INFECTION AND IMMUNITY, Nov. 1992, p. 4593–4597 Vol. 60, No. 11
0019-9567/92/114593-05$02.00/0

Colony-Stimulating Factors Activate Human Macrophages To Inhibit Intracellular Growth of *Histoplasma capsulatum* Yeasts

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Received 15 June 1992/Accepted 21 August 1992

Recombinant cytokines and colony-stimulating factors (CSFs) were tested for their abilities to activate human monocytes/macrophages (Mϕ) to inhibit the intracellular growth of or kill *Histoplasma capsulatum* yeasts. None of the cytokines or CSFs or combinations of cytokines and CSFs activated Mϕ fungistatic activity when they were added to Mϕ monolayers concurrently with yeasts. In contrast, culture of monocytes for 7 days in the presence of interleukin 3, granulocyte-Mϕ CSF, or Mϕ CSF stimulated Mϕ fungistatic (but not fungicidal) activity against *H. capsulatum* yeasts in a concentration-dependent manner. Optimal activation of Mϕ by CSFs required 5 days of coculture, and the cultures had to be initiated with freshly isolated peripheral blood monocytes. Culture of monocytes with combinations of CSFs or addition of CSFs during the 24 h of coculture with the yeasts did not further enhance Mϕ fungistatic activity for *H. capsulatum*. Addition of gamma interferon or tumor necrosis factor alpha to CSF-activated Mϕ also did not enhance Mϕ fungistatic activity. These results suggest that interleukin 3, granulocyte-Mϕ CSF, and Mϕ CSF may play a role in the cell-mediated immune response to *H. capsulatum* by enhancing monocyte/Mϕ fungistatic activity.

Human monocytes, cultured monocyte-derived macrophages (Mϕ), alveolar Mϕ, and polymorphonuclear neutrophils recognize unopsonized *Histoplasma capsulatum* yeasts and conidia via the CD18 family of adhesion-promoting glycoproteins (3, 16, 26). Attachment of yeasts and conidia to Mϕ is followed rapidly by ingestion (16). Phagocytosis of the yeasts stimulates the Mϕ respiratory burst (3) and phagolysosomal fusion (19). Despite exposure of yeasts to the Mϕ antifungal armamentarium, ingested yeasts multiply readily within human monocytes/Mϕ (5, 18).

Recently, we demonstrated that human monocytes and Mϕ could be activated to inhibit the intracellular growth of yeasts in response to phytohemagglutinin-generated cytokine(s) (18). Maximum inhibition was observed when cytokines were added to cell monolayers immediately after infection. Opsonization of yeasts in normal serum or *H. capsulatum*-immune serum did not affect the intracellular generation time of yeasts in either control Mϕ or cytokine-activated Mϕ (18).

In the present studies, we tested several recombinant cytokines and colony-stimulating factors (CSFs) for their abilities to activate human Mϕ to restrict the intracellular growth of *H. capsulatum* yeasts. None of the cytokines or CSFs or combinations of cytokines and CSFs rapidly activated Mϕ fungistatic activity against *H. capsulatum* yeasts in a manner similar to that of phytohemagglutinin-generated cytokines. However, interleukin 3 (IL-3), granulocyte-Mϕ CSF (GM-CSF), and Mϕ CSF (M-CSF) did activate human Mϕ fungistatic activity when they were present during the in vitro maturation of monocytes into Mϕ.

MATERIALS AND METHODS

Cytokines. Recombinant IL-1, IL-2, IL-3, GM-CSF, M-CSF, granulocyte CSF (G-CSF), and IL-6 were a generous gift from Immunex Corporation, Seattle, Wash. Gamma interferon (IFN-γ) was provided by Immunex and Genentech, San Francisco, Calif., or was purchased from Collaborative Research, Cambridge, Mass. Tumor necrosis factor alpha (TNF-α), transforming growth factor β, and platelet-derived growth factor were kindly provided by Genentech. Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co., St. Louis, Mo.

Yeasts. *H. capsulatum* G217B was maintained as described previously (16). Yeasts were grown in HMM medium (32) at 37°C with orbital shaking at 150 rpm. After 2 to 3 days, they were harvested by centrifugation, washed three times in Hanks' balanced salt solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 0.25% bovine serum albumin (BSA) (HBSA), and resuspended to 30 ml in HBSA. Large aggregates were removed by centrifugation at 200 × *g* for 5 min at 4°C. The top 2 ml was removed, and the single-cell suspension obtained was standardized to 10^6 cells per ml in RPMI 1640 (GIBCO, Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum and 10 μg of gentamicin (Sigma) per ml (18).

