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# Gamma Interferon Cooperates with Lipopolysaccharide To Activate Mouse Splenic Macrophages to an Antihistoplasma State

THOMAS E. LANE, BETTY A. WU-HSIEH, AND DEXTER H. HOWARD*

*Department of Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, California 90024*

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**Inhibition of the intracellular growth of *Histoplasma capsulatum* by murine resident red pulp splenic macrophages was examined. Splenic macrophages, unlike resident peritoneal macrophages, required a prolonged preincubation (18 h) with recombinant murine gamma interferon (rMuIFN-γ) for activation. To be fully activated, the splenic macrophages required incubation with rMuIFN-γ in combination with 0.1 μg of lipopolysaccharide (LPS) per ml. Splenic macrophages stimulated with rMuIFN-γ, LPS, or rMuIFN-γ and LPS produced tumor necrosis factor alpha (TNF-α), but recombinant murine TNF-α (rMuTNF-α) did not activate macrophages when used alone or as a second signal with rMuIFN-γ. Anti-TNF-α antibody did not block IFN-γ–LPS activation of splenic macrophages to any significant extent. One hundred micromolar ferrous sulfate antagonized IFN-γ–LPS activation of splenic macrophages, indicating that iron was involved in the fungistatic activity of cytokine-stimulated phagocytes. Our results indicate that (i) splenic macrophages differ significantly from peritoneal macrophages in their requirements for activation and (ii) the mechanism by which splenic macrophages exert their antifungal effects involves iron.**

The zoopathogenic fungus *Histoplasma capsulatum* is a facultative intracellular pathogen that invades the mononuclear phagocyte system of animals. Cell-mediated immunity appears to play a major role in host defense against *H. capsulatum* (8, 30, 31). $CD4^+$ T cells isolated from immune mice transfer immunity against infection by *H. capsulatum* to naive mice (8, 24, 26). In vitro studies have shown that recombinant murine gamma interferon (rMuIFN-γ) activates normal peritoneal macrophages to inhibit the intracellular growth of the fungus (30).

Studies on the interaction between murine macrophages and yeast cells of *H. capsulatum* have been conducted primarily with peritoneal macrophages (15, 27–30). However, peritoneal macrophages do not represent the type of phagocyte infected upon dissemination of the organism within the host. Following intravenous injection of *H. capsulatum*, yeast cells circulate through organs of the reticuloendothelial system such as the liver and the spleen (1) and come to reside in the macrophages of those organs. Splenic macrophages may play a key role in clearance of the fungus within the infected host. However, the interaction of splenic macrophages with *H. capsulatum* yeast cells has not been examined. Resident red pulp splenic macrophages share characteristics with peritoneal macrophages, such as expression of Fc receptors, Ia antigen, and the macrophage-specific antigen F4/80 (19). Therefore, we set out to determine if splenic macrophages could be activated to either kill or inhibit the intracellular growth of *H. capsulatum*.

Our results indicate that resident splenic macrophages are markedly different from peritoneal macrophages in their requirements for activation. Splenic macrophages require a prolonged preincubation with rMuIFN-γ to be activated, and in order to become fully activated the macrophages require a second signal, e.g., lipopolysaccharide (LPS) in the studies to be reported here. Splenic macrophages are stimulated by LPS to produce tumor necrosis factor alpha (TNF-α), but recombinant murine TNF-α (rMuTNF-α) did not combine with rMuIFN-γ to activate macrophages in vitro to inhibit *H. capsulatum*. Inclusion of 100 μM ferrous sulfate antagonized rMuIFN-γ–LPS-induced activation of splenic macrophages, indicating that iron is involved in the antifungal effects induced in cytokine-stimulated macrophages.

## MATERIALS AND METHODS

**Mice.** Inbred male C57BL/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Age-matched 6- to 8-week-old mice were used for all experiments.

**Fungus.** *H. capsulatum* 505, which has been used in experiments previously reported (15, 29–31), was employed in these studies. Yeast cells were grown on blood-cysteine-glucose agar slants at 37°C for 48 to 72 h prior to use.

**Reagents and media.** Splenic and peritoneal macrophage monolayers were cultured in modified Eagle's medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated defined fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, Utah) and supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) 15 mM glucose, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (SMEM). Phenol-extracted LPS from *Escherichia coli* serotype O11:B4 and ferrous sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. The rMuIFN-γ was supplied by Genentech Inc., South San Francisco, Calif. The rMuTNF-α and rabbit anti-mouse polyclonal anti-TNF-α antibody were purchased from Genzyme Corp., Cambridge, Mass. Possible contamination by extraneous endotoxin was monitored as follows. Each experiment contained a medium control without added stimulators, and activation of macrophages in these control situations was not observed. The FBS had 0.8 ng of endo-

* Corresponding author.

toxin per ml (supplier's assay), and the rMuIFN-γ had <10 pg of endotoxin per ml (supplier's assay).

