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MIF-driven activation of macrophages induces killing of intracellular *Trypanosoma cruzi* dependent on endogenous production of tumor necrosis factor, nitric oxide and reactive oxygen species

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ABSTRACT

The proinflammatory cytokine macrophage migration inhibitory factor (MIF) is a key player in innate immunity. MIF has been considered critical for controlling acute infection by the protozoan *Trypanosoma cruzi*, but the underlying mechanisms are poorly understood. Our study aimed to analyze whether MIF could favor microbicidal activity of the macrophage, a site where *T. cruzi* grows and the initial effector cell against this parasite. Using murine macrophages infected *in vitro*, we examined the effect of MIF on their parasiticidal ability and attempted to identify inflammatory agents involved in MIF-induced protection. Our findings show that MIF is readily secreted from peritoneal macrophages upon *T. cruzi* infection. MIF activates both primary and J774 phagocytes boosting the endogenous production of tumor necrosis factor-alpha via mitogen-activated protein kinase p38 signaling, as well as the release of nitric oxide and reactive oxygen species, leading to enhanced pathogen elimination. MIF can also potentiate the effect of interferon-gamma on *T. cruzi* killing by J774 and mouse peritoneal macrophages, rendering these cells more competent in reducing intracellular parasite burden. The present results unveil a novel innate immune pathway that contributes to host defense and broaden our understanding of the regulation of inflammatory mediators implicated in early parasite containment that is decisive for resistance to *T. cruzi* infection.

1. Introduction

Chagas disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is one of the most important public health problems in Latin America. The overall prevalence of this human infection is estimated at 8 million cases, with 100 million at risk for infection (Dias, 2015). During the acute phase, a high number of parasites circulate in the blood and invade diverse target tissues. Control of *T. cruzi* depends both on innate and acquired immune responses which are triggered during early infection, critical for host survival, and involve macrophages, natural killer cells, B cells, $CD4^+$ and $CD8^+$ T cells, as well as the production of proinflammatory cytokines such as interferon(IFN)-gamma, tumor necrosis factor(TNF)-α, interleukin(IL)-12 and IL-17 (Dutra and Gollob, 2008; Miyazaki et al., 2010; Savino et al., 2007). Nevertheless, there is still much to be learned about the innate immunity pathways responsible for protection against *T. cruzi* (Machado et al., 2012). IL-12 is known to enhance IFN-gamma production from NK cells and type I helper T cells whereas IL-17 is critical for immune cell activation through stimulated expression of proinflammatory cytokines. TNF-α cooperates with IFN-gamma for induction of resistance to *T. cruzi* infection by activating phagocytes to release high levels of reactive nitrogen intermediates, such as nitric oxide (NO), that are toxic to the parasite (Dutra and Gollob, 2008; Saeftel et al., 2001). Also, the production of reactive oxygen species (ROS), including superoxide radical and hydrogen peroxide, by macrophages has been pointed out as a crucial innate defense mechanism against this human pathogen (Guiñazú et al., 2010).

The proinflammatory cytokine macrophage migration inhibitory factor (MIF) participates in fundamental events of innate and adaptive immunity. MIF is a pleiotropic immunoregulator that is secreted by multiple cell types, including T cells,

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activated macrophages, and dendritic cells (Calandra et al., 1994; Matsumoto and Kanmatsuse, 2001; Popa et al., 2006). Upon release, MIF promotes the production of a number of proinflammatory moieties, including cytokines (TNF-α, IFN-gamma, IL-1β, IL-6 and IL-12), free radicals, inducible NO synthase (iNOS) and type 2 cyclooxygenase, and it orchestrates normal leukocyte influx into inflamed tissues (Balachandran et al., 2011; Calandra et al., 1994; Chuang et al., 2012; Cooke et al., 2009; Gregory et al., 2004; Xin et al., 2010). Moreover, MIF is required for protection against several protozoan infections (Rosado and Rodríguez-Sosa, 2011). Particularly, this immune mediator was found to be critical for controlling experimental acute Chagas' disease (Reyes et al., 2006; Terrazas et al., 2011), but in-depth characterization of the underlying effector mechanisms of MIF concurring to early resistance to *T. cruzi* infection is still pending. The aim of this study was therefore to analyze whether MIF is able to influence the trypanocidal activity of the macrophage, a cell that is probably one of the first to fight the infection, regardless serving as initial host for this pathogen (Stafford et al., 2002). Using murine macrophages *in vitro* infected with *T. cruzi,* we examined the effect of MIF on microbicidal effector functions of these phagocytes and also attempted to identify inflammatory mediators involved in MIF-induced intracellular parasite killing.

2. Materials and methods

2.1. Mice and parasites

Six- to eight-week-old female BALB/c mice were obtained from Centro Nacional de Energía Atómica (CNEA, Buenos Aires, Argentina) and maintained under standard conditions. Animals were housed in groups of five per cage and provided with food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and with the approval of the Research Ethics Committee of Hospital de Niños "Dr. Ricardo Gutiérrez" (Buenos Aires, Argentina).

The virulent RA strain of *T. cruzi* (Celentano and González Cappa, 1993) was used throughout these studies. The strain is maintained by serial passages in outbred mice at the University of Buenos Aires. Trypomastigotes were grown in monolayers of the Vero cell line with RPMI-1640 medium (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% vol/vol fetal calf serum, 200 UI/ml penicillin, 200 μg/ml streptomycin and 2×10^{-3} *M* L-glutamine (complete RPMI). The parasites were harvested from culture supernatant, repeatedly washed in RPMI medium (5000 × *g*, 15 min at 4 °C) and used immediately for infection of macrophage cultures.

2.2. MIF release from Trypanosoma cruzi-infected macrophages

Naïve BALB/c mouse macrophages were harvested from the peritoneal cavity three days after the injection of 1 ml of 3% sodium thioglycolate (Sigma-Aldrich, St. Louis, MO). The adherent cells were obtained after a 4-h incubation of single-cell suspensions (2×10^6 cells/ml) in 24-well plates with complete RPMI medium at 37 °C. The nonadherent cells were removed by exhaustive washing with Hank's solution. Culture RA trypomastigotes were added in several parasite-to-cell ratios (0:1, 1:1, 5:1 or 10:1) and incubated for 48 h at 37 °C. The supernatants were then harvested and used to determine MIF level by sandwich ELISA (Kamiya Biomedical, Seattle, WA) according to the manufacturer's specifications. The supplied standard was used to generate the standard curve. The assay sensitivity was 0.16 ng/ml.

2.3. Stimulation of macrophages, infection, and assessment of intracellular parasites

The J774 macrophage cell line was maintained by weekly passages in complete RPMI medium. The cells (10^5/well) were seeded in 8-well tissue culture plates (Lab-Tek™, Nunc, Thermo Fisher Scientific) and the adherence was allowed for 24 h. Macrophage activation was performed following a reported protocol with minor modifications (Jüttner et al., 1998). Initially, macrophages were incubated in complete RPMI medium or in medium containing cytokine(s) for variable stimulation regimens: recombinant interferon-gamma (rIFN-gamma, Pierce, Thermo Fisher Scientific) at 100 U/ml for 1 h and/or recombinant MIF (rMIF, R&D Systems, Minneapolis, MN) at 100, 500 or 1000 ng/ml for 2 h or 72 h. The adherent cells were then infected for 2 h with culture trypomastigote forms of *T. cruzi* RA at a 10:1 parasite-to-cell ratio. Thereafter, nonphagocytosed parasites were washed off, and the cultures were further maintained in complete RPMI for 48 h. Unstimulated cells infected with trypomastigotes served as control. Macrophages were washed with Hank's solution and fixed in formalin. After staining with Giemsa for 30 min, the number of intracellular parasites was determined by microscopic examination of stained cells. For each cytokine treatment, the percentage of infected cells as well as the absolute number of parasites per 100 macrophages were determined. Additionally, trypanocidal indexes were calculated as previously reported (Lima et al., 1997). The percent decrease of *T. cruzi* infection in macrophages was determined by the following formula: 100–(number of infected macrophages in the presence of MIF x 100/number of infected macrophages in the absence of MIF). Results were expressed as % uninfected cells. The percent decrease of *T. cruzi* uptake by macrophages was determined by the following formula: 100–(number of parasites per 100 macrophages in the presence of MIF x 100/number of parasites per 100 macrophages in the absence of MIF). Results were expressed as % parasite killing.