Monocyte preparation and culture. Human peripheral blood mononuclear cells were prepared under sterile conditions by dextran sedimentation and Ficoll-Hypaque centrifugation as described previously (20). The mononuclear cells were washed in Hanks' balanced salt solution containing 20 mM HEPES and 10 μg of gentamicin per ml (Hanks'-HEPES) and suspended to 3×10^6 to 4×10^6/ml in Hanks'-HEPES containing 0.1% autologous serum. One-tenth-milliliter volumes of mononuclear leukocytes were adhered in 96-well tissue culture plates (Corning, Cambridge, Mass.) for 1 h at 37°C in 5% CO_2–95% air. The adherent monocytes were washed vigorously with Hanks'-HEPES to remove the lymphocytes and then were cultured in M199 (GIBCO) containing 10% autologous serum and 10 μg of gentamicin per ml.

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Alternatively, human monocytes were purified from buffy coats via sequential centrifugation on Ficoll-Hypaque and Percoll (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) gradients (6). The monocytes were cultured in suspension in Teflon beakers at 10^6/ml in RPMI 1640 containing 12.5% human serum and 10 μg of gentamicin per ml (3, 18). After 5 to 7 days of culture, Mϕ were washed and suspended in HBSA containing 0.3 U of aprotinin per ml. Mϕ were suspended to 0.5×10^6 to 1.0×10^6/ml, and 0.1 ml was added to the wells of a 96-well plate. After 1 h of adherence, the monolayers were washed twice in media and either cultured in medium with or without cytokines or CSFs or tested immediately in the fungistasis assay.

Assay of monocyte/Mϕ fungistatic activity against *H. capsulatum* yeasts. Mϕ fungistatic activity against *H. capsulatum* was quantified by the incorporation of [^{3}H]leucine into remaining viable yeasts (31). Supernatant was removed from the wells of monocytes/Mϕ cultured under various conditions, and 5×10^3 viable yeasts in 0.1 ml of RPMI 1640 containing 5% heat-inactivated fetal calf serum–10 μg of gentamicin per ml were added to each well. After incubation for 24 h at 37°C, the plates were centrifuged at $931 \times g$. The supernatant was carefully aspirated through a 27-gauge needle, and 50 μl of [^{3}H]leucine (specific activity, 153 Ci/mmol; New England Nuclear, Boston, Mass.) in sterile water (1.5 μCi) and 5 μl of a 10× yeast nitrogen broth (Difco Laboratories, Detroit, Mich.) were added to each well. After further incubation for 24 h at 37°C, 50 μl of L-leucine and 50 μl of sodium hypochlorite were added to each well. The contents of the wells were harvested onto glass fiber filters with an automated harvester (Skatron, Sterling, Va.). The filters were placed into scintillation vials, scintillation cocktail was added, and counts per minute were determined with a Beckman LS 7000 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.).

Several control experiments were performed to demonstrate that the counts per minute obtained from the incorporation of [^{3}H]leucine accurately quantified the growth of yeasts. First, control wells containing Mϕ but no yeasts did not incorporate significant amounts of [^{3}H]leucine (<300 cpm). Second, adherent Mϕ were allowed to phagocytose 5×10^3 yeasts for 1 h at 37°C, the monolayers were washed, and the cells were pulsed with [^{3}H]leucine for 24 h. In a second set of wells, 5×10^3 yeasts alone also were pulsed with [^{3}H]leucine. In either case, the average count obtained was about 2,000 cpm. In contrast, when 5×10^3 yeasts were first incubated for 24 h, either in culture medium or within Mϕ, and then pulsed for 24 h with [^{3}H]leucine, the counts obtained were 7- to 20-fold higher. Thus, the assay clearly quantifies the intracellular and extracellular growth of *H. capsulatum* yeasts. Interestingly, counts per minute in control wells containing untreated Mϕ and yeasts were consistently higher than those in control wells containing yeasts only. Thus, yeasts grew better inside Mϕ than they did in the tissue culture medium.

