Proteasome-mediated Degradation of STAT1α following Infection of Macrophages with Leishmania donovani*

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Activation of the Janus-activated kinase 2 (JAK2)/STAT1α signaling pathway is repressed in Leishmania-infected macrophages. This represents an important mechanism by which this parasite subverts the microbial functions of the cell to promote its own survival and propagation. We recently provided evidence that the protein tyrosine phosphatase (PTP) SHP-1 was responsible for JAK2 inactivation. However, STAT1 translocation to the nucleus was not restored in the absence of SHP-1. In the present study, we have used B10R macrophages to study the mechanism by which this Leishmania-induced STAT1 inactivation occurs. STAT1α nuclear localization was shown to be rapidly reduced by the infection. Western blot analysis revealed that cellular STAT1α, but not STAT3, was degraded. Using PTP inhibitors and an immortalized bone marrow-derived macrophage cell line from SHP-1-deficient mice, we showed that STAT1 inactivation was independent of PTP activity. However, inhibition of macrophage proteasome activity significantly rescued Leishmania-induced STAT1α degradation. We further demonstrated that degradation was receptor-mediated and involved protein kinase Ca. All Leishmania species tested (L. major, L. donovani, L. mexicana, L. braziliensis), but not the related parasite Trypanosoma cruzi, caused STAT1α degradation. Collectively, results from this study revealed a new mechanism for STAT1 regulation by a microbial pathogen, which favors its establishment and propagation within the host.

Intracellular protozoan parasites of the Leishmania genus are the etiological agents of leishmaniasis, a condition that causes considerable worldwide morbidity and mortality, with pathologies ranging from disfiguring cutaneous to lethal visceral afflictions (1). Survival of these pathogens within the phagocytic cells of the host requires rapid alteration of macrophage signal transduction, resulting in abnormal immune functions (reviewed in Refs. 2 and 3). For instance, macrophage dysfunctions, such as failure to respond to interferon-γ (IFN-γ),1 have been linked to altered signaling cascades, including Ca2+-, PKC (protein kinase C)-, MAPK (mitogen-activated protein kinase)-, and JAK2 (Janus-activated kinase 2)-dependent pathways (4–11). Manipulation of these signaling pathways distorts the activities of transcription factors, such as STAT1α, which is important for the expression of IFN-γ-induced genes, such as inducible nitric-oxide synthase and major histocompatibility complex class II (12).

We have previously shown that the unresponsiveness of the JAK2 signaling pathway in Leishmania donovani-infected macrophages upon IFN-γ stimulation was due to the ability of the parasite to activate a particular protein tyrosine phosphatase (PTP), SHP-1 (4). SHP-1 is a known inhibitor of several tyrosine kinase-dependent pathways, including the JAK2/STAT1α pathway, where it dephosphorylates JAK2 (4, 13, 14). However, our observation that IFN-γ-stimulated STAT1α activity is also reduced in SHP-1-deficient macrophages following L. donovani infection indicates that Leishmania employs further mechanisms to inhibit STAT1 activity.2

Aside from SHP-1, numerous physiological and pathogen-induced proteins have been shown to regulate JAK/STAT pathways (15). Indeed, alternative splicing of the STAT1 mRNA itself produces a dominant negative variant, STAT1β, which binds the same promoter elements as STAT1α but lacks a crucial transcription activation domain (16, 17). The intracellular bacterium Mycobacterium avium induces STAT1β to inhibit macrophage STAT1α activity (18). Members of the suppressor of cytokine signaling (SOCS) family of proteins are induced by stimulation with various cytokines and provide negative feedback to the response of the cell to cytokine treatment (19, 20). SOCS proteins are capable of acting as ubiquitin ligases, and inhibition of signaling by proteasome-mediated receptor degradation has been proposed to contribute to inactivation of various STATs (21–27). Although IFN-γ-activated STAT1 has been shown to be ubiquitinated and protein levels have been stabilized by inhibitors of the proteasome, details of this mechanism and whether the SOCS family is involved have not been determined (28). SOCS family members have also been reported to prevent STAT phosphorylation by