2.4. Study of macrophage trypanocidal activity

J774 macrophages were infected with *T. cruzi* trypomastigotes in culture medium containing 0 or 1000 ng/ml rMIF. In a set of experiments with parasite-infected and MIF-stimulated cells, cultures were incubated in the presence of blocking antibodies to MIF (1 μg/ml) (Calandra et al., 1998) or TNF-α (20 μg/ml, Pharmingen, BD Biosciences, San Jose, CA), or pretreated for 30 min with selective inhibitors of iNOS (1×10^{-3} *M* aminoguanidine –AG-, Sigma-Aldrich) or NADPH-dependent ROS generation (3×10^{-4} *M* apocynin –APO-, Santa Cruz Biotechnology, Santa Cruz, CA). The growth of intracellular parasites in macrophages was evaluated by counting the absolute number of amastigotes per 100 cells at 72 h postinfection.

2.5. TNF-α measurement

J774 and peritoneal macrophages were infected for 2 h or 72 h with *T. cruzi* trypomastigotes in the presence or in the absence of 1000 ng/ml rMIF. For neutralization of cytokine activity, polyclonal antibody for MIF (1 μg/ml), or isotype-matched control immunoglobulin, was included in some experiments. TNF-α production was quantified in uninfected and parasite-infected cell supernatants collected at each time point using a sandwich ELISA (OptEIA™ Mouse TNF, Pharmingen, BD Biosciences) according to the manufacturer's instructions. The supplied standard was used to generate the standard curve. The assay sensitivity was 15 pg/ml.

2.6. Protein extraction and western blotting for total and phosphorylated MAPK

Macrophages were *T. cruzi*-infected for 24 h and then incubated in the presence of rMIF at 1000 ng/ml. At the indicated times poststimulation (0–120 min), the cells were washed and lysed in a modified RIPA buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1×10^{-3} *M* EDTA, and a protease/phosphatase inhibitor cocktail (Calbiochem, EMD Millipore, Danvers, MA). After centrifugation at 15,000 x *g* for 20 min, the supernatants of cell lysates containing 20 μg protein each were subjected to 10% SDS-PAGE and transferred onto Immobilon P membranes (EMD Millipore). The filters were probed with MAPK antibodies specific for total p38 and phosphorylated p38 (both at 1:1000 dilution; Santa Cruz Biotechnology), or total ERK1/2 (1:4000; Santa Cruz Biotechnology) and phosphorylated ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA). A HRP-conjugated appropriate secondary antibody was used to detect immunoreactive bands using an ECL system (GE Healthcare, Pittsburgh, PA).

2.7. Effects of p38 and ERK1/2 MAPK inhibitors on inflammatory response

SB203580 p38 inhibitor (Promega, Madison, WI) and U0126 ERK1/2 inhibitor (Promega) were added at 10^{-5} *M* concentration to J774 phagocytes 30 min prior to stimulation. Effects of p38 and ERK1/2 MAPK inhibitors on TNF-α production by macrophages infected with *T. cruzi* in the presence of MIF at 1000 ng/ml were evaluated by measuring the concentration of the cachexin in cell supernatants collected upon 72 h of incubation. DMSO-treated cells were used as a control.

2.8. Measurement of nitrite secretion

To determine the amount of NO released into the culture medium of cytokine-stimulated and/or parasite-infected J774 macrophages, nitrate was reduced to nitrite and this product was measured by a microplate assay method with Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) as described previously (Cardoni and Antúnez, 2004). Optical density at 540 nm was compared with a standard curve of $NaNO_2$. The assay sensitivity was $<1 \times 10^{-6}$ *M*. Supernatants from unstimulated, uninfected and MIF antibody-treated cells were also studied.

2.9. Quantification of intracellular ROS levels

ROS generation was measured by the DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate, Sigma-Aldrich) fluorescence method (Chuang et al., 2012). Briefly, J774 macrophages (10^6) were washed, suspended in 1 ml of phosphate buffered saline (PBS) and incubated with 10^{-5} *M* DCFH-DA for 30 min at 37 °C. The cells were then stimulated or not with rMIF (1000 ng/ml) or rIFN-gamma (100 U/ml) and *T. cruzi*-infected for 2 h or 72 h. Uninfected specimens and MIF antibody-treated cells were included as controls. Macrophages were then formalin-fixed for 15 min, harvested, repeatedly washed and analyzed by flow cytometry at a wavelength of 488 nm. Samples were acquired on a Sysmex Partec (Görlitz, Germany) PAS-III flow cytometer and data were analyzed using Cyflogic 1.2.1 software. Results are expressed as percentage of DCFH positive cells.

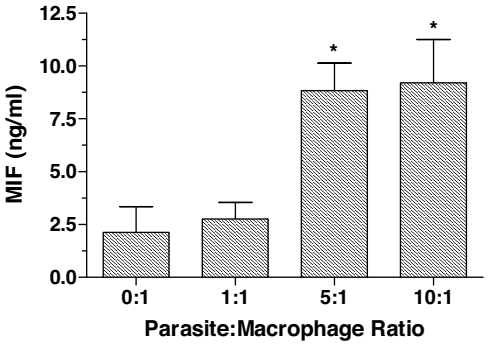


Fig. 1. MIF release from *T. cruzi*-infected macrophages. BALB/c mouse peritoneal macrophages were infected *in vitro* with RA strain trypomastigotes at different parasite-to-cell ratios (0:1, 1:1, 5:1 or 10:1). After 48 h, MIF levels in culture supernatants were determined by ELISA. The results represent means ± s.e.m. of three individual experiments assayed in triplicate. *$P < 0.05$ compared with uninfected macrophages.

2.10. Statistical analysis

Data analysis was carried out using GraphPad Prism 5.0 software. Arithmetic means and SEM were calculated. Significant differences among treatments were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's test or linear trend analysis. *P* values of 0.05 or less were considered to be significant.

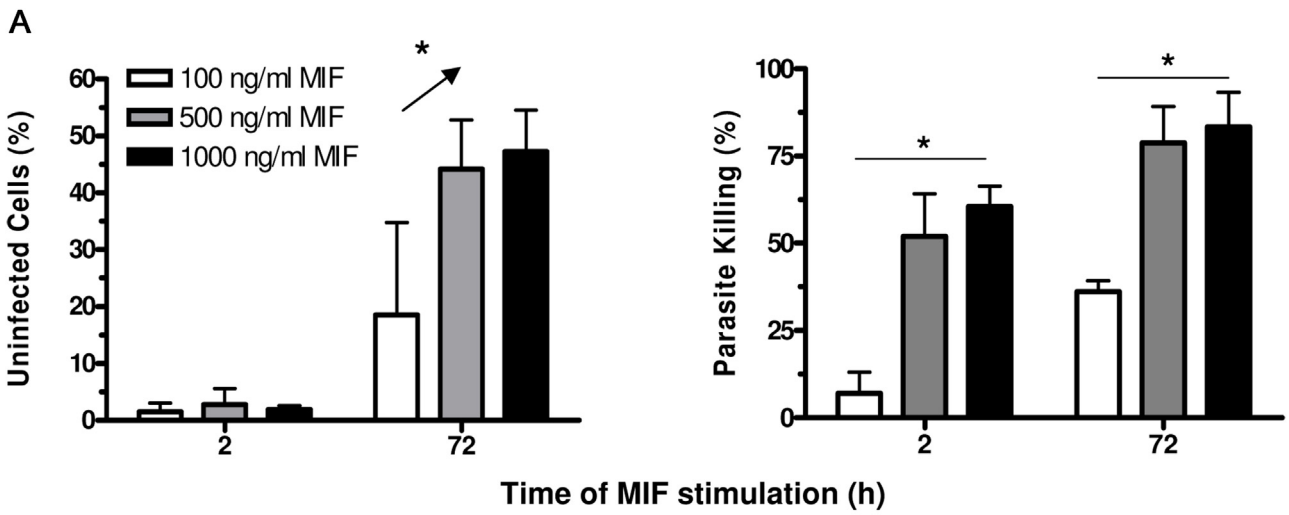
3. Results

3.1. Trypanosoma cruzi promotes MIF release by mouse peritoneal macrophages

Although a variety of cell types express MIF, macrophages are a major source (Calandra et al., 1994). As phagocytes are among the first cells to be parasitized by *T. cruzi*, we therefore investigated whether this protozoan could promote MIF production by mouse peritoneal macrophages. Live trypomastigotes induced MIF release by adherent cells depending on the parasite-to-cell ratio used for *in vitro* infection (Fig. 1). At a 1:1 ratio, MIF concentration in the 48-h supernatant from infected macrophages was low, akin to that detected in uninfected cultures. However, at ratios of 5:1 or 10:1, *T. cruzi* infection triggered increasing MIF secretion by the phagocytes at a significantly higher level than that recorded for controls (8.8 ± 1.1 and 9.2 ± 1.9 *vs* 2.1 ± 1.2 ng/ml, respectively; $P < 0.05$). These data provide evidence that *T. cruzi* is capable of inducing MIF liberation from murine macrophages.

