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Activation of TGF-β by *Leishmania chagasi*: Importance for Parasite Survival in Macrophages[1]

Kira R. Gantt,* Stacey Schultz-Cherry,¶ Nilda Rodriguez,‡ Selma M. B. Jeronimo,‖ Eliana T. Nascimento,‖ Todd L. Goldman,† Thomas J. Recker,§ Melissa A. Miller,§ and Mary E. Wilson[2]*†‡§

TGF-β is a potent regulatory cytokine that suppresses expression of inducible NO synthase and IFN-γ, and suppresses Th1 and Th2 cell development. We examined whether functionally active TGF-β is present in the local environment surrounding the invading protozoan *Leishmania chagasi*. Our prior data showed that TGF-β levels are significantly increased in *L. chagasi*-infected mice. In the current study, we found TGF-β was also abundant in bone marrows of humans with acute visceral leishmaniasis but not in those of uninfected controls. Furthermore, *L. chagasi* infection caused an increase in biologically active TGF-β in human macrophage cultures without changing the total TGF-β. Therefore, we investigated the means through which leishmania could augment activated but not total TGF-β. Incubation of latent TGF-β with *Leishmania* sp. promastigotes caused active TGF-β to be released from the latent complex. In contrast, the nonpathogenic protozoan *Crithidia fasciculata* could not activate TGF-β. TGF-β activation by leishmania was prevented by inhibitors of cysteine proteases and by the specific cathepsin B inhibitor CA074. Physiologic concentrations of TGF-β inhibited killing of intracellular *L. chagasi* in macrophages, although the phagocytosis-induced respiratory burst remained intact. In contrast, supraphysiologic concentrations of TGF-β had no effect on parasite survival. We hypothesize that the combined effect of abundant TGF-β stores at extracellular sites during infection, and the ability of the parasite to activate TGF-β in its local environment, leads to high levels of active TGF-β in the vicinity of the infected macrophage. Locally activated TGF-β could, in turn, enhance parasite survival through its effects on innate and adaptive immune responses. *The Journal of Immunology*, 2003, 170: 2613–2620.

Leishmania chagasi is an obligate intracellular protozoan that causes human visceral leishmaniasis, a disease characterized by suppression of cell-mediated immune responses and unchecked parasite replication in macrophages (1). TGF-β plays an important role in the progression of leishmaniasis in rodents (2, 3). The purpose of this study was to examine whether functionally active TGF-β may be present in the local environment surrounding the invading *Leishmania* promastigote.

TGF-β has been called a parasite escape mechanism through which leishmania survive in their mammalian host (3). An active role for the parasite in inducing host TGF-β has been implied but not directly shown. Nonetheless, there is ample evidence supporting a role for TGF-β in progression of leishmaniasis in vivo in experimental rodent models. There is an excess of TGF-β in hamsters infected with *Leishmania donovani*, and in mice infected with *L. donovani* or *L. chagasi* (4–6). Administration of neutralizing Ab to wild-type mice, or infection of BALB/c mice lacking the TGF-βRII on $CD4^+$ cells, decreases *Leishmania major*-induced lesion size (2, 7). Parasite Ag-specific Th2-type $CD4^+$ cells oppose the curative type-1 response to *L. major* infection in BALB/c mice, thereby promoting disease progression (8). In contrast, the type-1 immune response to *L. chagasi* is suppressed in susceptible BALB/c mice by TGF-β, which is derived in part from Th3-like TGF-β-producing $CD4^+$ T cells (5, 9).

Active TGF-β is augmented during infection of inflammatory mouse peritoneal macrophage cultures with several different *Leishmania* sp. in vitro (3, 10). The mechanism through which this occurs is unknown. TGF-β is released from cells noncovalently bound to the latency-associated peptide (LAP),[3] which renders it inactive. Extracellularly, the complex binds to the latency TGF-β binding protein (LTBP) and is stored in the extracellular matrix. To exert biological activity, latent TGF-β must be released from both LAP and LTBP. Activation can occur through various physicochemical means or enzymes that act on either LAP or LTBP (11).

Immune effects of TGF-β include promotion of chemotaxis, suppression of Th1 and Th2 development, and suppression of some macrophage microbicidal functions (12, 13). During the current study, we examined the hypotheses that *L. chagasi* induces the release of activated TGF-β by its effects on human macrophages and/or by acting on latent TGF-β itself. Our data showed an increase in the abundance of activated but not total TGF-β that was

*Interdisciplinary Immunology PhD Program, Departments of †Internal Medicine and ‡Microbiology, University of Iowa, Iowa City, IA 52242; §Veterans Affairs Medical Center, Iowa City, IA 52240; ¶Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706; and ‖Departamento de Bioquimica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil

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[2] Address correspondence and reprint requests to Dr. Mary E. Wilson, Department of Internal Medicine, University of Iowa, Iowa City, IA 52242. E-mail address: mary-wilson@uiowa.edu

[3] Abbreviations used in this paper: LAP, latency-associated peptide; LTBP, latency TGF-β binding protein; BMM, bone marrow macrophage; SFM, serum-free medium; MSFM, macrophrage SFM; NRK, non-neoplastic rat kidney; BCS, bovine calf serum; EGF, epidermal growth factor; CM, conditioned medium; PAI, plasminogen activator inhibitory; MDM, monocyte-derived macrophage; Man-LAM, mannose-lipoarabinomannan; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DCF, dichlorodihydrofluorescein diacetate; iNOS, inducible NO synthase.

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due, at least in part, to activation of latent TGF-β by the parasite itself. Likely, a combination of parasite-derived and induced host factors lead to an increase in the local concentration of biologically active TGF-β in vivo. TGF-β could be one means through which the parasite manipulates its immediate microenvironment to its own survival advantage.