In a third set of controls, various numbers of yeasts were added in triplicate to the wells of a 96-well plate. The yeasts were pulsed with [^{3}H]leucine for 24 h, and the counts per minute obtained were plotted against the number of yeasts added to the wells. For 1×10^3 to 3×10^4 yeasts per well, there was a linear relationship in the counts per minute obtained and the number of yeasts per well. In addition, there was a linear relationship between the counts per minute obtained and the number of CFU obtained from the wells after the 24-h incubation period.

Finally, in numerous experiments, we find that the results obtained with the [^{3}H]leucine assay directly correlate with the results obtained with assays in which the intracellular growth of yeasts is quantified by counting the number of yeasts per infected Mϕ via phase-contrast microscopy (18).

As there was considerable variation in the counts per minute obtained for yeasts multiplying in untreated Mϕ, the data are presented as mean ± standard error of the mean (SEM) percent inhibition, which is defined as [1 − (cpm in activated Mϕ/cpm in control Mϕ)] × 100. All experimental procedures were performed in triplicate or quadruplicate, and all experiments were performed at least three times with cells from different donors.

RESULTS

Cytokines and CSFs do not rapidly activate cultured Mϕ to inhibit the intracellular growth of *H. capsulatum* yeasts. Previous studies (18) in our laboratory had demonstrated that a phytohemagglutinin-generated cytokine supernatant could activate cultured human monocyte-derived Mϕ to inhibit the intracellular growth of *H. capsulatum* yeasts. Optimal activation required that the cytokines be added to the Mϕ simultaneously with yeasts. Preincubation of Mϕ for 24 h with the cytokine supernatant actually resulted in less inhibition of the intracellular multiplication of yeasts (18).

Therefore, we tested the abilities of purified recombinant cytokines and CSFs, both singly and in various combinations, to rapidly activate Mϕ fungistasis against *H. capsulatum* yeasts. Suspension-cultured Mϕ were adhered in 96-well plates, washed, and then incubated with 5×10^3 viable yeasts in medium with or without various cytokines. After 24 h of culture, Mϕ fungistatic activity was quantified as described in Materials and Methods. Again, none of the factors tested activated Mϕ to inhibit the intracellular growth of yeasts. The cytokines tested included IL-1, IL-2, IL-3, IL-6, GM-CSF, M-CSF, G-CSF, IFN-γ, TNF-α, LPS, transforming growth factor β, and platelet-derived growth factor. Combinations of cytokines and growth factors that were without effect included IFN-γ plus TNF-α, IFN-γ plus IL-3, IFN-γ plus GM-CSF, IFN-γ plus LPS, IFN-γ plus M-CSF, GM-CSF plus IL-3, and M-CSF plus IL-3.

Activation of monocytes by CSFs during in vitro differentiation into Mϕ. We next attempted to induce Mϕ antifungal activity by culturing monocytes in the presence of various cytokines and CSFs for 7 days. Table 1 shows that of the cytokines and CSFs tested, only IL-3, GM-CSF, and M-CSF activated Mϕ to inhibit significantly ($P < 0.005$) the intracellular growth of yeasts. Activation of Mϕ fungistatic activity against *H. capsulatum* yeasts by these CSFs was concentration dependent (Fig. 1) and required 5 days of in vitro culture (Fig. 2). In contrast, when Mϕ cultured in suspension for 5 to 7 days were adhered in 96-well tissue culture plates and then further cultured for 1 to 7 days in the presence of CSFs, inhibition of yeast growth was never greater than 25% (Table 2).

CSFs did not affect the number of Mϕ that were present after 7 days of culture. In two experiments performed in triplicate, medium was removed from the wells of Mϕ cultured in medium with or without CSFs and the Mϕ were lysed with ZAP-OGLOBIN II (Coulter Diagnostics). The released cell nuclei then were quantified on a Coulter Counter (20). The mean number of Mϕ in control wells was 32,787. The mean numbers of Mϕ from wells cultured in the presence of IL-3, GM-CSF, and M-CSF were 37,873, 36,827, and 30,417, respectively.