3.2. Exogenously added rMIF activates J774 macrophages to kill Trypanosoma cruzi

J774 macrophages were incubated for 2 h with rMIF at different concentrations ranging from 0 to 1000 ng/ml and then infected with *T. cruzi* trypomastigotes. Two hours later, comparable low rates of noninfected cells in the cytokine-treated and control series were found. However, a positive trend ($P < 0.05$) in the percentage of parasite-free cells in response to increasing amounts of rMIF added could be observed upon a 72-h incubation period before infection (18.5 ± 16.3, 44.2 ± 8.7 and 47.3 ± 7.2% at 100, 500 and 1000 ng/ml, respectively; Fig. 2A). Also, in a dose-dependent fashion, macrophages stimulated with rMIF, for either 2 or 72 h, contained significantly less ($P < 0.05$) intracellular parasites than untreated infected controls (4.1 ± 3.1, 27.9 ± 6.4 and 32.6 ± 3.3% at 100, 500 and 1000 ng/ml, respectively, after 2 h; 19.4 ± 1.6, 42.4 ± 5.5 and 44.9 ± 5.6% killing of *T. cruzi* amastigotes at 100, 500 and 1000 ng/ml, respectively, after 72 h; Fig. 2B and C). The analysis



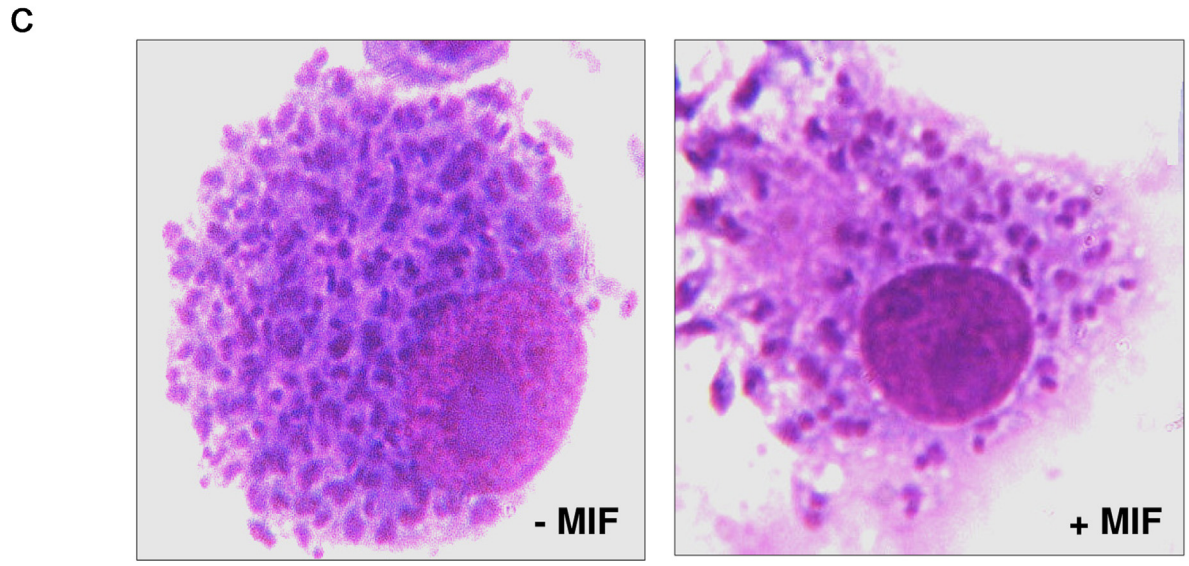


Fig. 2. Effect of MIF stimulation of macrophages on subsequent cell infection with *T. cruzi*. Murine J774 macrophages were primed with recombinant MIF at 100, 500 or 1000 ng/ml for 2 or 72 h and then infected with RA strain trypomastigotes (10 parasites/cell). The percentages of uninfected cells (A) and intracellular parasite killing (B) were estimated upon 48 h of infection. The results represent means ± s.e.m. of three individual experiments assayed in triplicate. $^{*}P < 0.05$ for positive increasing trend and 100 *vs* 1000 ng/ml MIF dose. (C) Microphotographs (original magnification, X 100) showing the reduction in amastigote load of MIF-treated macrophages. Cells were initially incubated in the presence (+MIF) or in the absence (−MIF) of proinflammatory cytokine for 72 h. Thereafter, the phagocytes were *T. cruzi*-infected for 48 h, formalin-fixed and stained with hematoxylin-eosin as described under Materials and methods. Images are representative of three independent experiments.

of MIF-exposed, *T. cruzi*-infected peritoneal macrophages isolated from naïve BALB/c mice showed similar outcome (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.imbio.2016.08.007).

3.3. *Coactivation of macrophages by rMIF and rIFN-*gamma *leads to enhanced killing of intracellular* Trypanosoma cruzi

IFN-gamma has been demonstrated to play a critical role in early resistance against *T. cruzi* infection, mainly through the activation of effector mechanisms in macrophages to destroy both internalized trypomastigotes and amastigotes (Lykens et al., 2010; Savino et al., 2007). We herein investigated whether the combined stimulation with rIFN-gamma and rMIF rendered J774 cells more effective in eliminating intracellular parasites. Pre-treatment of macrophages with rIFN-gamma at 100 U/ml induced trypanocidal activity and resulted in an increased percentage of *T. cruzi*-free cells after 48 h of infection. Addition of rMIF at 100 ng/ml was unable to potentiate rIFN-gamma-mediated protection (Fig. 3A and B). In contrast, when cell cultures were primed with rIFN-gamma and subsequently incubated with rMIF at higher doses (500–1000 ng/ml), synergistic cooperation between both cytokines significantly ($P < 0.05$) augmented the percentage of uninfected cells (18.9 ± 4.8% *vs* 83.1 ± 8.3% for rIFN-gamma- and [rIFN-gamma + 1000 ng/ml rMIF]-fostered cultures, respectively) and killing of parasites by J774 macrophages under dual stimulation (39.9 ± 7.2% *vs* 82.1 ± 3.5% and 86.0 ± 5.5% for rIFN-gamma-,[rIFN-gamma + 500 ng/ml rMIF] and [rIFN-gamma + 1000 ng/ml rMIF]-treated cells, respectively) (Fig. 3A and B). A similar activation profile was also observed for

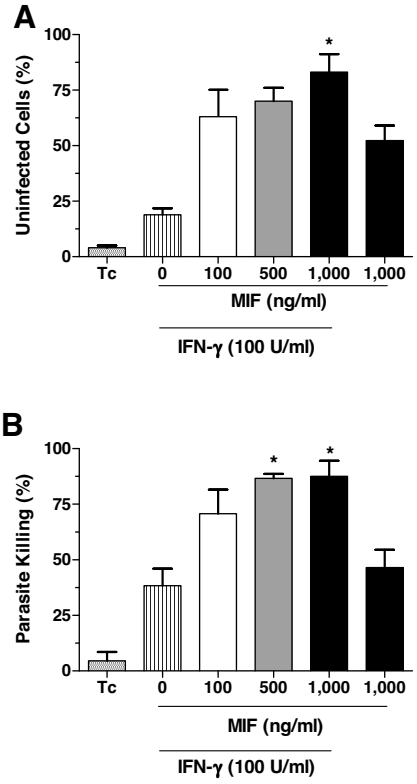


Fig. 3. Effect of IFN-gamma plus MIF cotreatment of macrophages on subsequent cell infection with *T. cruzi*. Phagocytic J774 cells were initially stimulated or not with recombinant IFN-gamma (100 U/ml, 1 h). Macrophages were further treated or not with recombinant MIF at 100, 500 or 1000 ng/ml for 72 h and then infected with RA strain trypomastigotes (10 parasites/cell). Unstimulated controls (Tc) were infected as well. The percentages of uninfected cells (A) and intracellular parasite killing (B) at 48 h postinfection were calculated. The results represent means ± s.e.m. of three individual experiments assayed in triplicate. *$P < 0.05$ for (500–1000 ng/ml MIF + 100 U/ml IFN-gamma) *vs* Tc and 100 U/ml IFN-gamma without MIF addition.