Materials and Methods

Cells

Human PBMC were isolated on Ficoll-Hypaque as described (14). Bone marrow macrophages (BMMs) from C3H/HeJ or BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were cultured in RPMI 1640, 10% FCS, and 20% L929 cell supernatant (American Type Culture Collection, Manassas, VA). A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669) was maintained in Syrian hamsters (15). Promastigotes in serum-free medium (SFM) were used in the stationary phase of growth for all experiments (16). Promastigotes were opsonized with 5% human or mouse serum (26°C, 30 min) for macrophage experiments. This caused no discernable loss of viability according to flagellar motility and growth. *Crithidia fasciculata* (American Type Culture Collection) was cultured in SFM with 20 μg/ml hemin.

L. chagasi or *C. fasciculata* at 3×10^8/ml in 10 mM Tris/5 mM $CaCl_2$ (4°C) were lysed by freeze-thaw. Unlysed parasites and nuclei were removed at $2{,}000 \times g$ (10 min, 4°C). Soluble and microsomal fractions were separated at $100{,}000 \times g$ (60 min, 4°C).

Non-neoplastic rat kidney (NRK) soft agar assay

NRK cells (NRK-49FCRL-1570; American Type Culture Collection) were grown in 24-well plates in 5 mM L-glutamine, DMEM (Cellgro/Mediatech, Herndon, VA), 10% bovine calf serum (BCS), 0.3% noble agar, and 5 ng/ml epidermal growth factor (EGF; Calbiochem, San Diego, CA) (17). Latent TGF-β was provided by conditioned medium (CM) from MDCK or RM-1 cells (American Type Culture Collection) grown in 0.1% BSA in DMEM for 6–8 h. Wells contained medium alone, CM treated with 0.1 M HCl to activate TGF-β, rTGF-β, 1×10^7 promastigotes/ml, untreated CM, or CM that had been preincubated with promastigotes (30 min, 37°C). Protease inhibitors were added during the CM-promastigote preincubation (4 nM cystatin C (Calbiochem), 8 nM CA074 (Peptides International, Louisville, KY), 10 μM leupeptin, 10 μM bestatin (Sigma-Aldrich, St. Louis, MO)). After 3–5 days at 37°C and 5% CO_2, colonies >62 μm in diameter were quantified microscopically. The colony number is proportional to the concentration of activated TGF-β. Conditions were done in triplicate.

Plasminogen activator inhibitory (PAI) luciferase assay

Mink lung fibroblasts (MvLu) stably expressing a TGF-β-responsive PAI promoter-luciferase construct were a kind gift from D. Rifkin (New York University, New York, NY) (18). MvLu in 0.1% BSA/DMEM were incubated in different combinations of medium, parasites, TGF-β, latent TGF-β (CM), and/or Abs for 16–18 h. Luciferase activity was assayed with the Promega (Madison, WI) luciferase system. Control wells contained medium alone, untreated CM, HCl-treated CM, or a standard curve of 1–1000 fg/ml TGF-β. Conditions were done in triplicate.

Immunohistochemistry

Bone marrow smears from Brazilians with visceral leishmaniasis or normal Iowan donors (kindly provided by Dr. R. Gingrich (University of Iowa)) were fixed and processed for immunohistochemistry as described (14). Slides were stained with rabbit anti-TGF-β (R&D Systems, Minneapolis, MN) and biotinylated anti-rabbit IgG. They were developed with streptavidin-alkaline phosphatase plus alkaline phosphatase substrate and levamisole (Vector Laboratories, Burlingame, CA) and counterstained with nuclear fast red as described.

Macrophage infections

BMMs were maintained in macrophage SFM (MSFM) with 2% non-heat-inactivated BCS (HyClone, Logan, UT), which contains minimal TGF-β. For microscopic assays, 5×10^5 BMMs/ml in MSFM (Invitrogen, Carlsbad, CA) were infected synchronously (1200 rpm, 2 min, 4°C) with promastigotes at a multiplicity of infection of five promastigotes to one macrophage. TGF-β or 12 μg/ml neutralizing anti-TGF-β Ab (R&D Systems) were added to some conditions. After 4 h, parasites were rinsed off and medium plus TGF-β was replaced. Slides were stained with Diff-Quik (Dade Behring, Newark, DE). Parasites associated with 500 macrophages/condition were counted.

TGF-β was measured in supernatants from 2×10^6 monocyte-derived macrophages (MDMs)/ml in MSFM with 2% BCS. Cells were incubated with *L. chagasi* or the positive controls mannose-lipoarabinomannan (ManLAM) from *Mycobacterium tuberculosis* (provided by L. Schlesinger (University of Iowa)) or LPS (Sigma-Aldrich), each at 10 μg/ml. RNA was extracted using RNA-STAT (Tel-Test, Friendswood, TX). RNase protection assays were performed using kits from BD PharMingen (San Diego, CA) and quantified by densitometry.

Spin trapping

MDMs (1×10^6) in 1 ml of HBSS were infected for 30 min at 37°C and 5% CO_2 with opsonized promastigotes plus 100 μM diethylenetriaminepentaacetic acid to chelate iron (Sigma-Aldrich) and the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, 100 mM; Sigma-Aldrich). Electron paramagnetic resonance spectra of supernatants were obtained at room temperature using a Bruker ESP 300 spectrometer as described (14). Superoxide dismutase or catalase (Sigma-Aldrich) was added to some macrophage cultures to differentiate between DMPO-OH generated from O_2^- or hydroxyl radical, respectively.

Dichlorodihydrofluorescein diacetate (DCF) assay

MDMs were preloaded with 25 μM DCF in HBSS (26°C and 45 min; Molecular Probes, Eugene, OR). Opsonized *L. chagasi* promastigotes (5:1), 40 μg/ml opsonized zymosan, or buffer was added to triplicate wells containing 1×10^6 MDMs in 200 μl. Fluorescent emissions at 37°C were detected every 5 min at 485 nm excitation and 538 nm emission on a BMG FLUOstar 403 microplate spectrofluorometer (BMG Lab Technologies, Durham, NC).

