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SHORT REPORT: INHIBITION BY TUMOR NECROSIS FACTOR-α–ACTIVATED MACROPHAGES OF THE TRANSITION OF *PARACOCCIDIOIDES BRASILIENSIS* CONIDIA TO YEAST CELLS THROUGH A MECHANISM INDEPENDENT OF NITRIC OXIDE

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Abstract. It is known that peritoneal murine macrophages activated with interferon-γ exert a fungicidal effect against *Paracoccidioides brasiliensis* conidia by a nitric oxide (NO)–mediated mechanism. This NO-mediated effect can also be induced by other cytokines such as tumor necrosis factor-α (TNF-α). The aim of this study was to determine if TNF-α–activated peritoneal murine macrophages infected with *P. brasiliensis* were able to show fungistatic/fungicidal effects mediated by NO. The results indicated that although macrophage activation with TNF-α did not result in NO production, these cells played an important role in inhibiting the conidia from becoming yeast cells. *In vivo*, the NO-independent inhibitory effect would prove of importance for the establishment of *P. brasiliensis* in host tissues.

Paracoccidioidomycosis (PCM) is one of the most important endemic mycoses in Latin America, and Colombia is one of the countries where the disease is frequently diagnosed.[1] This disease is a progressive, chronic disorder caused by the inhalation of the infective particles (conidia) produced by the mycelial form of the fungus *Paracoccidioides brasiliensis.* The mycosis affects primarily the lungs, but it can disseminate into other organs and systems.[1]

Interferon-γ (IFN-γ), one of the strongest stimuli for macrophage activation, plays a key role in killing intracellular parasites through a nitric oxide (NO)–mediated mechanism.[2] This effect has been demonstrated in murine peritoneal macrophages activated with IFN-γ against *P. brasiliensis* conidia.[3] Additionally, the killing by IFN-γ-activated murine macrophages could not be abrogated by superoxide dismutase, catalase, or azide, suggesting that the fungicidal mechanism was independent of the oxidative burst products.[4]

Tumor necrosis factor-α (TNF-α) has also been associated with the anti-microbial activity of macrophages. This cytokine acts as an autocrine inductor and provides a second signal that induces cytotoxic activity in IFN-γ activated-macrophages, stimulating NO production.[2] It has been reported to have an important role in preventing fungal infections caused by *Candida* and *Histoplasma*.[5,6]

We explored TNF-α activity in PCM. *Paracoccidioides brasiliensis* was co-cultured with murine macrophages as follows. Isolate ATCC 60855 (American Type Culture Collection, Manassas, VA), previously shown to sporulate freely on special media, was used. The fungus was maintained at 18°C in its mycelial form by successive transfers on both modified Sabouraud dextrose agar (Mycosel®; Becton Dickinson, Sparks, MD) and the synthetic McVeight Morton modified agar.[7] The production, collection, and purification of conidia were done according to previously standardized procedures.[8] Only inocula with a conidial viability > 90% were used in the experiments.

Adult male BALB/c mice (8–12 weeks old) were used to obtain non-induced peritoneal macrophages by washings with RPMI 1640 medium. Cells collected were counted in a hemocytometer, and their viability determined by trypan blue exclusion. The cellular suspension was adjusted to a concentration of 1×10^6 viable cells/mL.[3] Aliquots of 0.25 mL of peritoneal cell suspensions were deposited in each well of a Laboratory-Tek-chamber slide (catalog number 177402; Nalgene Nunc International, Naperville, IL). The chambers were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for two hours to allow cells to adhere; non-adherent cells were then removed by aspiration, and the adherent monolayers rinsed with RPMI 1640 medium. The number of non-adherent cells was calculated and subtracted from the total number of peritoneal cells deposited in the chamber. A series of macrophages were activated with 10 ng/mL of TNF-α (catalog number T-7539; Sigma, St. Louis, MO), while others were activated with 50 units/mL of recombinant IFN-γ (catalog No. 19301U; PharMingen, San Diego, CA). The latter served as positive controls both for the inhibition of the conidia-to-yeast transition and nitrite (NO_2) production. A monoclonal antibody to TNF-α (30 ng/mL) was used to determine the specificity of this cytokine (catalog number 18120D; Pharmingen). The monolayers corresponding to either activated or non-activated macrophages were infected with 20 μL of the conidial suspension at a macrophage: conidium ratio of 10:1. The chambers were incubated again at 37°C in an atmosphere of 5% CO_2 and 95% air for 96 hours. The supernatant in each well was then collected and kept at –70°C for later detection of NO. The slides were fixed with absolute methanol, air-dried, and stained with modified silver methenamine or modified Wright stains.[3] The monolayers were observed by light microscopy and the number of intracellular fungal propagules was counted, distinguishing between conidia (non-transformed cells) and yeasts (transformed cells). More than 200 intracellular propagules were counted and the percentage of conidia converted into yeast cells was calculated. Percent conversion was calculated as the number of intracellular yeast cells/200 intracellular fungal cells × 100.[3] The concentration of NO_2 in culture supernatants was measured with Griess reagent (1% sulfanilamide [S-9251; Sigma], 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride [N-5889; Sigma], and 2.5% phosphoric acid [P-6560; Sigma]) and taken as indicator of the generation of NO.[9]