rIFN-gamma- and/or rMIF-primed, parasite-infected peritoneal macrophages (Supplementary Fig. S2 in the online version at DOI: 10.1016/j.imbio.2016.08.007). Our findings indicate that the anti-*T. cruzi* effect of rIFN-gamma on macrophages is markedly enhanced by the cooperation of rMIF.

3.4. MIF potentiates TNF-α secretion from infected macrophages through the p38 mitogen-activated protein kinase signaling pathway

MIF has been largely recognized as a potent activator of phagocytic cells (Lolis and Bucala, 2003). In a first approach, we examined MIF ability to enhance the production of TNF-α, a major macrophage product displaying trypanostatic and trypanocidal properties (Silva et al., 1995). Upon incubation with rMIF (1000 ng/ml) for 24 h, parasite-free macrophages produced significantly ($P < 0.01$) augmented levels of the proinflammatory factor (Fig. 4A). Of note, in comparison to IFN-gamma-mediated activation, MIF proved to be a stronger ($P < 0.05$) inducer of TNF-α (957.4 ± 97.2 *vs* 564.5 ± 35.7 pg/ml). We next determined the kinetics of TNF-α secretion from MIF-primed *T. cruzi*-harboring macrophages. Upon 2 h of infection, cytokine levels in rMIF-treated and untreated cultures remained low and undistinguishable (Fig. 4B). Nonetheless, after 72 h of infection, *T. cruzi* promoted an increase in the production of TNF-α, much more pronounced ($P < 0.05$) in rMIF-stimulated and parasite-infected J774 cells (74.6 ± 5.2 pg/ml *vs* 610.3 ± 10.6 pg/ml for rMIF-untreated and treated infected samples, respectively; Fig. 4C). Addition of MIF neutralizing Ab drastically ($P < 0.001$) reversed the stimulatory effect of rMIF on the induction of TNF-α response at 72 h of infection, demonstrating the specificity of the observed macrophage reactivity (Fig. 4C). Enhanced TNF-α release from rMIF-exposed and *T. cruzi*-infected peritoneal macrophages was detected as well (Supplementary Fig. S3 in the online version at DOI: 10.1016/j.imbio.2016.08.007).

Furthermore, we verified that MIF potentiated the activation of infected J774 macrophages via the p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway. Phosphorylation of p38 MAPK was detectable at 30 min after MIF stimulation, reaching maximal levels at 60 min and slightly declining thereafter, without changes in their total protein levels (Fig. 4D). On the other hand, induction of the ERK1/2 MAPK cascade could not be detected in stimulated/infected phagocytes. Inhibition of the intracellular p38 MAPK signal was tested to show its involvement in MIF plus *T. cruzi*-driven induction of TNF-α production. Increased cytokine levels in the supernatant from rMIF- and *T. cruzi*-receiving macrophages were significantly ($P < 0.01$) reduced by pretreatment of cells with the p38 MAPK-specific inhibitor SB203580 (576.3 ± 23.1 *vs* 51.7 ± 12.6 pg/ml for J774 cells in the absence or in the presence of SB203580, Fig. 4E). No substantial downregulation was achieved using the ERK1/2 MAPK-specific inhibitor U0126. There was no toxic effect of inhibitors on cell viability. These results suggest that MIF-induced upregulation of TNF-α expression in infected macrophages is mediated, at least in part, through the p38 MAPK signaling route.

3.5. MIF upregulates NO and ROS production in infected J774 phagocytes

We next asked whether MIF treatment could further generate trypanocidal mediators other than TNF-α in J774 macrophages. Uninfected cell priming with rMIF at 1000 ng/ml led to augmented production of NO and ROS ($P < 0.05$; Fig. 5A and B, left panels). Moreover, MIF-dependent induction resulted in greater ROS formation (44.4 ± 0.2 *vs* 19.2 ± 1.0% of $DCFH^+$ cells) and lower NO secretion (4.6 ± 0.7 *vs* $12.5 \pm 0.4 \times 10^{-6}$ *M*) than those triggered upon stimulation with rIFN-gamma at 100 U/ml. Interestingly, *T. cruzi* infection itself did not drive an immediate inflammatory response in this cell line. However, treatment with rMIF readily elicited a significant ($P < 0.05$) ROS response in phagocytes infected for 2 h, though NO remained at levels equivalent to those produced by mock-treated cultures (Fig. 5A and B, central panels). After 72 h of infection, the macrophages displayed increased ($P < 0.05$) production of both inflammatory agents, which was even more enhanced in J774 cells previously primed with rMIF (44.3 ± 0.8 *vs* 70.3 ± 6.3% of ROS-$DCFH^+$ cells; and 15.7 ± 0.4 *vs* $21.5 \pm 1.5 \times 10^{-6}$ *M* NO, respectively, for mock- *vs* rMIF-receiving infected cultures). To confirm that more efficient activation of parasiticidal agents in *T. cruzi*-harboring macrophages was dependent on preliminary addition of exogenous rMIF, stimulation was performed in the presence of cytokine-specific blocking antibody. A significant ($P < 0,05$) reduction of both NO and ROS responses in infected cells was observed as a direct consequence of MIF neutralization (Fig. 5A and B; right panels).

3.6. MIF induces elimination of internalized Trypanosoma cruzi from macrophages through endogenous production of TNF-α, NO and ROS

In an attempt to verify that MIF-induced TNF-α, NO axis and ROS effectively exerted trypanocidal actions on intracellular *T. cruzi* forms, parasite burden was determined in stimulated and infected J774 cells in the presence of iNOS and ROS inhibitors, or neutralizing antibodies to each of the above proinflammatory cytokines. Upon 48-h infection, rMIF (1000 ng/ml)-stimulated phagocytes

