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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.