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1 The abbreviations used are: IFN-γ, interferon-γ; CR3, complement receptor 3; EMSA, electrophoretic mobility shift assay; GAS/ISRE, γ-activated sequence/interferon stimulation response element; JAK, Janus-activated kinase; PKC, protein kinase C; PTP, protein tyrosine phosphatase; PIAS-1, protein inhibitor of activated STAT1; SOCS-1, suppressor of cytokine signalling-1; STAT, signal transducer and activator of transcription; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; bpV(phen), potassium bispexoxol(1, 10-phenanthroline) oxovanadate.

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binding upstream receptors and preventing autophosphorylation or by competing for STAT binding (29–31). Viruses have developed a wide range of mechanisms for inhibiting IFNγ signaling, including production of decoy receptors and dominant negative intermediates (32). Of particular interest, paramyxovirus-encoded proteins have been shown to cause specific degradation of STAT1 by the proteasome by recruiting a cellular E3-ubiquitin-protein isopeptide ligase (33–35). However, other groups have reported that this degradation is cell-type-specific and suggested that inhibition of STAT1 phosphorylation is a more important mechanism (32, 36). To our knowledge, no microbial pathway has been reported to repress STAT1α by proteasome-mediated degradation.

In the present study, we investigated the mechanisms whereby Leishmania infection causes macrophage STAT1α inactivation. Our results show that abnormal STAT1α nuclear translocation in Leishmania-infected macrophages could be the consequence of rapid and sustained STAT1α protein diminution and that this may contribute to the lack of response to IFNγ. The depletion was shown to be specific to STAT1α, as STAT3 levels were unchanged, and was triggered by the early interaction between the pathogen and host cell. Our study further implicates protein kinase Ca (PKCa)-dependent signaling and proteasomes (but not PTPs) in this process. Together, these results argue for a direct role of the proteasome pathway in the specific proteolysis of STAT1α in macrophages infected by the protozoan parasite Leishmania, representing a new mechanism whereby microbial pathogens can subvert microbicidal activity.

EXPETIMENTAL PROCEDURES

Cell Culture—L. donovani strains 2211 and 182D, Leishmania major Friedlin strain (MHOM/IL/S8/Friedlin), Leishmania mexicana, and Leishmania (viantina) braziliensis promastigotes, Trypanosoma cruzi strain ECUAD0 trypomastigotes and immortalized murine bone marrow-derived cell lines B10R, me-3 (SHP-1 II receptor; α-CDR) and CD16/CD32 (FcγIII/II receptor; α-FcyR) were purchased from Pharmingen, and H-34 (SH-2/3 receptor; α-CR3) and CD16/CD32 (FcγIII) receptor; α-FcyR) were purchased from Santa Cruz Biotechnology, from a generous gift from Dr. Paola Allavena (Mario Negri Institute, Milan, Italy). The cells were first infected for 6 h, washed three times, then left to rest for 2 h before the addition of IFNγ (100 units/ml).

Electrophoretic Mobility Shift Assay (EMSA) and Western Blotting—Electrophoretic mobility shift assay (EMSA) was performed as described previously (43). The oligonucleotide sequence for EMSA (GAS/ISRE consensus) was 5'-AAG-TAC-TTT-CAG-TTT-CAT-ATT-CTA-3'. α-Phosphoysrine-STAT1α and a-phosphophorysine-STAT3 were kindly provided from Dr. David Frank (Dana Farber Institute). α-STAT1α and a-STAT3 were obtained from Sigma, and α-PKCa from Cell Signaling Technology, and α-PKCa from BD Transduction Laboratories (catalogue numbers sc-591, sc-483, 2261, and 610107, respectively). All experiments are representative of three.