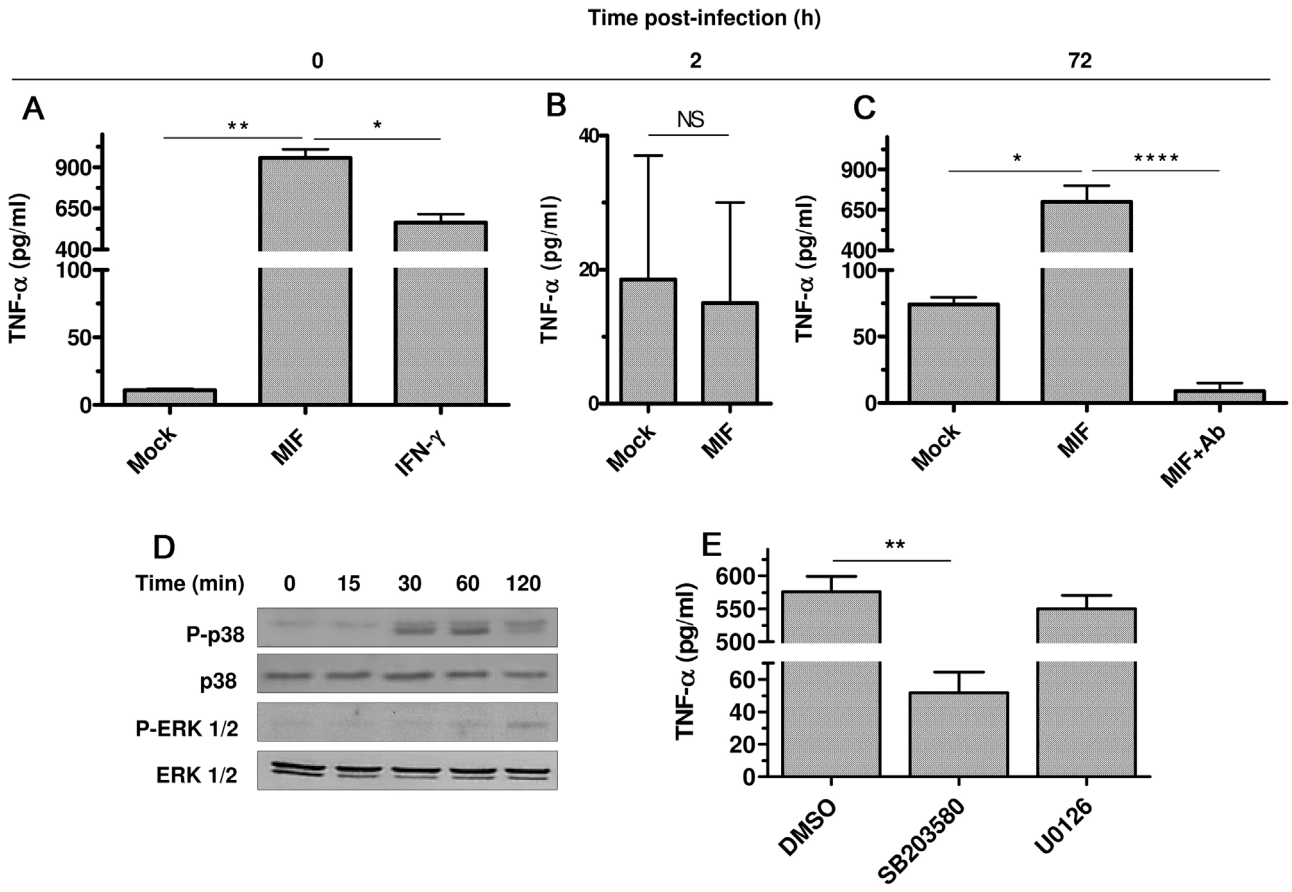


Fig. 4. TNF-α secretion from MIF-stimulated macrophages, before and after *T. cruzi* infection. (A-C) Following incubation with rMIF (1000 ng/ml) or rIFN-gamma (100 U/ml) for 24 h, J774 macrophages were infected with *T. cruzi* trypomastigotes. MIF neutralizing antibody (1 μg/ml) was included in some experiments (MIF + Ab). Mock-treated cells were used as control. TNF-α levels (mean ± s.e.m.) in the supernatants of unprimed and cytokine-primed cells, collected prior to infection (0 h) and 2 h and 72 h later, were quantified using a commercial ELISA. The results shown are representative of three independent experiments performed in triplicate. NS, nonsignificant difference; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. (D) Induction of MAPK signaling pathway. Macrophages were *T. cruzi*-infected for 24 h and then treated with rMIF at 1000 ng/ml. At the indicated times postreatment (0–120 min), the cells were washed and lysed. The lysates were analyzed by Western immunoblotting using antibodies to total (p38 and ERK1/2) and phosphorylated (P-p38 and P-ERK1/2) kinases. (E) Modulation of TNF-α response through MAPK inhibition. J774 macrophages were treated for 30 min with either p38 (SB203580) or ERK1/2 (U0126) specific inhibitor at 10 μM each. Vehicle-treated controls (DMSO) were included for testing. Subsequently, the cells were infected with *T. cruzi* in the presence of MIF (1000 ng/ml). TNF-α levels (mean ± s.e.m. of three independent experiments) in 72-h culture supernatants were measured by ELISA. ** $P < 0.01$.

succeeded in controlling *T. cruzi* growth, sharply reducing the number of amastigotes per 100 cells ($P < 0.01$; Fig. 6). Stimulation in the presence of anti-MIF resulted in almost complete blocking of cytokine-driven parasiticidal activity. As evidence of iNOS/NO and TNF-α involvement, we found that either AG or TNF-α neutralizing mAb caused a significant ($P < 0.05$) reversal of the MIF-potentiated inhibition of *T. cruzi* survival within macrophages (135.3 ± 24.6 and 119.1 ± 15.2 *vs* 46.2 ± 6.1 parasites/100 cells, for AG- or TNF-α blocking mAb-treated *vs* uninhibited, stimulated/infected cultures, respectively). Likewise, abolishing ROS generation by preincubation with Apo resulted in a substantial elevation of pathogen load in macrophages compared to that recorded for rMIF-stimulated and *T. cruzi*-infected phagocytes devoid of inhibitory pretreatment (112.3 ± 4.4 *vs* 46.2 ± 6.1 amastigotes/100 cells, respectively; $P < 0.05$). Altogether, these findings show that TNF-α, NO and ROS are key factors mediating MIF-dependent control of *T. cruzi* multiplication inside host phagocytes.

4. Discussion

Macrophages play important roles in *T. cruzi* infection by serving either as host and effector cells against the parasite. The present study shows that the proinflammatory cytokine MIF induces the microbiostatic/microbicidal ability of murine macrophages that impairs multiplication of *T. cruzi* forms living within the invaded cells. Exogenous rMIF promoted a significant dose-dependent decrease in the number of internalized parasites after 48-h infection of J774 cells and murine peritoneal macrophages, compared to the infection alone. Also, the percentage of uninfected cells augmented as consequence of gradual increment in the amount of rMIF added to culture medium. The autocrine/paracrine effects of phagocyte-derived MIF could further enhance trypanocidal activity as *T. cruzi* proved capable of triggering MIF liberation from mouse macrophages. In our hands, a high dose of rMIF is required to provoke a significant trypanocidal effect on infected macrophages. This is not surprising, given that MIF concentration necessary for phagocyte activation to kill *Leishmania major in vitro* has been found to exceed 1 μg/ml (Jüttner et al., 1998). Approximately equivalent doses of exogenous MIF have proven capable of triggering TNF-α, NO and ROS production in cultured cells (Alam et al., 2012; Calandra et al., 1998; Chuang et al., 2012). Of note, this cytokine level falls within the wide range of MIF concentrations measured in pathophysiologic conditions and known to promote inflammation *in vivo*. Both systemic and local concentrations of cytokines are hard to define accurately and can be much greater than expected. For instance, plasma MIF levels up to 0.56-3.20 μg/ml were demonstrated in patients with disseminated intravascular coagulation and sepsis, whereas MIF was also