Azocasein assay

Stationary-phase promastigotes (1×10^7) in 100 μl were incubated for 2 h at 37°C in 20 mg of azocasein (Sigma-Aldrich) in HBSS as described (19). Released dye (366 nm) reflects protease activity. Control wells contained azocasein without protease or 3 mg/ml trypsin. Conditions were tested in triplicate.

Immunoblots

Promastigote lysates or fractions were separated on 9% polyacrylamide gels, transferred to nitrocellulose, and incubated with 1:2000 polyclonal antiserum to *L. major* cathepsin B (kindly provided by Dr. J. Sakanari (Sonoma State University, Rohnert Park, CA)) (20) or 1:1000 mAb to β-tubulin (E7; Developmental Studies Hybridoma Bank, University of Iowa). Secondary Abs were anti-rabbit IgG-HRP (Calbiochem) or anti-mouse IgG-HRP (Bio-Rad, Hercules, CA), each diluted 1:20,000 (16). Autoradiograms were developed by ECL (Amersham, Piscataway, NJ).

Statistical analyses were done using Sigma Stat 2.03 software (SPSS, Chicago, IL).

Results

TGF-β in human visceral leishmaniasis

We previously demonstrated an increase in the total amount of TGF-β in cultured liver or spleen cells from mice infected with *L. chagasi*, and others have shown that TGF-β is increased during infection of mice or hamsters with *L. donovani* (2, 3, 5, 6, 9, 10). Using immunohistochemistry, we also found a dramatic increase in TGF-β in human bone marrow aspirates from Brazilians with acute visceral leishmaniasis. Fig. 1 shows representative stains of bone marrow smears from one of five visceral leishmaniasis patients, and one of five uninfected controls either from Brazil (one person with hepatocarcinoma) or Iowa (four healthy bone marrow donors). We were unable to obtain bone marrow samples from healthy Brazilians. TGF-β staining was observed both intracellularly and extracellularly, consistent with the fact that latent TGF-β is stored in extracellular matrix (11).

Using sensitive bioassays, we investigated whether *L. chagasi* infection induces human macrophages to produce TGF-β in vitro (Fig. 2). Human MDMs were infected with stationary-phase promastigotes, LPS, or the *M. tuberculosis* surface glycolipid manLAM for 4 h. Supernatants were collected 48 h later. Active TGF-β was detected with the MvLu-PAI luciferase bioassay (18). There was no apparent toxicity of these agents for MvLu cells

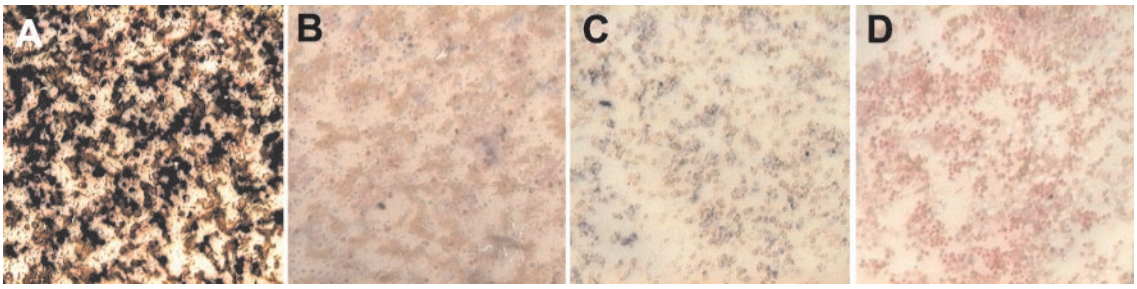

FIGURE 1. Immunohistochemical detection of TGF-β in human samples. Bone marrow aspirates were obtained from five Brazilians with visceral leishmaniasis and five uninfected controls. Representative TGF-β stains are shown from a patient with visceral leishmaniasis (*A* and *B*) or a healthy Iowan (*C* and *D*). Abs were polyclonal rabbit anti-TGF-β followed by anti-rabbit IgG (*A* and *C*) or anti-rabbit IgG alone (*B* and *D*). Slides were counterstained with nuclear fast red to stain nuclei. TGF-β appears as a heavy purple stain.

according to trypan blue. Ten micrograms per milliliter LPS or man-LAM have each been shown to stimulate release of activated TGF-β from murine peritoneal macrophages or human monocytes, respectively (21, 22).

There was a significant increase in active TGF-β in the supernatants of human macrophages infected with promastigotes, although total TGF-β did not change (Fig. 2). In contrast, both activated TGF-β and TGF-β mRNA increased after incubation of human monocytes with man-LAM or LPS, similar to published reports (22). Four replicate RNase protection assays showed there was not a significant change in TGF-β1 mRNA in MDMs infected with *L. chagasi* (data not shown), suggesting the mechanism through which MDMs up-regulate TGF-β during leishmania infection may differ from that activated by bacterial LPS and mycobacterial man-LAM.

Activation of latent TGF-β by promastigotes

An increase in activated TGF-β in the face of constant total TGF-β observed in MDM cultures deserves explanation. Therefore, using the sensitive NRK cell colony assay, we examined the hypothesis that promastigotes directly activate latent TGF-β in vitro. As a source of latent TGF-β, we used CM from RM-1 or MDCK cell lines, which release latent TGF-β into extracellular medium. Each assay included negative control wells containing NRK cells plus EGF, and positive controls with activated TGF-β added. In the presence of EGF, the number of NRK colonies is a direct measure of the concentration of active TGF-β (17). Variations in absolute colony numbers between experiments are due to the fact that longer incubations allow more colonies to grow to significant size.