Effects of combinations of IL-3, GM-CSF, and M-CSF on

TABLE 1. Inhibition of intracellular growth of *H. capsulatum* yeasts by monocytes cultured with cytokines or CSFs

Cytokine or CSF	% Inhibition of intracellular growth (no. of expts)[a]
IL-1	13.1 ± 6.6 (6)
IL-2	1.1 ± 1.0 (3)
IFN-γ	11.3 ± 5.2[b] (16)
TNF-α	6.0 ± 6.0 (5)
IL-3	61.0 ± 3.7[c] (12)
GM-CSF	61.4 ± 4.8[c] (12)
M-CSF	39.3 ± 4.7[c] (12)
G-CSF	21.7 ± 6.2 (7)

[a] Values are means ± SEM. All experiments were performed with cells from different donors.
[b] Combined results from experiments using IFN-γ from three commercial sources.
[c] Statistically significant ($P < 0.005$, *t* test) compared with control values. Results with other cytokines were not statistically different from control values ($P > 0.1$).

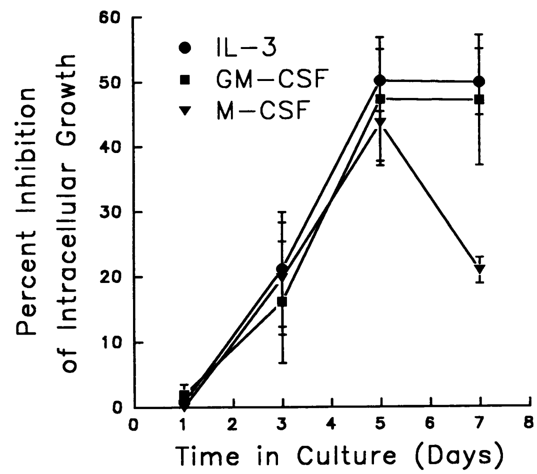

FIG. 2. Time course of CSF induction of human Mϕ fungistatic activity against *H. capsulatum* yeasts. The culture conditions were as described in the legend to Fig. 1, except that the concentration of CSFs used was 100 ng/ml. The data are means ± SEM from four experiments with cells from different donors.

monocyte antifungal activity. To determine whether Mϕ antifungal activity against *H. capsulatum* could be enhanced further, adherent monocytes were cultured for 7 days in the presence of IL-3, GM-CSF, or M-CSF or various combinations of these. The data in Table 3 show that combinations of CSFs did not enhance monocyte antifungal activity compared with that obtained with a single factor.

In additional experiments, monocytes were cultured for 7 days in the presence of IL-3, GM-CSF, or M-CSF. At the end of the culture period, the Mϕ were washed and then medium, the same CSF, or a different CSF was added to the differentiated Mϕ along with the yeasts. After a further 24 h of culture, Mϕ antifungal activity was quantified. The addition of various CSFs at the time of infection did not enhance Mϕ fungistatic activity compared with that obtained with a single factor (data not shown).

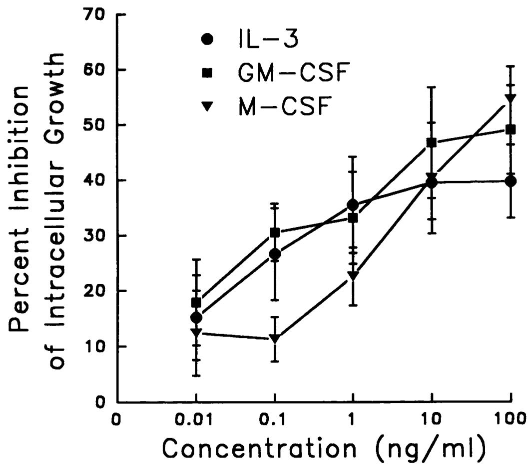

FIG. 1. Concentration-dependent CSF activation of human Mϕ to inhibit the intracellular growth of *H. capsulatum* yeasts. Adherent monocytes were cultured in medium with or without various concentrations of IL-3, GM-CSF, or M-CSF. After 7 days of culture, the Mϕ were washed and then incubated for 24 h with 5 × 10^3 yeasts. The percent inhibition of intracellular growth was calculated by comparing the counts per minute for control Mϕ cultures with the counts per minute for Mϕ cultured in the presence of CSFs, as described in Materials and Methods. The data are means ± SEM from five experiments with cells from different donors.