Statistical analyses were done using the Student *t*-test and one-way analyses of variance. The program used was Statistic for Windows, version 5.0 (Statsoft Inc., Tulsa, OK). The level of significance was assumed to be $P < 0.05$.

When compared with untreated controls, marked inhibition of the conidia-to-yeast transition was observed in macrophages previously treated with TNF-α (mean ± SD = 5.5 ± 2.4% versus 61.3 ± 5.8%) ($P < 0.00001$) (Figure 1). Additionally, when the percent inhibition of the transition in macrophages activated with IFN-γ was determined, a marked inhibition of the process (8.1 ± 3.0%) was observed. The addition of monoclonal antibody to TNF-α to macrophages activated with the corresponding cytokine resulted in a significant suppression of the inhibitory effect (41.5 ± 13.1%; $P < 0.00001$), indicating the specificity of this cytokine (Figure 1).

Unstimulated macrophages infected with *P. brasiliensis* conidia produced basal levels of NO_2^- (mean ± SD = 2.5 ± 0.4 μM). Since there was no difference in NO_2 levels between TNF-α–stimulated co-cultures and those containing both TNF-α and antibody to TNF-α, clearly this cytokine did not induce NO_2 production. Conversely, macrophages infected with *P. brasilienis* conidia and activated with IFN-γ, produced high levels of NO_2 (76.2 ± 4.2 μM), as had been previously observed (Figure 2).

These results illustrate for the first time that TNF-α-activated macrophages are capable of inhibiting the conidia-to-yeast transition in *P. brasiliensis* by an NO-independent pathway. The inhibitory process proved to be reversible by the addition of monoclonal antibody to TNF-α, suggesting that this cytokine plays an important role in the macrophage fungicidal mechanism against *P. brasiliensis* conidia.

It has been demonstrated that *in vitro* TNF-α plays a role in the induction of NO production, thus providing a second signal for the induction of inducible nitric oxide synthase (iNOS) and for the microbiocidal activity of the IFN-γ-activated macrophages against *Leishmania major* and *Trypanosoma cruzi*.[10–12] We have not seen this effect on peritoneal macrophages activated with IFN-γ.[13] Dorger and others studied the production of NO and iNOS expression in both rat and hamster alveolar macrophages.[14] They showed a species difference in the production of NO, and observed that when these cells were stimulated with IFN-γ, lipopolysaccharide (LPS), or TNF-α, they differed in iNOS expression. Thus, rat alveolar macrophages express iNOS and produced NO in a dose-dependent manner after treatment with IFN-γ and LPS, but not when treated with TNF-α. Conversely, in hamster alveolar macrophages, production of NO and iNOS expression were not detected after similar treatment.[14]

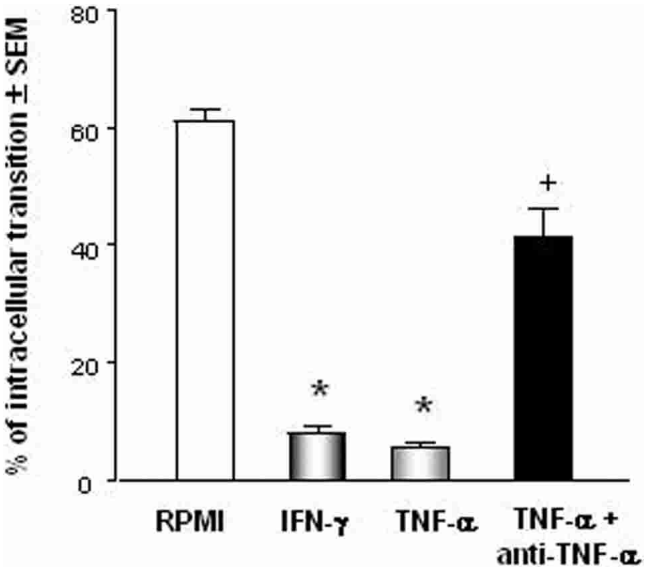

FIGURE 1. Effect of the addition of tumor necrosis factor-α (TNF-α) on the intracellular conidia-to-yeast transition of *Paracoccidioides brasiliensis* in murine peritoneal macrophages after 96 hours of co-culture at 37°C (n = 6). Bars show the mean ± SEM. *Significant differences ($P < 0.000001$) between non-stimulated macrophages and interferon-γ (IFN-γ) (50 units/mL) or TNF-α (10 ng/mL)–activated macrophages. +Significant difference ($P < 0.00001$) between TNF-α-activated macrophages and TNF-α activated macrophages plus monoclonal antibody to TNF-α (anti-TNF-α). RPMI = RPMI 1640 medium.