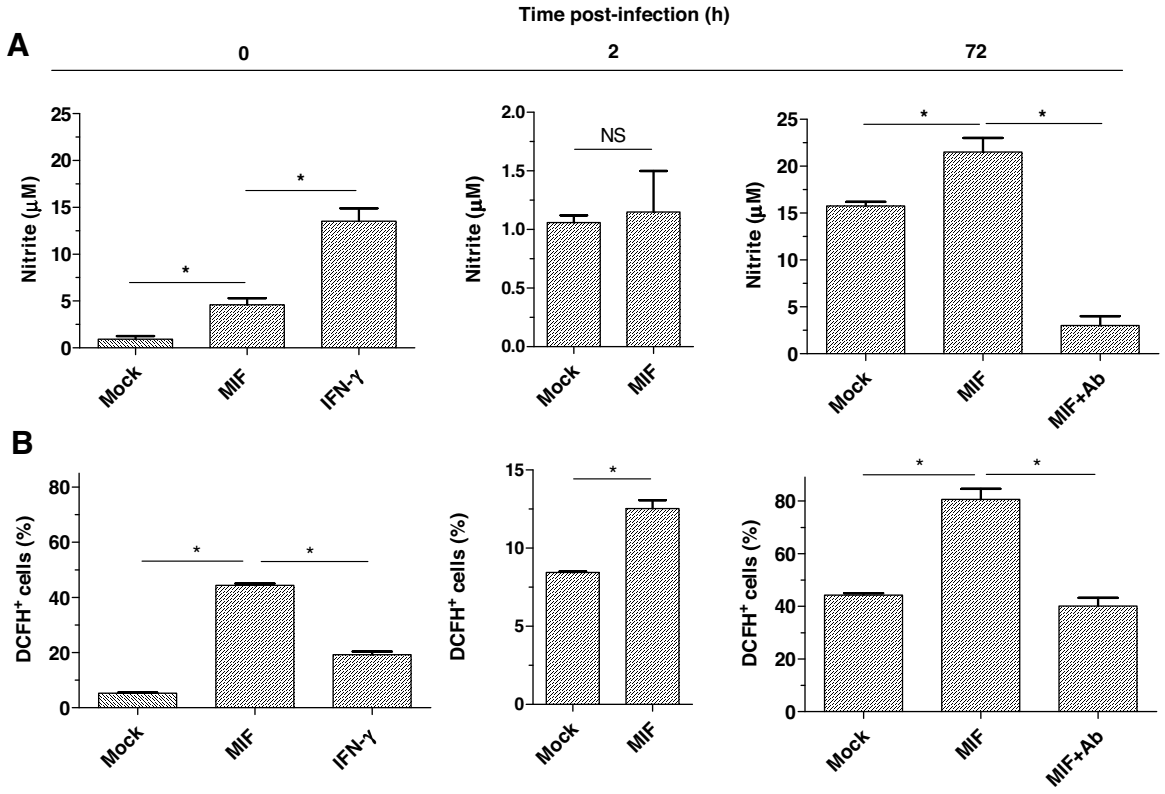


Fig. 5. Production of reactive nitrogen and oxygen species in MIF-primed macrophages, before and after *T. cruzi* infection. J774 macrophages were infected with *T. cruzi* trypomastigotes in the presence or in the absence of rMIF (1000 ng/ml) or rIFN-gamma (100 U/ml). MIF neutralizing antibody (1 μg/ml) was included in some experiments (MIF + Ab). Mock-treated cells were used as control. Cell specimens and culture supernatants were collected at 0, 2 or 72 h of infection. (A) Nitrite levels measured by the Griess method as described under Materials and methods. (B) Quantification of ROS generation by the DCFH-DA fluorescence procedure as described under Materials and methods. Values are expressed as percentage of DCFH positive cells. All results shown represent means ± s.e.m. of three individual experiments assayed in triplicate. NS, nonsignificant difference; * $P < 0.05$.

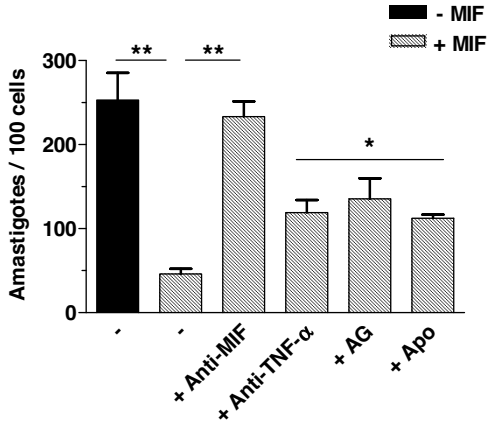


Fig. 6. MIF-driven mediators of macrophage trypanocidal activity. J774 macrophages were infected with *T. cruzi* trypomastigotes in culture medium containing 0 (−MIF) or 1000 ng/ml rMIF (+MIF). In a set of experiments with parasite-infected and MIF-stimulated cells, cultures were incubated in the presence of blocking antibodies to MIF (1 μg/ml, Anti-MIF) or TNF-α (20 μg/ml, Anti-TNF-α), or pretreated for 30 min with selective inhibitors of inducible NO synthase (1×10^{-3} *M* aminoguanidine, AG) or NADPH-dependent ROS generation (3×10^{-4} *M* apocynin, APO). The growth of intracellular parasites in macrophages was evaluated by counting the absolute number of amastigotes per 100 cells at 72 h postinfection. Results (means ± s.e.m.) are representative of three independent experiments performed in triplicate. * $P < 0.05$, antibody/inhibitor-treated cells *vs* *T. cruzi*-infected and MIF-stimulated cultures; ** $P < 0.01$.

found to sharply increase (200–440 ng/ml) at the inflammation site during rheumatoid arthritis and experimental autoimmune uveitis (Emonts et al., 2007; Kitaichi et al., 2000; Onodera et al., 1999). It remains to be determined whether variable MIF concentration is linked to differential expression of receptor complex types on target cells, displaying particular binding affinities that determine distinct thresholds for the diverse biological effects of this factor.

As reported for an *in vitro* study of phagocyte-dependent killing of *L. major* (Jüttner et al., 1998), addition of rMIF to effector cells before become infected is required to fuel the anti-*T. cruzi* machinery in phagocytes. In the early course of natural infection, the production of a set of inflammatory mediators, including MIF, by innate immune cells is promoted by *T. cruzi*. Remarkably, during this process, *in vivo* priming of parasite-free macrophages by proinflammatory cytokines may indeed occur, as observed for mycobacterial infection in mice (Flesch et al., 1995). In the context of Chagasic infection *in vivo*, it is very likely that *T. cruzi*-invaded phagocytes may secrete a significant amount of MIF that can reach macrophages in the vicinity of parasitized cells before the trypomastigote does. Consequently, paracrine MIF actions would enable priming of uninfected macrophages leading to effective activation before pathogen internalization (Onodera et al., 1997). Besides, preformed MIF intracellular stores could be promptly released in response to multiple proinflammatory agents (Calandra et al., 1994) triggered at the onset of infection and then exert further autocrine/paracrine priming effects on the non-invaded macrophage subpopulation.

Furthermore, exogenous MIF was able to potentiate IFN-gamma-mediated activation of trypanocidal mechanisms in J774 cells. Stimulation of the macrophage with IFN-gamma is known to mainly activate the iNOS biochemical system within this cell, leading to the control of parasite proliferation (Bergeron and Olivier, 2006). In our series, synergistic cooperation between IFN-gamma

and MIF at increasing concentrations resulted in a significant reduction in the numbers of infected phagocytes and intracellular parasites, more efficient than that achieved with IFN-gamma alone. These findings are in agreement with previous reports showing improved control of *T. cruzi* growth in macrophages by treatment with IFN-gamma combined with other cytokines (Golden and Tarleton, 1991; Muñoz-Fernández et al., 1992). Interestingly, MIF stimulation of anti-*T. cruzi* activity seems to operate primarily with macrophages as it had no effect on infected murine cardiomyocytes (unpublished result).

The main effector mechanisms that limitate infection by *T. cruzi* depend on activation of macrophages. Upon parasite invasion, these cells are activated by mechanisms that are modulated by a variety of cytokines and chemokines. The ability of MIF to promote the synthesis and release of diverse mediators of inflammation has been well documented (Alam et al., 2012; Chuang et al., 2012). Particularly, our study reveals that TNF-α is one of the main trypanocidal agents elicited upon MIF priming of J774 cells. Macrophage-derived TNF-α has a key role in the response against *T. cruzi* by indirectly inducing expression of the enzyme iNOS and subsequent NO release. Further, TNF-α acts in an autocrine/paracrine fashion triggering macrophage activation and boosts IFN-gamma-mediated effects and its own production, as well as that of other cytokines (Gutierrez et al., 2007). We herein verified that MIF treatment promotes TNF-α overproduction by infected J774 phagocytes resulting in enhanced restraint of parasite proliferation. Additionally, as reported for other MIF targets (Aeberli et al., 2006; Cheng et al., 2010), our findings suggest that the regulatory pathway underlying MIF-dependent upregulation of TNF-α expression involves p38 MAPK intracellular signaling.

Once activated, macrophages, as well as other cells of the innate immune system, produce several oxidative molecules, such as NO and ROS. These free radicals, alone or combined, kill the intracellular pathogen by chemically modifying the structural properties of parasite target proteins, disturbing their functions, and inactivating catalytic sites of trypanosome enzymes (Gutierrez et al., 2007). MIF has been demonstrated to induce NO-dependent killing of the intracellular protozoan *Leishmania major* by macrophages (Jüttner et al., 1998). Similarly, in our *in vitro* model, MIF-driven activation of iNOS and NADPH oxidase that gave rise to endogenous overproduction of NO and ROS contributed to elimination of *T. cruzi* amastigotes from macrophages. Unlike IFN-gamma, which triggers mainly the generation of nitrogen intermediates, MIF appears to be a major activator of oxygen-dependent trypanocidal mechanisms in macrophages. Of note, MIF-mediated boosting of ROS generation was observed as early as 2 h of infection. This initial elevation can favor an even greater increase in NADPH phagocyte oxidase-derived ROS in the course of parasite infection that may become determinant for host protection (Santiago et al., 2012; Zorov et al., 2000).