Effect of IFN-γ and TNF-α on CSF-induced Mϕ antifungal activity. IFN-γ (33) and TNF-α (11, 27) appear to play a role in host defense against *H. capsulatum* in the murine system. However, the data presented here indicate that neither of these cytokines is capable of activating human Mϕ fungistatic activity individually or in combination with each other. Therefore, we explored the possibility that IFN-γ and/or TNF-α might augment the Mϕ antifungal activity obtained when monocytes are cultured in the presence of CSFs. Monocytes were cultured for 7 days in medium or in the presence of IL-3, GM-CSF, or M-CSF. At the end of the culture period, the monolayers were washed and medium, IFN-γ, or TNF-α was added to the Mϕ along with the yeasts. Mϕ antifungal activity then was quantified after a further 24 h of culture. Neither IFN-γ nor TNF-α enhanced the ability of CSF-stimulated Mϕ to inhibit the intracellular growth of *H. capsulatum* yeasts (Table 4).

DISCUSSION

IL-3, GM-CSF, and M-CSF activate human monocyte/Mϕ antimicrobial activity against several intracellular pathogens, including *Trypanosoma cruzi* (23), *Leishmania donovani* (30), *Leishmania amazonensis* (9, 10), and *Mycobacterium avium* complex (1). GM-CSF and IL-3 also enhance the fungicidal activity of fresh monocytes against *Candida albi-*

TABLE 2. Lack of activation of differentiated Mϕ by culture with IL-3, GM-CSF, and M-CSF

Days in culture with cytokines	% Inhibition of intracellular growth with[a]: IL-3	GM-CSF	M-CSF
1	8.3 ± 2.9	13.3 ± 4.0	12.7 ± 1.9
3	7.8 ± 3.7	10.0 ± 6.4	12.0 ± 2.7
5	25.3 ± 11.5	22.7 ± 11.5	20.7 ± 11.3
7	12.2 ± 5.7	11.4 ± 6.1	10.3 ± 5.3

[a] Values are means ± SEM. $n = 6$.

TABLE 3. Lack of further enhancement of monocyte antifungal activity by combinations of IL-3, GM-CSF, and M-CSF

Additional cytokine (0.1 ng/ml)	% Inhibition of intracellular growth with[a]:		
	IL-3	GM-CSF	M-CSF
None	49.3	51.4	53.3
IL-3		64.4	53.5
GM-CSF	57.1		58.6
M-CSF	62.0	58.0	

[a] Values are means of two experiments from different donors. Cytokine concentration, 1.0 ng/ml.

cans (28, 29) and maintain this antifungal activity through 5 days of culture (29).

The present studies demonstrate that human Mϕ fungistatic activity against *H. capsulatum* yeasts is induced when monocytes are cultured in the presence of IL-3, GM-CSF, or M-CSF. Stimulation of Mϕ fungistatic activity by CSFs is concentration dependent and requires 3 to 5 days of culture. Interestingly, CSFs do not stimulate Mϕ fungistatic activity against *H. capsulatum* yeasts when monocytes are first cultured for 5 to 7 days in standard tissue culture medium and then further cultured in the presence of the CSFs. Thus, it appears that CSFs must be present during the process of monocyte differentiation into mature Mϕ for the CSFs to induce Mϕ fungistatic activity against *H. capsulatum* yeasts.

Maximum antifungal activity is obtained when monocytes are cultured with IL-3, GM-CSF, or M-CSF individually. Culture of monocytes with various combinations of two CSFs or addition of a second CSF during the 24-h incubation with yeasts does not enhance Mϕ antifungal activity compared with that obtained with a single CSF.

The data for human monocytes/Mϕ presented here contrast with the in vitro and in vivo data reported for the murine system which suggest that IFN-γ and TNF-α both may play a role in the activation of Mϕ antihistoplasma activity (11, 27, 33). Thus, regardless of whether human monocytes/Mϕ were cultured for 24 h or up to 7 days, neither IFN-γ nor TNF-α nor a combination of the two cytokines activated Mϕ to inhibit the intracellular growth of *H. capsulatum* yeasts. Furthermore, addition of IFN-γ or TNF-α to CSF-activated Mϕ during the 24-h incubation with yeasts also did not enhance Mϕ fungistatic activity.