CM had some baseline TGF-β activity (Fig. 3). However, preincubation of CM with *L. chagasi* promastigotes caused a significant increase in active TGF-β compared with CM alone ($p < 0.001$, $n = 7$ independent assays). Addition of neutralizing anti-TGF-β during the 30-min parasite-CM preincubation decreased the colony formation to background levels (Fig. 3*B*; $p = 0.008$, paired *t* test). Furthermore, supernatants from promastigote cultures grown in SFM activated latent TGF-β (Fig. 3*A*), whereas SFM alone did not (not shown). Latent TGF-β in CM was also activated by HCl treatment. Promastigotes without EGF did not stimulate colony growth (0 ± 0 vs 87 ± 1 colonies for promastigotes vs promastigotes plus EGF, respectively ($n = 3$)).

Preincubation of parasites with CM at different temperatures before addition to NRK cells revealed that TGF-β activation was temperature dependent (Fig. 3*C*), suggesting that activation occurred during the preincubation and not during the 3- to 5-day NRK assay. Taken together, these data indicate that incubation of latent TGF-β with either promastigotes or secreted products of promastigotes causes the release of activated TGF-β.

To verify these observations with an alternate bioassay, TGF-β was quantified by the MvLu-PAI luciferase assay (18). As in the NRK soft agar assay, incubation of CM with promastigotes significantly increased the active TGF-β from 0 ± 22 pg/ml in CM alone to 7306 ± 783 pg/ml in promastigote-exposed CM (mean $\pm$ SE; $p = 0.01$, paired *t* test). Active TGF-β was reduced to 1679 ± 507 pg/ml TGF-β by addition of neutralizing Ab ($p < 0.05$, paired *t* test; $n = 3$ experiments). Microscopic examination revealed that the promastigotes did not infect MvLu fibroblasts.

Mechanism of promastigote-mediated TGF-β activation

Based upon the hypothesis that *Leishmania* proteases are responsible for activating TGF-β, we tested a variety of protease inhibitors for a lack of toxicity for *L. chagasi* promastigotes, NRK cells, and MvLu cells. Among those that were not toxic for eukaryotic cells, 10 μM leupeptin, an inhibitor of cysteine and serine proteases, inhibited both the parasite-mediated and the parasite supernatant-mediated activation of latent TGF-β (Fig. 3, *A* and *B*). Cystatin, an inhibitor of cysteine proteases (23), also prevented parasite-induced activation of TGF-β. In contrast, bestatin, which inhibits the major promastigote surface protease GP63 or MSP (Refs. 24 and 25, and K. Leidal and M. Wilson, unpublished data), did not prevent parasite-mediated TGF-β activation. A dose-response study indicated that promastigote-mediated TGF-β activation was significantly inhibited by 0.4 nM cystatin (Fig. 3*C*).

The above inhibitor data suggested a cysteine protease is partially responsible for the promastigote-induced release of active TGF-β from the latent complex. CA074 specifically inhibits the activity of purified *L. major* or *L. chagasi* cathepsin B enzymes but

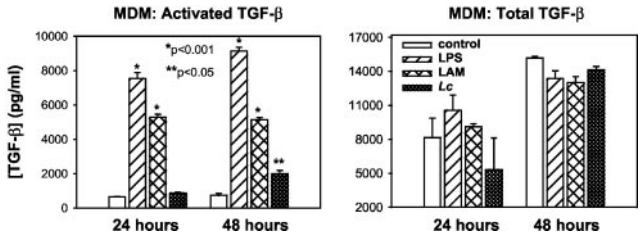

FIGURE 2. Macrophage TGF-β production during infection of human macrophages with *L. chagasi*. Supernatants were collected from human MDMs 24 or 48 h after infection with *L. chagasi* promastigotes. The MvLu-PAI luciferase assay was used to quantify activated TGF-β. Results were quantified by comparison to a standard curve made with rTGF-β. LPS and man-LAM were positive controls that have been shown to cause TGF-β release (21, 22). Data are the means of three macrophage infection experiments, each with triplicate conditions. Statistical analyses, utilizing a paired *t* test, compare TGF-β in treated vs control supernatants.