On the other hand, a slight decline in TNF-α and NO intermediate concentration observed upon 2 h of infection is likely due to concomitant transforming growth factor-β (TGF-β) production in the infected macrophages. The host cell invasion by *T. cruzi* is strictly dependent on the activation of the TGF-β signaling pathway, while subsequent parasite-driven production of TGF-β (together with IL-10) modulates macrophage activation by *T. cruzi* in order to avoid self-destruction and tissue damage (Bogdan and Röllinghoff, 1999). Previous findings demonstrated that trypomastigote invasion of phagocytic cells is readily able to induce an early peak of TGF-β release (Silva et al., 1998) which in turn may downregulate TNF-α and NO secretion (Dunham et al., 1990; Gazzinelli et al., 1992) during the first hours of infection. Conversely, lack of depression in macrophage ROS generation at 2 h postinfection could be attributed to the ability of TGF-β to act as an inducer, rather than inhibitor, of intracellular reactive oxygen intermediates (Ishikawa et al., 2014). An additional, or eventually alternative, explanation for poor induction of inflammatory mediators in recently infected macrophages could be that *T. cruzi* is unique in eliciting very few changes in host cell gene expression at early time points of infection (2–6 h). As a result of parasite internalization, an initial lag phase in which the invaded cells display a marked delay in their transcriptional responses has been verified (Costales et al., 2009; Vaena de Avalos et al., 2002). Our data show that enhanced generation of macrophage-derived trypanocidal factors becomes evident later (72 h) in the course of infection.

MIF-mediated immunoprotection against protozoan pathogens has been found to greatly rely upon activation of macrophage microbicidal machinery (Rosado and Rodriguez-Sosa, 2011). Particularly, endogenous MIF plays an important role in the host defense against *T. cruzi*, in part by promoting production of the proinflammatory cytokines IL-12, IL-18, IL-1β, and TNF-α from phagocytic cells during the early phase of infection (Reyes et al., 2006). Moreover, MIF interaction with trypanosomal antigens has been shown to synergistically enhance maturation of dendritic cells through activation of the p38 MAPK pathway and upregulated IL-12 release, thus favoring anti-*T. cruzi* effector mechanisms (Terrazas et al., 2011). Accordingly, recently infected MIF$^{-/-}$ mice displayed impaired innate cytokine expression and inefficient intracellular killing of parasites resulting in exacerbated susceptibility to experimental Chagas disease (Reyes et al., 2006). Overall, these data are coincident with our present results and strongly advocate for MIF to be given consideration as an effective inducer of macrophage-derived mediators of inflammatory response critically implicated in the elimination of *T. cruzi in vivo*.

In conclusion, our current study draws attention to a newly described innate immune mechanism, based upon MIF-mediated induction of parasiticidal agents, allowing enhanced amastigote killing within phagocytic cells. Taken together, our findings show that MIF is a primary component of proinflammatory response elicited upon *T. cruzi* infection, which may activate macrophages via the classical pathway and potentiate the endogenous production of TNF-α, NO and ROS, as well as their trypanostatic/trypanocidal actions, thus favoring intracellular pathogen elimination. The present results broaden our understanding of the regulation of inflammatory mediators implicated in early parasite containment that is decisive for resistance to Chagasic infection.

Competing interests

All authors recognize no actual or potential conflict of interest including any commercial, personal, financial or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, their work. The authors alone are responsible for the content and writing of their paper.

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References

Aeberli, D., Yang, Y., Mansell, A., Santos, L., Leech, M., Morand, E.F., 2006. Endogenous macrophage migration inhibitory factor modulates glucocorticoid sensitivity in macrophages via effects on MAP kinase phosphatase-1 and p38 MAP kinase. FEBS Lett. 580, 974.

Alam, A., Haldar, S., Thulasiram, H.V., Kumar, R., Goyal, M., Iqbal, M.S., Pal, C., Dey, S., Bindu, S., Sarkar, S., Pal, U., Maiti, N.C., Bandyopadhyay, U., 2012. Novel anti-inflammatory activity of epoxyazadiradione against macrophage migration inhibitory factor: inhibition of tautomerase and proinflammatory activities of macrophage migration inhibitory factor. J. Biol. Chem. 287, 24844.

Balachandran, S., Gadekar, P.K., Parkale, S., Yadav, V.N., Kamath, D., Ramaswamy, S., Sharma, S., Dagia, N.M., 2011. Synthesis and biological activity of novel MIF antagonists. Bioorg. Med. Chem. Lett. 21, 1508.

Bergeron, M., Olivier, M., 2006. *Trypanosoma cruzi*-mediated IFN-gamma-inducible nitric oxide output in macrophages is regulated by iNOS mRNA stability. J. Immunol. 177, 6271.

Bogdan, C., Röllinghoff, M., 1999. How do protozoan parasites survive inside macrophages? Parasitol. Today 15, 22.

Calandra, T., Bernhagen, J., Mitchell, R.A., Bucala, R., 1994. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J. Exp. Med. 179, 1895.

Calandra, T., Spiegel, L.A., Metz, C.N., Bucala, R., 1998. Macrophage migration inhibitory factor is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria. Proc. Natl. Acad. Sci. U. S. A. 95, 11383.

Cardoni, R.L., Antúnez, M.I., 2004. Circulating levels of cyclooxygenase metabolites in experimental *Trypanosoma cruzi* infections. Mediators Inflamm. 13, 235.

Celentano, A.M., González Cappa, S.M., 1993. *In vivo* macrophage function in experimental infection with *Trypanosoma cruzi* subpopulations. Acta Trop. 55, 171.

Cheng, Q., McKeown, S.J., Santos, L., Santiago, F.S., Khachigian, L.M., Morand, E.F., Hickey, M.J., 2010. Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. J. Immunol. 185, 1238.

Chuang, Y.C., Su, W.H., Lei, H.Y., Lin, Y.S., Liu, H.S., Chang, C.P., Yeh, T.M., 2012. Macrophage migration inhibitory factor induces autophagy via reactive oxygen species generation. PLoS One 7, e37613.

Cooke, G., Armstrong, M.E., Donnelly, S.C., 2009. Macrophage migration inhibitory factor (MIF), enzymatic activity and the inflammatory response. Biofactors 35, 165.

Costales, J.A., Daily, J.P., Burleigh, B.A., 2009. Cytokine-dependent and −independent gene expression changes and cell cycle block revealed in *Trypanosoma cruzi*-infected host cells by comparative mRNA profiling. BMC Genomics 10, 252.

Dias, J.C., 2015. Evolution of Chagas disease screening programs and control programs: historical perspective. Glob. Heart 10, 193.

Dunham, D.M., Arkins, S., Edwards 3rd., C.K., Dantzer, R., Kelley, K.W., 1990. Role of interferon-gamma in counteracting the suppressive effects of transforming growth factor-beta 2 and glucocorticoids on the production of tumor necrosis factor-alpha. J. Leukoc. Biol. 48, 473.

Dutra, W.O., Gollob, K.J., 2008. Current concepts in immunoregulation and pathology of human Chagas Disease. Curr. Opin. Infect. Dis. 21, 287.

Emonts, M., Sweep, F.C., Grebenchtchikov, N., Geurts-Moespot, A., Knaup, M., Chanson, A.L., Erard, V., Renner, P., Hermans, P.W., Hazelzet, J.A., Calandra, T., 2007. Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenal response, and early death in patients with severe sepsis. Clin. Infect. Dis. 44, 1321.

Flesch, I.E., Hess, J.H., Huang, S., Aguet, M., Rothe, J., Bluethmann, H., Kaufmann, S.H., 1995. Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon gamma and tumor necrosis factor alpha. J. Exp. Med. 181, 1615.

Gazzinelli, R.T., Oswald, I.P., Hieny, S., James, S.L., Sher, A., 1992. The microbicidal activity of interferon-gamma-treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. Eur. J. Immunol. 22, 2501.