Immunofluorescence Staining—Experiments were performed in chamber slides. Cells (1 × 10⁶) were gently washed three times with cold phosphate-buffered saline and fixed with 200 μl of methanol at −20 °C for 10 min. The slides were blocked with 200 μl of phosphate-buffered saline + 0.5% bovine serum albumin and then treated with 100 μl of anti-STAT1α in phosphate-buffered saline + 0.5% bovine serum albumin for 1 h at room temperature in a humid, dark chamber, washed three times, and then treated with fluorescein isothiocyanate-conjugated secondary antibody for 1 h. After the final washes, coverslips were mounted on the chamber slides using 90% glycerol, 10% phosphate-buffered saline, and 2.5% DABCO (1,4-diazabicyclo(2,2,2)octane). Pictures were taken using an immunofluorescence microscope at 400x magnification.

Two-dimensional Electrophoresis—After infection, 5 × 10⁶ cells were washed and lysed in 1 ml of buffer (50 mM Tris, pH 8, 137 mM NaCl, 1% Triton X-100, and Complete™ protease inhibitor mixture (Roche Applied Science)). STAT1α-containing complexes were precipitated with 1 μg of α-STAT1α overnight at 4 °C and AG plus-agarose beads (Santa Cruz Biotechnology) for 1 h, all at 4 °C on a rotating platform. Immunoprecipitates were then washed four times with immunoprecipitation lysis buffer, and the remaining bead complexes were eluted in 45 μl of 2D rehydration buffer (40 mM Tris, 8 M urea, 4% (w/v) CHAPS) for 30 min at 37 °C. Isoelectric focusing was performed using the IPGPhor isoelectric focusing system 18-cm Immobiline immobilized pH gradient strips, pH 3–10 (Amersham Biosciences), according to manufacturer’s instructions. Proteins were resolved by 12% SDS-PAGE and visualized by Vorum silver staining.

RESULTS

Effect of L. donovani Infection on STAT1α Nuclear Localization—Previous studies have reported that JAK2/STAT1 signaling is altered in Leishmania-infected macrophages (4, 6). Although JAK2 tyrosyl phosphorylation was shown to be inhibited, the effect on STAT1α activity has not been reported. We therefore first established whether STAT1α activation by IFNγ stimulation was affected in Leishmania-infected macrophages. As shown in Fig. 1, EMSA allowed us to observe increased nuclear STAT1 DNA binding activity in response to IFNγ. However, this induction was prevented in Leishmania-infected macrophages. Interestingly, the STAT1α nuclear signal in non-stimulated macrophages was substantially diminished in infected cells in comparison to uninfected ones.

It was important to determine the time frame in which this STAT1 down-regulation occurred. As indicated in Fig. 2A, STAT1 nuclear signal faded rapidly in L. donovani-infected cells in the absence of IFNγ, and was almost completely undetectable 6 h post-infection. This contrasted with IFNγ treatment of uninfected cells, which resulted in a significant, time-dependent increase in STAT1 translocation.

Lower nuclear levels of STAT1 during infection could result from its retention in the cytoplasm, for example, due to a failure to phosphorylate tyrosine 701. It could also reflect a reduction in total cellular STAT1 protein. To address these two possibilities, we monitored STAT1α protein level as well as its phosphorylation on residue Tyr-701 in whole cell lysates. Consistent with the EMSA, we observed that Leishmania infection, unlike IFNγ stimulation, did not lead to STAT1α Tyr-701 residue phosphorylation (Fig. 2B). Importantly, we noticed that...
STAT1α protein levels decreased rapidly in the presence of Leishmania and in some cases disappeared completely by the third hour of infection. This observation permitted us to conclude that Leishmania-mediated STAT1α degradation is responsible for the absence of STAT1 from the nucleus. Of utmost interest, it appeared that this phenomenon was specific to STAT1α, because STAT3 protein level was unchanged by *L. donovani* infection.