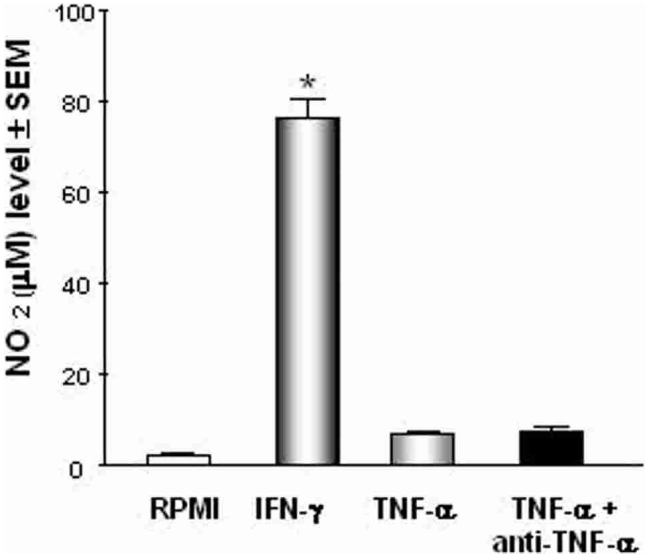

FIGURE 2. Nitrite (NO_2) production by murine peritoneal macrophages infected with *Paracoccidioides brasiliensis* conidia 96 hours after activation with interferon-γ (IFN-γ) (50 units/mL) or tumor necrosis factor-α (TNF-α) (10 ng/mL) (n = 6). Bars show the mean ± SEM. *Significant difference ($P < 0.00001$) between IFN-γ-activated macrophages and non-stimulated macrophages. RPMI = RPMI 1640 medium; anti-TNF-α = monoclonal antibody to TNF-α.

Paris-Fortes and others, using an experimental hamster model of PCM, found that during the early periods post-*P. brasiliensis* yeast infection, macrophages from infected animals had a higher spreading ability associated with increased TNF-α production and enhanced fungicidal activity that limited fungal dissemination.[15] *In vivo*, Souto and others used knockout mice for either IFN-γ (GKO) or TNF-α receptor (p55KO) and infected them with *P. brasiliensis* yeasts.[16] They showed that the animals were unable to control the infection, suggesting that IFN-γ and TNF-α receptors mediate resistance to infection with *P. brasiliensis*. Also, Calich and Kashino studied mice resistant (A/Sn) or susceptible (B10.A) to *P. brasiliensis*, and found that peritoneal macrophages from the susceptible strain secreted low levels of TNF-α in comparison with those from the resistant animals.[17] Additionally, it was shown that early after inoculation, a high level of secretion of TNF-α coincided with IFN-γ production by lymph node cells, and that this was followed by sustained or continued secretion of interleukin-12 (IL-12). Conversely, low concentrations of these cytokines in the early stages of infection were associated with the production of IL-5, IL-10, and transforming growth factor-β, all of which characterize the progressive illness in the susceptible animals.[17] These in-

vestigators suggested that production of such cytokines is crucial in resistance against *P. brasiliensis*.[17]

Bocca and others obtained different results when using inhibitors of NO *in vivo*.[18] They found that bronchoalveolar and peritoneal macrophages from animals thus treated did not produce NO_2^-, spontaneously released TNF-α, and exhibited no disturbance of the lymphoproliferatve capacity of spleen cells. These results suggested that although NO is important for the fungal killing, its production may also result during the course of infection in immunosupression.[18] In addition, Nascimento and others also observed that macrophages from susceptible mice to PCM (B10.A) produced high and persistent NO levels, while in resistant animals (A/Sn), TNF-α production predominated, indicating that either TNF-α or NO expression is associated with resistance and susceptibility, respectively.[19]

Our results suggest that TNF-α plays an important role in a fungicidal and/or fungistatic mechanism against *P. brasiliensis* conidia, and that this mechanism is independent of the L-arginine NO pathway. These findings also indicate that an alternative antifungal mechanism different from the oxidative pathways should exist. We have shown, however, that TNF-α participates in the control of the infection, probably influencing the pathogenesis of PCM. More studies are needed to determine this point.

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