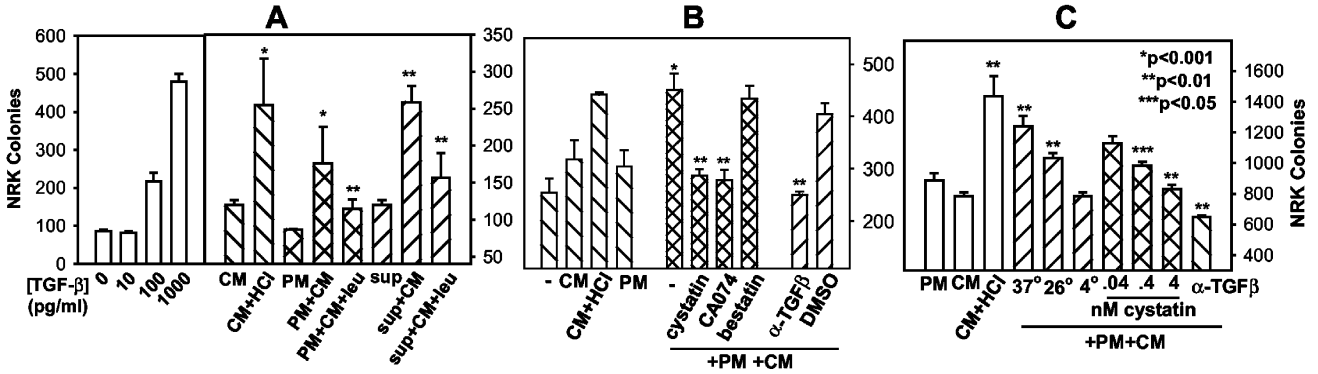

FIGURE 3. Activation of TGF-β by promastigotes. *A*, NRK colony counts are shown after incubation in medium alone or with increasing amounts of rTGF-β, latent TGF-β provided by CM, acid-activated CM (CM+HCl), or *L. chagasi* promastigotes (PM). Conditions tested included CM incubated for 30 min with promastigotes, supernatants from promastigotes grown for 16 h in SFM (sup), or conditions with leupeptin (leu). *B*, NRK cells were grown in the presence of CM activated by *L. chagasi* promastigotes (+PM+CM) without or with protease inhibitors. Cystatin inhibits cysteine proteases, CA074 inhibits cathepsin B, and bestatin inhibits GP63 activity (60). Control wells included neutralizing Ab to TGF-β or the CA074 solvent DMSO. *C*, NRK cells were grown with CM that was preincubated with PM either at different temperatures or with different concentrations of the cysteine protease inhibitor cystatin. All panels contain results from three assays. All conditions shown contained EGF. Statistical analyses (paired *t* test) compare CM incubated without vs with promastigotes, or CM plus promastigotes alone versus with protease inhibitors or Ab.

not *L. major* cathepsin L or other cysteine proteases (20, 26–28). According to the NRK soft agar assay, there was a significant decrease in TGF-β activation when promastigotes were incubated with CM in the presence of CA074 ($p \leq 0.001$, paired *t* test) but not with the CA074 solvent DMSO (Fig. 3*B*). Neither leupeptin, cystatin, bestatin, nor CA074 inhibited the growth of NRK colonies stimulated by TGF-β in acid-activated CM (data not shown). The efficacy of protease inhibitors was verified by their inhibition of the promastigote-induced lysis of azocasein over 2 h (Table I).

Latent TGF-β activation by Leishmania *sp. but not* C. fasciculata

All species of *Leishmania* tested caused the release of active TGF-β from the latent complex (Fig. 4*A*). In contrast, *C. fasciculata*, a kinetoplastid in the same family as *Leishmania*, did not activate TGF-β (Fig. 4*B*). *L. chagasi* promastigotes and *C. fasciculata* were lysed and organelles separated into soluble (S) and microsomal fractions (M; Fig. 4*C*). The TGF-β releasing capacity localized in total lysates (L) and in soluble but not in microsomal fractions of *L. chagasi*. In contrast, neither the total lysate nor subcellular fractions of *C. fasciculata* activated latent TGF-β. Thus, the ability to activate latent TGF-β is neither a property of all kinetoplastids nor of all fractionated cells. Immunoblots incubated with Ab to *L. major* cathepsin B showed that cathepsin B was present in the total promastigote lysate and the soluble fraction, consistent with the protease inhibitor data (Fig. 4*D*) (20). The mechanism through which this enzyme has access to extracellular latent TGF-β is not clear, although the majority of protease activity released by *L. chagasi* promastigotes into extracellular medium was inhibited by cysteine protease inhibitors (data not shown).

TGF-β prolongs survival of leishmania in murine macrophages

We previously showed that genetically susceptible BALB/c mice have high levels, and genetically resistant C3H/HeJ mice have low levels, of TGF-β during *L. chagasi* infection (5). To discern the effect of TGF-β on infected macrophages, we preincubated C3H/HeJ BMMs overnight with 0.1–5000 pg/ml TGF-β before infection with promastigotes (Fig. 5). The use of macrophages from a mouse model in which parasites are killed allowed us to determine whether TGF-β antagonizes microbicidal activities. The survival of intracellular leishmania was significantly and repeatedly prolonged in macrophages preincubated with a physiologically achievable concentration of TGF-β (100 pg/ml) but not with higher concentrations. Neutralizing Ab to TGF-β decreased parasite survival back to baseline levels (2.48-fold decrease at 24 h). The effects of the cytokine would have been missed or misinterpreted if only one TGF-β concentration had been tested. These data are consistent with the fact that many activities of TGF-β are observed only at low to intermediate, but not at high concentrations (29–31). They also suggest that, although TGF-β reverses some microbicidal function, it does not globally arrest all macrophage microbicidal activities. For instance, TGF-β did not augment the level of macrophage phagocytosis (Fig. 5; 0 time point). We therefore investigated whether TGF-β influences macrophage oxidative responses.

Effects of TGF-β on oxidative responses

It has been reported that generation of H_2O_2 by inflammation-elicited mouse peritoneal macrophages in response to PMA is suppressed by 1 ng/ml TGF-β. However, superoxide generation was

Table I. *Azocasein assay of protease activity in the presence of protease inhibitors*

Source of Protease	No Inhibitor	Cystatin	CA074	Bestatin	Cystatin + Bestatin	Cystatin + CA074
Trypsin	2.45 ± 0.5[a]					
L. chagasi promastigotes	0.138 ± 0.01	0.0435 ± 0.015† (68%)[a]	0.0350 ± 0.004* (75%)	0.0452 ± 0.015† (89%)	0.0250 ± 0.002* (82%)	0.0267 ± 0.002* (81%)

[a] Mean ± SE absorbance units at 366 nm; $n = 3$ experiments (percent inhibition compared to that of *L. chagasi* promastigotes without inhibitors).
*, $p < 0.001$ compared to *L. chagasi* without protease inhibitor.
†, $p < 0.005$ compared to *L. chagasi* without protease inhibitor.

