHP, or SHPTP1) that is expressed in macrophages as well as in other myeloid cells and that is known to negatively regulate Jak-dependent signaling (5). While there is no direct support for this possibility, indirect support is provided by the finding that at least two dual-specificity, stress-activated phosphoprotein phosphatases, PAC-1 (19) and MKP-1 (6), are induced during leishmania infection of macrophages (13a).

In summary, the results of the present study provide direct evidence for the attenuation of gamma interferon-induced activation of Jak1 and Jak2 and phosphorylation of Stat1 in U-937 cells infected with *L. donovani*. Evidence that this effect is selective is provided by the finding that gamma interferon-induced changes in whole-cell tyrosine phosphorylation are not impaired in infected cells. This result also implies that gamma interferon-mediated cell signaling in mononuclear phagocytes leading to changes in the levels of protein tyrosine phosphorylation involves protein tyrosine kinases in addition to Jak1 and Jak2, protein tyrosine phosphatases with changes in their activities, or both. The findings presented also suggest that differentiated U-937 cells can be used as a model system to study the effects of infection with *L. donovani* as well as with other intracellular microbes on gamma interferon-regulated cell signaling. This conclusion is supported by the finding that infection of human peripheral blood monocytes with *L. donovani* also results in marked attenuation of the gamma interferon-induced activation of the Jak-Stat1 pathway.

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