Golden, J.M., Tarleton, R.L., 1991. *Trypanosoma cruzi*: cytokine effects on macrophage trypanocidal activity. Exp. Parasitol. 72, 391.

Gregory, J.L., Leech, M.T., David, J.R., Yang, Y.H., Dacumos, A., Hickey, M.J., 2004. Reduced leukocyte-endothelial cell interactions in the inflamed microcirculation of macrophage migration inhibitory factor-deficient mice. Arthritis Rheum. 50, 3023.

Guiñazú, N., Carrera-Silva, E.A., Becerra, M.C., Pellegrini, A., Albesa, I., Gea, S., 2010. Induction of NADPH oxidase activity and reactive oxygen species production by a single *Trypanosoma cruzi* antigen. Int. J. Parasitol. 40, 1531.

Gutierrez, F.R.S., Mariano, F.S., Miranda-Santos, I.K.F., Silva, J.S., 2007. Effector mechanisms of macrophages infected with *Trypanosoma cruzi*. In: Denkers, E.Y., Gazzinelli, R.T. (Eds.), Protozoans in Macrophages. Landes Biosciences, Austin, pp. 207–220.

Ishikawa, F., Kaneko, E., Sugimoto, T., Ishijima, T., Wakamatsu, M., Yuasa, A., Sampei, R., Mori, K., Nose, K., Shibanuma, M., 2014. A mitochondrial thioredoxin-sensitive mechanism regulates TGF-β-mediated gene expression associated with epithelial-mesenchymal transition. Biochem. Biophys. Res. Commun. 443, 821.

Jüttner, S., Bernhagen, J., Metz, C.N., Röllinghoff, M., Bucala, R., Gessner, A., 1998. Migration inhibitory factor induces killing of *Leishmania major* by macrophages: dependence on reactive nitrogen intermediates and endogenous TNF-alpha. J. Immunol. 161, 2383.

Kitaichi, N., Matsuda, A., Kotake, S., Namba, K., Tagawa, Y., Sasamoto, Y., Ogasawara, K., Iwabuchi, K., Onoé, K., Matsuda, H., Nishihira, J., 2000. Inhibition of experimental autoimmune uveoretinitis with anti-macrophage migration inhibitory factor antibodies. Curr. Eye Res. 20, 109.

Lima, M.F., Zhang, Y., Villalta, F., 1997. β-chemokines that inhibit HIV-1 infection of human macrophages stimulate uptake and promote destruction of *Trypanosoma cruzi* by human macrophages. Cell. Mol. Biol. (Noisy-le-grand) 43, 1067.

Lolis, E., Bucala, R., 2003. Macrophage migration inhibitory factor. Expert Opin. Ther. Targets 7, 153.

Lykens, J.E., Terrell, C.E., Zoller, E.E., Divanovic, S., Trompette, A., Karp, C.L., Aliberti, J., Flick, M.J., Jordan, M.B., 2010. Mice with a selective impairment of IFN-gamma signaling in macrophage lineage cells demonstrate the critical role of IFN-gamma-activated macrophages for the control of protozoan parasitic infections in vivo. J. Immunol. 184, 877.

Machado, F.S., Dutra, W.O., Esper, L., Gollob, K., Teixeira, M.M., Factor, S.M., Weiss, L.M., Nagajyothi, F., Tanowitz, H.B., Garg, N.J., 2012. Current understanding of immunity to *Trypanosoma cruzi* infection and pathogenesis of Chagas disease. Semin. Immunopathol. 34, 753.

Matsumoto, K., Kanmatsuse, K., 2001. Increased production of macrophage migration inhibitory factor by T cells in patients with IgA nephropathy. Am. J. Nephrol. 21, 455.

Miyazaki, Y., Hamano, S., Wang, S., Shimanoe, Y., Iwakura, Y., Yoshida, H., 2010. IL-17 is necessary for host protection against acute-phase *Trypanosoma cruzi* infection. J. Immunol. 185, 1150.

Muñoz-Fernández, M.A., Fernández, M.A., Fresno, M., 1992. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha and IFN-gamma through a nitric oxide-dependent mechanism. Immunol. Lett. 33, 35.

Onodera, S., Suzuki, K., Matsuno, T., Kaneda, K., Takagi, M., Nishihira, J., 1997. Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. Immunology 92, 131.

Onodera, S., Tanji, H., Suzuki, K., Kaneda, K., Mizue, Y., Sagawa, A., Nishihira, J., 1999. High expression of macrophage migration inhibitory factor in the synovial tissues of rheumatoid joints. Cytokine 11, 163.

Popa, C., van Lieshout, A.W., Roelofs, M.F., Geurts-Moespot, A., van Riel, P.L., Calandra, T., Sweep, F.C., Radstake, T.R., 2006. MIF production by dendritic cells is differentially regulated by Toll-like receptors and increased during rheumatoid arthritis. Cytokine 36, 51.

Reyes, J.L., Terrazas, L.I., Espinoza, B., Cruz-Robles, D., Soto, V., Rivera-Montoya, I., Gómez-García, L., Snider, H., Satoskar, A.R., Rodríguez-Sosa, M., 2006. Macrophage migration inhibitory factor contributes to host defense against acute *Trypanosoma cruzi* infection. Infect. Immun. 74, 3170.

Rosado, J.D., Rodríguez-Sosa, M., 2011. Macrophage migration inhibitory factor (MIF): A key player in protozoan infections. Int. J. Biol. Sci. 7, 1239.

Saeftel, M., Fleischer, B., Hoerauf, A., 2001. Stage-dependent role of nitric oxide in control of *Trypanosoma cruzi* infection. Infect. Immun 69, 2252.

Santiago, H.C., Gonzalez Lombana, C.Z., Macedo, J.P., Utsch, L., Tafuri, W.L., Campagnole-Santos, M.J., Alves, R.O., Alves-Filho, J.C., Romanha, A.J., Cunha, F.Q., Teixeira, M.M., Radi, R., Vieira, L.Q., 2012. NADPH phagocyte oxidase knockout mice control *Trypanosoma cruzi* proliferation, but develop circulatory collapse and succumb to infection. PLoS Negl. Trop. Dis. 6, e1492.

Savino, W., Villa-Verde, D.M., Mendes-da-Cruz, D.A., Silva-Monteiro, E., Perez, A.R., Aoki, M.delP., Bottasso, O., Guiñazú, N., Silva-Barbosa, S.D., Gea, S., 2007. Cytokines and cell adhesion receptors in the regulation of immunity to *Trypanosoma cruzi*. Cytokine Growth Factor Rev. 18, 107.

Silva, J.S., Vespa, G.N., Cardoso, M.A., Aliberti, J.C., Cunha, F.Q., 1995. Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect. Immun. 63, 4862.

Silva, J.S., Aliberti, J.C., Martins, G.A., Souza, M.A., Souto, J.T., Pádua, M.A., 1998. The role of IL-12 in experimental *Trypanosoma cruzi* infection. Braz. J. Med. Biol. Res. 31, 111.

Stafford, J.L., Neumann, N.F., Belosevic, M., 2002. Macrophage-mediated innate host defense against protozoan parasites. Crit. Rev. Microbiol. 28, 187.

Terrazas, C.A., Huitron, E., Vazquez, A., Juarez, I., Camacho, G.M., Calleja, E.A., Rodríguez-Sosa, M., 2011. MIF synergizes with *Trypanosoma cruzi* antigens to promote efficient dendritic cell maturation and IL-12 production via p38 MAPK. Int. J. Biol. Sci. 7, 1298.

Vaena de Avalos, S., Blader, I.J., Fisher, M., Boothroyd, J.C., Burleigh, B.A., 2002. Immediate/early response to *Trypanosoma cruzi* infection involves minimal modulation of host cell transcription. J. Biol. Chem. 277, 639.

Xin, D., Rendon, B.E., Zhao, M., Winner, M., McGhee Coleman, A., Mitchell, R.A., 2010. The MIF homologue D-dopachrome tautomerase promotes COX-2 expression through beta-catenin- dependent and −independent mechanisms. Mol. Cancer Res. 8, 1601.

Zorov, D.B., Filburn, C.R., Klotz, L.O., Zweier, J.L., Sollott, S.J., 2000. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J. Exp. Med. 192, 1001.