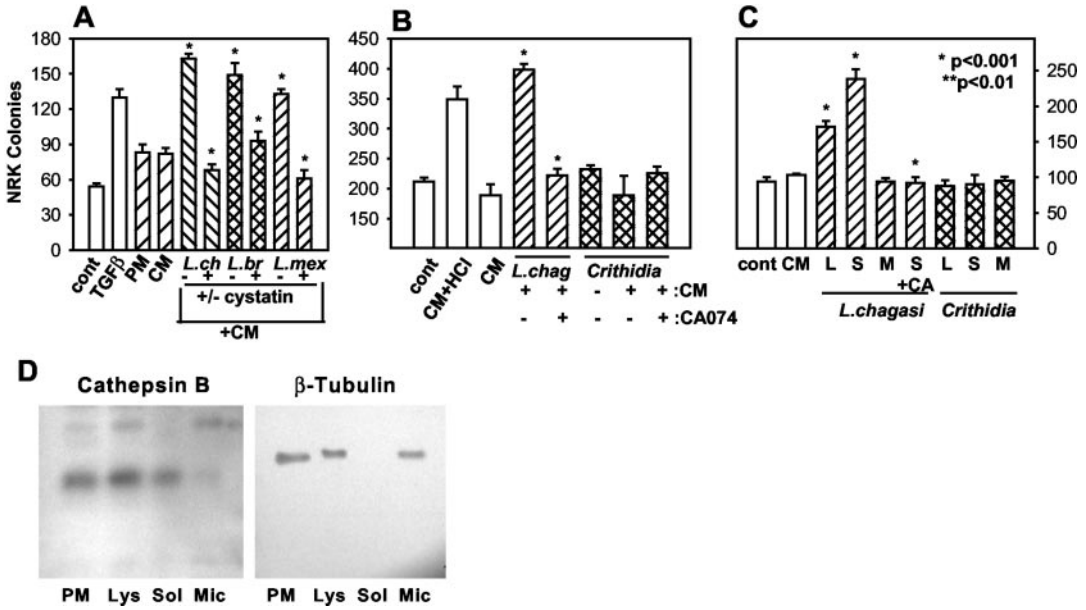

FIGURE 4. Activation of TGF-β by *Leishmania* sp. *A*, NRK cells were grown in the presence of TGF-β (1000 pg/ml), untreated CM as a source of latent TGF-β, *L. chagasi* promastigotes (PM), or CM preincubated with promastigotes of *L. chagasi*, *L. braziliensis*, or *L. mexicana* in the absence or presence of cystatin. *B*, NRK colonies were formed in the presence of EGF alone (cont), untreated or HCl-activated CM, or CM preincubated with *L. chagasi* promastigotes (*L. chag*) or *C. fasciculata*, with or without the cathepsin B inhibitor CA074. *C*, NRK cells were grown in EGF alone (cont), or CM preincubated with buffer alone (CM), or with total lysates (L) or the soluble (S) or microsomal (M) fractions of *L. chagasi* or *C. fasciculata*. +CA, CA074 was added to the condition. Results are representative of three separate assays, each with conditions in triplicate. Statistical analyses (paired *t* test) compare CM vs CM+PM, or CM+PM alone vs CM+PM with protease inhibitors or Ab. All comparisons that are not marked were nonsignificant. *D*, Immunoblots of total promastigote cells, promastigote lysate, or the soluble and microsomal fractions of *L. chagasi* lysates were incubated with an Ab to *L. major* cathepsin B or to β-tubulin. Note that β-tubulin is found in the microsomal but not the soluble fraction of leishmania.

not affected, suggesting that TGF-β does not affect the NADPH oxidase activity in mouse cells (32). Another report indicated that cocaine suppresses the superoxide produced by PMA-stimulated freshly isolated human PBMC, and this suppression is mediated through TGF-β (33). As there has been a paucity of studies showing the effect of TGF-β on phagocytosis-induced macrophage microbicidal activity, we studied the effects of TGF-β on the respiratory burst of human macrophages in response to particle ingestion. MDMs were treated overnight with TGF-β, after which they were allowed to phagocytose promastigotes (Fig. 6*A*) or opsonized zymosan (not shown) in the presence of the spin trap DMPO. Similar to our prior reports, a 1:2:2:1 electron paramagnetic resonance spectrum was observed, reflecting O_2^- reacting with DMPO to generate DMPO-OH (Fig. 6*A*). DMPO-OH formation was prevented by membrane-impermeable superoxide dismutase but unaffected by catalase (not shown), indicating it was derived from superoxide likely formed at the phagocytic cup rather than from hydroxyl radical (14, 16). TGF-β did not suppress superoxide generation during phagocytosis of leishmania or opsonized zymosan at any concentration, and at 300 pg/ml TGF-β potentially increased superoxide (Fig. 6*A*). In replicate assays, the mean peak amplitudes generated during leishmania phagocytosis were 1.90 ± 0.48 in untreated cells compared with 3.20 ± 0.77 in cells exposed to 300 pg/ml TGF-β (mean $\pm$ SD; $n = 3$, $p < 0.04$, paired *t* test). Negative controls (DMPO alone, macrophages plus DMPO, parasites plus DMPO) generated negligible amounts of DMPO-OH (not shown).

As an alternate measure of NADPH oxidase products, we infected 2-day human macrophages in the presence of DCF, a membrane-permeant probe that is deacetylated intracellularly. The fluorescence of DCF increases upon oxidation by hydrogen peroxide in the presence of cellular peroxidases, and provides a quantitative measure of the phagocyte respiratory burst (34). These data showed there was no significant change in H_2O_2 generated in the presence of TGF-β (Fig. 6*B*).