**Macrophages.** Splenic macrophages were isolated by the method described by Nusrat et al. (19). Briefly, spleens were removed from mice and perfused with collagenase (170 U/ml, type D; Boehringer Mannheim, Indianapolis, Ind.) and DNase (20 μg/ml, type I; Boehringer Mannheim) dissolved in prewarmed (37°C) Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.9 mM $CaCl_2$–5.5 mM D-glucose (DPBSG) and adjusted to pH 7.3 (19). Following perfusion, the cell suspension was added to a 50-ml Teflon beaker and mixed by pipette for 8 min at 37°C. The cell suspension was passed through a 40-μm-pore-size nylon mesh, spun at 250 × *g* for 10 min at 4°C, and resuspended in a 63.6% isotonic Percoll gradient (Pharmacia, Uppsala, Sweden) in $Ca^{2+}$-$Mg^{2+}$-free DPBS containing 9% heat-inactivated defined FBS and 36 μg of DNase I per ml. The gradient was overlaid with 1.5 ml of DPBS and centrifuged at 1,800 × *g* for 15 min at 4°C. The interface was collected and washed twice with DPBSG containing 1% FBS, and the cells were resuspended in medium at a final concentration of $4 \times 10^7$ per ml. One hundred microliters of the suspension was seeded onto 15-mm-diameter Formvar-coated glass coverslips (Bellco Glass Inc., Vineland, N.J.) in 24-well tissue culture plates (Corning Glass Works, Corning, N.Y.) and incubated at 37°C in a 5% $CO_2$ atmosphere for 1 h. The process was repeated once more for a total of two seedings for each coverslip. The coverslips were then vigorously washed with prewarmed Hanks' balanced salt solution to remove nonadherent cells. Greater than 80% of adherent cells expressed the macrophage-specific F4/80 antigen (13, 19) as measured by immunofluorescence (data not shown).

Peritoneal cells were harvested from the peritoneal cavities of mice as previously described (15, 29, 30). The peritoneal cells were then subjected to the same isolation technique as described for splenic macrophages. The peritoneal cells were suspended in medium at a concentration of $10^6$ per ml. One hundred microliters of the cell suspension was seeded onto 15-mm-diameter Formvar-coated glass coverslips in 24-well tissue culture plates. Only a single seeding was done for peritoneal cells. The cells were incubated at 37°C in a 5% $CO_2$ atmosphere for 2 h and vigorously washed to remove nonadherent cells. This technique resulted in a confluent monolayer of peritoneal macrophages.

**Assay for macrophage activation.** Splenic and peritoneal macrophages were isolated and seeded onto Formvar-coated glass coverslips as described above and used immediately for the macrophage assay. In brief, macrophage monolayers were incubated in 1 ml of SMEM with or without the activating factors (rMuIFN-γ, rMuTNF-α, and LPS) for either a 3.5- or 18-h preincubation. At the completion of the preincubation, yeast cells of *H. capsulatum* were washed and approximately $2 \times 10^5$ to $3 \times 10^5$ cells were added to the macrophage monolayer. Two hours were allowed for phagocytosis, and then extracellular yeasts were washed away. Two coverslips that were not exposed to any activating agent(s) were fixed in methanol in order to determine the number of yeast cells per macrophage at the start of incubation. Control studies showed that preexposure to cytokine or LPS did not increase phagocytosis, and macrophages, in the presence of activating agents, had nearly the same number of yeast cells per macrophage as did those unexposed to activating agents. The remaining monolayers were overlaid with either medium alone or medium containing the activating agent(s) corresponding to the preincubation protocol and incubated for an additional 18 h at 37°C. After the second incubation period the monolayers were washed and fixed in methanol. Monolayers were stained with periodic acid-Schiff stain, and the mean number of yeast cells within infected macrophages was determined by counting the yeast cells in a minimum of 100 infected cells. The net growth was calculated by subtracting the mean number of yeast cells per infected macrophage at zero time from the mean number of yeast cells per macrophage at the conclusion of the experiment. The percent inhibition of growth was calculated from the following formula: [1 − (growth within macrophages in various test media/growth within macrophages in control medium)] × 100.

**Assay for antagonistic effect of iron on activated splenic macrophages.** Macrophage monolayers were incubated as described for the macrophage activation assay except that the monolayers treated with IFN-γ–LPS were exposed to 100 μM $FeSO_4$ prior to and following exposure to yeast cells of *H. capsulatum*. The antagonistic effect of iron on IFN-γ–LPS-induced activation of splenic macrophages was determined by counting the number of intracellular yeast cells as described above.