Disappearance of STAT1α from the nucleus was further reinforced by microscopic observation using immunofluorescence staining. Cells were infected with *Leishmania* or treated with IFNγ (100 units/ml; positive control for STAT1α activation) for 30 min. STAT1α protein seemed equally distributed in resting macrophages (Fig. 3, top left panel). As expected, IFNγ stimulation led to clustering of STAT1α in the nucleus (Fig. 3, top right panel). Importantly, in *Leishmania*-infected macrophages (Fig. 3, bottom left panel), a different pattern was revealed in that nuclear STAT1α was less abundant with diffuse localization in the cytoplasm, suggesting that STAT1α degradation may start in the nucleus.

Protein Tyrosine Phosphatases and SHP-1 Are Not Involved in STAT1α Inactivation—Many studies have reported evidence for a role of PTPs in STAT1 regulation (21, 22, 25, 44). As we have previously shown, Leishmania infection leads to PTP and SHP-1 activation (4, 37, 40). It was legitimate to test whether the STAT1α inactivation seen here was dependent upon these molecules. As shown in Fig. 4A, our results demonstrated that inhibition of PTPs by bpV(phen) did not prevent the rapid disappearance of STAT1 triggered by IFNγ. In addition, we further demonstrated that the PTP SHP-1 is not involved in STAT1α inactivation by comparing immortalized macrophages from motheaten (SHP-1−/−) mice with their wild-type equivalents (37). As seen in Fig. 4B, the pattern of STAT1 nuclear degradation was similar between wild-type and SHP-1-deficient cells, except for the basal translocation in resting cells. Indeed, the STAT1 signal was stronger in the absence of SHP-1. This is consistent with the fact that SHP-1 is known to regulate JAK2 phosphorylation (13). Thus, in its absence, higher JAK2 activity increases STAT1 nuclear translocation.

*STAT1α Is Degraded via the Proteasome Pathway—*Because STAT1α proteins apparently underwent proteolysis during Leishmania infection, we addressed the role of proteasomes in this process. Macrophages were treated with increasing doses of MG-132 and clasto-lactacystin β-lactone (c-lactacystin), a more specific proteasome inhibitor, before infection with *Leishmania*. As seen in EMSA experiments in Fig. 5A, STAT1 reappeared in the nucleus when MG-132 and c-lactacystin were used. Moreover, these inhibitors also restored STAT1α protein level in whole cell lysates during infection (Fig. 5B). These
results allowed us to conclude that, in the case of *Leishmania* infection, STAT1α is degraded via the proteasome.

**Implication of Different Leishmania Species and Macrophage Receptors in STAT1α Inactivation**—The present study has demonstrated so far that infection of macrophages with *L. donovani* causes STAT1α inactivation by proteasomal degradation. It was important to verify whether other *Leishmania* species or other protozoan pathogens had the same ability. As seen in Fig. 6, A and B, all tested *Leishmania* species (*L. donovani*, *L. major*, *L. mexicana*, and *L. braziliensis*) caused STAT1α degradation. However, *T. cruzi*, a protozoan of the same Trypanosomatidae family as *Leishmania*, had no effect on STAT1. Moreover, phagocytosis of the malaria pigment hemozoin by macrophages had no effect either. Thus, the interaction of *Leishmania* species with the macrophages specifically affects STAT1α degradation and cannot be generalized to other similar pathogen/macroage interactions or other forms of phagocytosis.