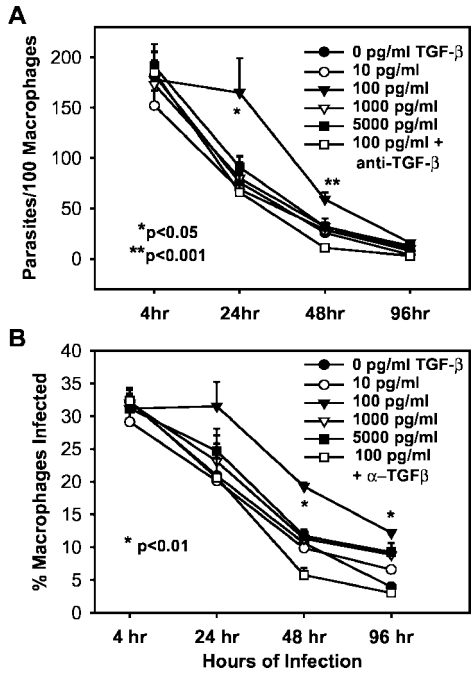

FIGURE 5. Effect of TGF-β on survival of *L. chagasi* in macrophages. BMMs from C3H/HeJ mice were preincubated overnight with TGF-β and infected with *L. chagasi* promastigotes. The ratio of intracellular parasites to macrophages was enumerated between 4 and 96 h later. Shown are the mean $\pm$ SE of three experiments, each with duplicate conditions. Statistical analyses utilized the paired *t* test.

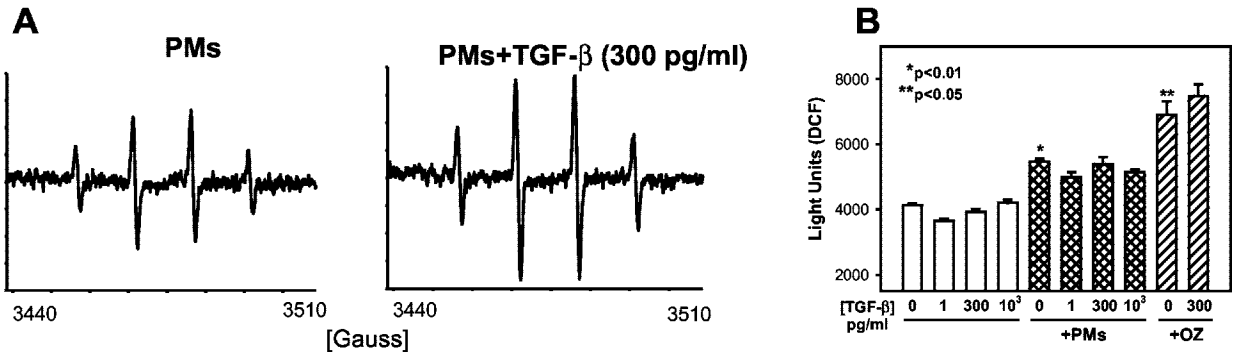

FIGURE 6. Effect of TGF-β on macrophage generation of reactive oxygen intermediates. *A*, MDMs were treated overnight with 0, 0.1, 1, 10, 300, or 1000 pg/ml TGF-β, and then challenged with promastigotes in the presence of the spin trap DMPO. Superoxide was detected using electron paramagnetic resonance. Shown is a DMPO-OH spectrum from controls and from the TGF-β concentration that elicited the most change (300 pg/ml). Gauss is the measurement of magnetic flux. Three replicate assays showed similar changes. *B*, Hydrogen peroxide was detected using DCF. Human monocytes were treated overnight with 0–1000 pg/ml TGF-β, loaded with DCF, and challenged with promastigotes (PMs), opsonized zymosan (OZ), or buffer. Fluorescence at 485 nm excitation and 538 nm emission was monitored every 5 min. Data shown are the mean ± SE of fluorescence emissions over the first 30 min in three assays, each with triplicate conditions. The paired *t* test was used for statistical analyses of infected vs uninfected wells. There was no significant difference between light units generated without vs with TGF-β during PM or OZ infection.

Discussion

TGF-β has potent immunosuppressive properties that can affect the outcome of infectious and autoimmune diseases (35). TGF-β enhances the progression, or prevents the cure of leishmaniasis in murine models (2, 3, 5, 7–10, 36). We investigated TGF-β in the local environment surrounding the leishmania-infected macrophage and the live *L. chagasi* promastigote. High levels of total TGF-β were previously reported in tissues and cultured immune cells from mice and hamsters infected with *L. donovani* or *L. chagasi* (2, 3, 5, 6, 9, 10). Extending these observations to human disease, we found greater amounts of total TGF-β in bone marrow aspirates from Brazilians with visceral leishmaniasis than in controls. *L. chagasi* infection of human macrophages in vitro did not result in an increase in the amount of total (latent plus active) TGF-β protein or in TGF-β1 mRNA. However, there was an increase in biologically active TGF-β in infected human macrophage cultures. This suggested there may be local activation of TGF-β in these cultures. Therefore, we investigated whether TGF-β was activated by the parasite or by parasite-derived factors. Our results showed that live promastigotes of several *Leishmania* sp. and promastigote culture supernatants were able to directly activate latent TGF-β. Activation occurred at least in part through the cysteine family protease, cathepsin B. The combined effect of abundant TGF-β stores at extracellular sites, and the ability of the parasite to activate TGF-β in its local environment, would lead to high levels of active TGF-β in the vicinity of the infected macrophage.

Using a model in which *L. chagasi* are killed intracellularly, TGF-β was able to prolong intracellular parasite survival. This occurred at low (physiologic) but not at high TGF-β concentrations. Many other activities of TGF-β also exhibit biphasic kinetics, a fact that may account for the ability of TGF-β to both promote and inhibit inflammatory responses (37, 38). For instance, low-dose TGF-β1 is a chemoattractant for monocytes, T cells, and neutrophils (39–42), whereas higher doses suppress innate and adaptive immune responses (29, 31). TGF-β alters the innate macrophage phenotype by inhibiting TNF-α, IFN-γ, and inducible NO synthase (iNOS), and enhancing arginase expression (29, 43–45). This diversion of arginine from iNOS to arginase leads to decreased $NO^{\cdot}$ and enhanced polyamines, which could in turn enhance parasite growth (12, 45, 46).