Our data are in agreement with the studies of Fleischmann et al. (5) with regard to the inability of IFN-γ to activate human Mϕ fungistatic activity against *H. capsulatum* yeasts. However, the findings of both studies differ considerably from the report of Brummer et al. (2) that human monocytes actually killed intracellular *H. capsulatum* yeasts by an oxygen-dependent mechanism when cultured for 3 days with 500 U of IFN-γ per ml. This result is based on a 2-h challenge with yeasts followed by lysis of the Mϕ and plating of the yeasts on brain heart infusion agar containing 10% sheep blood. In contrast, both the present study and that of Fleischmann et al. (5) examined the fate of yeasts after 24 h of incubation with Mϕ.

One explanation for these discrepant results is that although some of the ingested histoplasmas may be killed shortly after ingestion by Mϕ, the majority survive and multiply. Thus, after 24 h, the rapid growth of surviving organisms obscures the fact that some are killed during the early stages of infection. However, the data of Brummer et al. (2) are difficult to interpret, since the authors do not account for all of the yeasts that are added to the Mϕ. Thus, of 2,000 fungal U added per Mϕ monolayer, 384 CFU was determined to be noningested or nonadherent and 240 CFU was recovered from lysed, nonactivated control Mϕ. Therefore, only 624 CFU (31%) was recovered from the initial 2,000 CFU added to the Mϕ.

The mechanism(s) by which CSFs stimulate human Mϕ fungistatic activity against *H. capsulatum* yeasts is unknown. However, it is unlikely that fungistasis is mediated through an oxygen-dependent mechanism, as has been suggested for *L. amazonensis*, *L. donovani*, *T. cruzi*, and *C. albicans* (9, 23, 24, 28). *H. capsulatum* yeasts activate the respiratory burst of human monocytes/Mϕ and neutrophils upon phagocytosis (3, 25, 26) but are not killed (5, 18, 25). Furthermore, the intracellular growth of *H. capsulatum* yeasts proceeds at a similar rate both in freshly isolated monocytes and in cultured Mϕ that have no myeloperoxidase and a decreased ability to produce toxic oxygen metabolites (12, 21, 24). In addition, we have found that all of the fungistatic activity of human neutrophils is mediated by a nonoxidative mechanism(s) (15). Therefore, these data suggest that yeasts are resistant to toxic oxygen metabolites produced by human phagocytes.

Nitric oxide produced from L-arginine metabolism has been reported to be involved in the killing of several intracellular parasites by mouse peritoneal Mϕ (8, 14). Nitric oxide also may play a role in inhibiting the intracellular growth of *H. capsulatum* yeasts in activated murine splenic Mϕ (11). However, regardless of the cytokines used to stimulate human Mϕ, nitrite (quantified by using the Greiss reagent [7]) is not detected in the culture medium (17). Moreover, N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthetase (22), does not affect the intracellular growth of *H. capsulatum* yeasts within human Mϕ (17). These results are in agreement with the reports of others that nitric oxide does not appear to play a role in the antimicrobial activity of human Mϕ against *Toxoplasma gondii*, *Chlamydia psittaci*, *L. donovani* (13), or *Cryptococcus neoformans* (4). Therefore, the inhibition of the intracellular growth of yeasts by cytokine-activated monocytes/Mϕ probably is mediated by an oxygen-independent pathway. Further studies will be required to define the mechanism(s) by which activated human Mϕ mediate fungistatic activity against *H. capsulatum* yeasts.

TABLE 4. Lack of enhancement by IFN-γ and TNF-α of monocyte activation by IL-3, GM-CSF, and M-CSF

Secondary cytokine[a]	% Inhibition of intracellular growth with[b]:			
	Medium	IL-3	GM-CSF	M-CSF
None (medium)		67.9 ± 6.8	73.4 ± 3.6	44.7 ± 2.0
IFN-γ	7.6 ± 7.5	58.2 ± 12.5	65.4 ± 4.4	38.9 ± 2.2
TNF-α	5.9 ± 5.8	65.3 ± 8.8	63.2 ± 3.5	53.0 ± 3.6

[a] Present during the 24-h incubation with yeasts.
[b] Values are means ± SEM. $n = 3$.

ACKNOWLEDGMENTS

We thank George Deepe for help with the statistics and Ward Bullock for critical reading of the manuscript.

This work was supported by Public Health Service grant AI-28392 from the National Institute of Allergy and Infectious Diseases and a grant from the Western-Southern Foundation.

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