The degradation that we observed started as early as 30 min after parasite contact, an insufficient time for parasite internalization. We therefore asked whether macrophage receptors previously implicated in *Leishmania/macrophage interactions* had a role to play in STAT1α inactivation. Proteasomal targeting of STAT1α could hence be triggered by receptor signaling. Several receptors, such as the complement receptor 3 (CR3), the receptor for the Fc portion of immunoglobulins γ (FcγR), and the mannose-fucose receptor (mannose R) are known to bind *Leishmania* (45–47). Using blocking antibodies against these three receptors, we inhibited their ligation to *Leishmania* and observed the effects on STAT1 nuclear translocation. Results suggested that all three had a potential role to play in STAT1 inactivation (Fig. 6C), because the translocation of
STAT1α inactivation is not specific to L. donovani and is receptor-dependent. Effect of different species of Leishmania (donovani (Ld), major (Lm), mexicana (Lmex), braziliensis (Lb)) as well as of T. cruzi (Tc) and the malaria pigment hemozoin (Hz) (25 μg/ml) on STAT1α (all infections for 3 h) shown by EMSA (A) and Western blot (B). C, effect of receptor blocking (1 h) prior to Ld infection (3 h) on the nuclear translocation of STAT1 using blocking antibodies against CR3, FcγR, and mannose R. IFNγ was used as a positive control (3 h of stimulation). Nil, uninfected macrophages.

**Fig. 6.**

**DISCUSSION**

*Leishmania* is known for its ability to down-regulate numerous macrophage functions that are detrimental to its survival and persistence (3, 10, 50–52). Some of these are generally induced by cytokines such as IFNγ (e.g. NO, major histocompatibility complex, interleukin-12). Thus, the parasite has evolved ways to inhibit the different signaling pathways activated by such stimulation, namely the JAK2/STAT1α and mitogen-activated protein kinase extracellular signal-regulated kinase-1/2 pathways (4–7). Previous studies (4, 7) have demonstrated that the inhibition of JAK2 and extracellular signal-regulated kinase-1/2 during infection was mediated by the PTP SHP-1.2 The scope of the present study was to identify mechanisms that were responsible for this inactivation.

We observed that both the IFNγ-dependent STAT1 nuclear translocation and basal STAT1α protein level were inhibited in a time-dependent manner in *Leishmania*-infected macrophages. This occurred following infection with both New and Old World species of *L. donovani*, major, *mexicana*, and *braziliensis*, arguing for a conserved evolutionary trait among the genus. The fact that we observed no disappearance of STAT1α following phagocytosis of the malarial pigment hemozoin or infection with a different trypanosomatid (T. cruzi) indicates that the phenomenon is specific to *Leishmania* infection and is not a result of phagocytosis or general cell stress.
Some studies have attributed inhibition of STATs by various stimuli to the action of a PTP on JAK proteins (21, 22, 25) or a nuclear PTP dephosphorylating STAT1 directly (44). This last report is further reinforced by a recent study describing a nuclear PTP dephosphorylating STAT1 directly (44). This last observation of STAT1 phosphorylation (α-p-PKC) during L. donovani (Ld) infection (from 10 to 60 min). α-PKCα was immobilized on the stripped membrane to verify equal protein loading. B. effect of inhibition of PKC (inhibitor G6976, from 3 to 25 nM) before Leishmania-infection (for 3 h) on STAT1α nuclear translocation. Nil, uninfected macrophages; − , untreated infected macrophages.

FIG. 7. Role of PKCα in the inactivation of STAT1α. A, increased PKCα phosphorylation (α-PKCα) during L. donovani (Ld) infection (from 10 to 60 min). α-PKCα was immobilized on the stripped membrane to verify equal protein loading. B. effect of inhibition of PKC (inhibitor G6976, from 3 to 25 nM) before Leishmania-infection (for 3 h) on STAT1α nuclear translocation. Nil, uninfected macrophages; − , uninfected infected macrophages.

Members of the SOCS family, especially SOCS1, have been shown to repress STAT1α by binding to, and causing dephosphorylation of, upstream molecules, such as JAK2, and/or by recruiting a ubiquitin ligase complex, leading to proteasome-mediated degradation (15, 19). One could therefore propose that Leishmania infection induces expression of SOCS1, which binds to STAT1α, resulting in its ubiquitination and degradation. However, a number of observations conflict with this theory. First, no direct interaction between SOCS proteins and STAT1α has been reported. Repression appears to occur by targeting upstream molecules. Second, SOCS1 is known to induce degradation of JAK2 (19, 20), but the JAK2 protein level remains unchanged following infection (4, 6). Third, we have been unable to detect increased SOCS1 expression in infected cells by Northern blot (data not shown). Another family member, SOCS3, has been shown to be transiently induced by Leishmania in human monocyte-derived macrophages (58). However, data from SOCS3 knock-out mice suggest that SOCS3 regulates STAT1α by mechanisms other than its stability (59, 60). Taken together, these studies suggest that Leishmania-induced degradation of STAT1α is unlikely to be mediated by SOCS induction.