The fact that TGF-β delayed but did not abrogate leishmania killing by murine macrophages suggested that some microbicidal events remained intact despite TGF-β exposure. Recent reviews state that TGF-β suppresses phagocyte oxidative responses (13). However, the primary data show suppression in limited situations. Prior incubation of murine macrophages with TGF-β1 suppresses the PMA-induced release of H_2O_2 by caseinate-induced peritoneal macrophages, but this does not occur through suppressing the NADPH oxidase because the report specifically shows that superoxide production is not affected (32, 33). Although a mechanism is not shown, possibilities would include enhanced expression of a cellular peroxidase. In human cells, PMA-induced superoxide production by PBMCs is suppressed by cocaine, and this suppression is mediated by TGF-β (33). PBMCs contain monocytes but not macrophages, and the phenotype of monocytes changes dramatically as they differentiate to macrophages including down-regulation of TGF-β receptors I and II. Thus, even the cocaine effects should not be generalized from one cell type to the other.

As it had not previously been documented, we examined the effects of TGF-β on the respiratory burst elicited during phagocytosis of a microbe (*L. chagasi*) or particle (zymosan) by human macrophages. We discovered that TGF-β1 did not suppress superoxide or H_2O_2 production during phagocytosis of either promastigotes or opsonized zymosan by human macrophages. The mechanism through which TGF-β prolongs parasite survival in macrophages may involve mechanisms distinct from the NADPH oxidase. Whether there is a shift of macrophage arginine metabolism toward arginase and away from iNOS remains to be determined (12).

The abundance of activated TGF-β is controlled at two points: first, intracellularly by the rates of transcription and translation, and second, extracellularly through release of bioactive TGF-β from the latent complex. Rifkin and coworkers (21) previously reported that thioglycollate-elicited mouse peritoneal macrophages activate latent TGF-β when stimulated with LPS, whereas resident peritoneal macrophages do not. This led them to conclude that pre-eliciting or stimulating macrophages is necessary to activate TGF-β. Consistent with this conclusion, another group infected thioglycollate-elicited mouse peritoneal macrophages with *Leishmania mexicana* or *Leishmania braziliensis* and found an increase in activated TGF-β in culture supernatants. Parasite effects on resting macrophages or on total TGF-β were not reported (3, 10, 29), although another report showed an increase in TGF-β1 mRNA upon infection with *L. donovani* (47). In the current study, we

demonstrated that phagocytosis of *L. chagasi* increases the amount of biologically active TGF-β in human macrophage cultures without changing the total amount of TGF-β or TGF-β1 mRNA synthesized. It is possible that, in some studies of macrophage infection, leishmania directly activate the abundant latent TGF-β stores in FCS-containing medium, but we purposely omitted FCS and used TGF-β-low medium in macrophage cultures. Human or murine macrophages could have different inherent abilities to activate TGF-β, just as inflammatory vs resting murine macrophages differ in this capacity. Indeed, TGF-β activation can be influenced by the abundance of several molecules that vary depending on activation state of the macrophage (LTBP production, type II transglutaminase, plasmin, urokinase plasminogen activator binding to the urokinase plasminogen activator receptor, M6P/insulin-like growth factor receptor II) (31).

TGF-β is released from most cells noncovalently bound to LAP, which is the homodimeric TGF-β propeptide. It must be liberated from this complex before it can ligate TGF-βR (11). Latent TGF-β is linked through disulfide bonds to LTBP, which mediates storage in extracellular matrix. Activation is a primary means of TGF-β regulation in vivo. Activated TGF-β is released from LAP by physical and chemical exposures including heat, acid pH, chaotropic agents, plasmin, subtilisin-like peptidases, and thrombospondin (11, 48–51). Human cathepsin B and lysosomal cathepsin D each activate latent TGF-β (48, 50). Influenza virus neuraminidase has been found to activate latent TGF-β (17), and pathologic conditions that cause tissue remodeling or destruction such as invasive tumors, influenza and paramyxovirus infection, and cutaneous leishmaniasis can also lead to activation (3, 10, 11, 17, 37).

Data in the current report support the hypothesis that leishmania cysteine proteases are partly responsible for TGF-β activation. Cathepsins L and B have been cloned from *L. mexicana*, *L. donovani*, *L. major*, and *L. chagasi* (20, 52–54). Studies of null mutants have documented a role for each cathepsin in *L. mexicana* virulence (53, 55–58). Treatment of infected mice with specific inhibitors suggests that cathepsin B of the parasite (or host) promotes disease progression (23, 27, 59). The mechanism through which the cathepsins promote parasite virulence was previously unknown. However, Somanna et al. (28) recently cloned the cathepsin B homolog from *L. chagasi* and showed this is expressed in both the amastigote and promastigote forms of the parasite. They found that both parasite lysates and recombinant *L. chagasi* cathepsin B were able to release activated TGF-β from the latent precursor. Using sensitive bioassays, we found that live promastigotes and products released from promastigotes activate latent TGF-β, and TGF-β can prolong parasite survival in cultured primary macrophages capable of parasite killing. Studies of the mechanism suggest this may not occur through suppression of the NADPH oxidase. Cathepsin B may not be the only mechanism through which *Leishmania* sp. activate TGF-β. Other activating molecules could include secreted acidic compounds, secreted glycoconjugates that compete for TGF-β binding to LAP, deglycosylation, or alternate parasite proteases. Nonetheless, the effects of cystatin and CA074 suggest a role for the cysteine proteases, and cathepsin B in particular, in activating latent TGF-β in the vicinity of the invading promastigote. Given the roles of TGF-β in suppressing innate and adaptive immune responses, it seems likely that local activation of TGF-β provides a survival advantage for the parasite upon initial inoculation into the host environment.

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