Two studies have described proteasome-mediated degradation of STATs following cytokine stimulation. One study showed that, following phosphorylation in response to IFNγ, STAT1α is ubiquitinated and degraded (28). A second study showed that STAT3 was degraded during ciliary neurotrophic factor or 12-O-tetradecanoylphorbol-13-acetate stimulation (49). In both cases, the STAT was protected by inhibition of the proteasome. We report here that the use of two different proteasome inhibitors, MG-132 and the more specific clasto-lactacystin β-lactone, rescued STAT1α nuclear translocation as well as restored its general protein level in Leishmania-infected macrophages. We conclude that Leishmania infection results in proteasome-mediated degradation of STAT1α. This contrasts
with the normal mechanisms of repression of STAT1α following cytokine stimulation, where activity is reduced but stability is not (15, 21–25). Instead, it is reminiscent of the action of various paramyxoviral proteins, which cause ubiquitination and degradation of STAT1 by the proteasome (33–35). However, these viral proteins are expressed within the cell where they are able to recruit a cellular ubiquitin ligase complex by physical interaction (35). The rapidity of Leishmania–activated degradation means that it is unlikely to be caused by a parasite protein gaining access to the cell. Instead, the degradation means that it is unlikely to be caused by a parasite physical interaction (35). The rapidity of this nonspecific result could be that Leishmania activates a cellular ubiquitin ligase complex by recruiting a cellular ubiquitin ligase complex by allowing the degradation of STAT1α by using specific knock-out parasite strains. The absence of either molecule did not affect the nuclear translocation was re-infection. This is consistent with the hypothesis, it has been demonstrated that Leishmania must bind to STAT1α inactivation by using specific knock-out parasite strains. The absence of either molecule did not affect STAT1α nuclear translocation (data not shown). However, this result does not rule out their possible involvement in this process, because both lipophosphoglycan and gp63 can bind to all three receptors and so may be redundant. In the absence of double knock-out parasites, we could not verify this hypothesis.

Both CR3 and FcγR ligation lead to PKC activation (48). Thus, CR3 could be important in STAT1α inactivation. Results showed that PKCα was activated in macrophages from 10 to 60 min following infection. This is consistent with the initiation of STAT1α proteolysis at 30 min post-infection. Using a PKC inhibitor (Go6976), we were able to demonstrate PKC involvement in our specific STAT1α nuclear inactivation. This is in accordance with a previous report showing PKC-dependent STAT1α degradation by the proteasome (49).

Finally, analysis of STAT1α immunoprecipitates by two-dimensional electrophoresis allowed us to see differences between patterns of proteins associated with STAT1α in resting macrophages and macrophages infected with Leishmania for 30 min. This is a good indication that Leishmania infection alters protein complex formation in macrophages, and the pattern is particularly suggestive of changes in the phosphorylation state of a number of proteins. Further identification of these spots will enable us to better understand the mechanisms underlying STAT1α degradation by the proteasome.

Overall, this study has shed light on a novel mechanism utilized by Leishmania to alter macrophage signaling, i.e. STAT1α degradation by the proteasome in the early stages of infection. This phenomenon seems to start by the triggering of three macrophage receptors (CR3, FcγR, and the mannose-fucose receptor) by the parasite and subsequent PKCα induction. By allowing the degradation of STAT1α, in addition to the inhibition of JAK2 by SHP-1, the parasite is able to inhibit the response of the host cell to IFNγ. This favors its persistence within the cell and propagation of the disease.

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