**TNF-α production and assay.** Splenic macrophages were seeded onto Formvar-coated coverslips and incubated with IFN-γ (2,000 U/ml), LPS (0.1 μg/ml), or both in 1 ml of SMEM for 36 h. Yeast cells were added following an 18-h incubation period. Two hours were allowed for phagocytosis, and then unphagocytized yeast cells were washed away. Macrophage monolayers were incubated for an additional 18 h in medium containing activating factors to which the cells were previously exposed. Cell-free supernatants from wells were collected at 4, 8, 18, 20, and 36 h following adherence to coverslips. Supernatants were assayed for TNF-α according to the method described by Merrill et al. (18). The murine L929 cell line (American Type Culture Collection), retrieved in log growth phase by trypsinization, was washed several times, labelled with chromium in a total volume of 1.0 ml with 0.25 mCi of $^{51}Cr$ (Amersham International) for 1 h, and washed three times in medium. Labelled target cells were plated with test sample dilutions in 96-well U-bottom plates (Linbro Titertek; Flow Laboratories, McLean, Va.) at a concentration of $5 \times 10^5$ cells per ml with 5 μg of actinomycin D per ml. Eighteen hours later, plates were centrifuged at 600 × *g*, 50 μl of supernatant was aspirated, and radioactivity was counted in a gamma counter. rMuTNF-α standards were the generous gift of Cetus Corp. (Norwalk, Conn.). These were titrated from $10^4$ U/ml (25 nM) to $10^{-3}$U/ml (2.5 fM).

**Statistical analyses.** Statistical analyses were performed by the Student *t* test.

## RESULTS

**IFN-γ alone fails to fully activate splenic macrophages to an antihistoplasma state.** Preexposure to rMuIFN-γ is essential for activation of normal peritoneal macrophages to an antihistoplasma state (30). We first studied whether resident splenic macrophages had the same requirements as those of peritoneal macrophages for activation to an antihistoplasma state. Peritoneal and splenic macrophages were isolated from the same mice and incubated with either 10 or 2,000 U of rMuIFN-γ per ml for either 3.5 or 18 h prior to the addition of *H. capsulatum*. Following exposure to yeast cells, the macrophages were incubated for an additional 18 h in the presence of rMuIFN-γ and their activated state was measured as described in Materials and Methods. The results recorded in Table 1 indicate that a preexposure to

TABLE 1. rMuIFN-γ does not fully activate murine splenic macrophages to an antihistoplasma state

| Macrophages | rMuIFN-γ (U/ml) | Time (h) of preexposure[a] | No. of yeast cells/infected macrophage[b] | P[c] | Growth[d] | % Inhibition[e] |
|---|---|---|---|---|---|---|
| Peritoneal | 0 | | 6.9 ± 1.6 | | 5.0 | 0 |
| | 10 | 3.5 | 3.7 ± 1.3 | ≤0.05 | 1.8 | **64** |
| | 10 | 18 | 2.6 ± 0.6 | ≤0.02 | 0.7 | **86** |
| | 2,000 | 3.5 | 3.1 ± 1.0 | ≤0.05 | 1.2 | **76** |
| | 2,000 | 18 | 2.3 ± 0.9 | ≤0.001 | 0.4 | **92** |
| Splenic | 0 | | 7.9 ± 1.2 | | 5.7 | 0 |
| | 10 | 3.5 | 7.9 ± 0.9 | | 5.8 | 0 |
| | 10 | 18 | 5.0 ± 0.4 | ≤0.02 | 2.9 | **50** |
| | 2,000 | 3.5 | 6.8 ± 1.1 | NS | 4.7 | 19 |
| | 2,000 | 18 | 5.0 ± 0.7 | ≤0.02 | 2.9 | **50** |

[a] Time of preexposure indicates the time for which macrophage monolayers were exposed to rMuIFN-γ prior to the addition of *H. capsulatum*. Following exposure to the yeast cells, the monolayers were washed and incubated for an additional 18 h in medium containing the same concentration of rMuIFN-γ as that to which the monolayers were previously exposed. Both splenic and peritoneal macrophages were collected from the same mice for all experiments.

[b] The results are means ± standard deviations for five experiments. The mean ± standard deviation at zero time was 1.9 ± 0.3 yeast cells per macrophage for peritoneal macrophages and 2.1 ± 0.7 yeast cells per macrophage for splenic macrophages. The zero time readings were nearly the same after exposure to rMuIFN-γ for both peritoneal and splenic macrophages.

[c] Significance was determined by the Student *t* test for the numbers of yeast cells per infected macrophage. Values for macrophages exposed to IFN-γ were compared with the values for macrophages unexposed to IFN-γ (controls).

[d] Growth is recorded as the net growth, which was calculated by subtracting the zero time mean number of yeast cells per infected macrophage from the mean number of yeast cells per infected macrophage after 18 h of incubation at 37°C.

[e] Percent inhibition represents the percent reduction of the growth of yeast cells within infected macrophages incubated in various test media compared with the growth of yeast cells within infected macrophages incubated in control medium. Statistically significant data are indicated in boldface.

TABLE 2. LPS cooperates with rMuIFN-γ to activate murine splenic macrophages to an antihistoplasma state

| Row | rMuIFN-γ (U/ml) | LPS (μg/ml) | Time (h) of preexposure[a] | No. of yeast cells/infected macrophage[b] | P[c] | Growth[d] | % Inhibition[e] |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | | 7.9 ± 1.2 | | 5.7 | 0 |
| 2 | 10 | 0 | 3.5 | 7.2 ± 0.9 | NS | 5.0 | 12 |
| 3 | 10 | 0 | 18 | 4.8 ± 0.4 | ≤0.05 | 2.6 | **54** |
| 4 | 2,000 | 0 | 3.5 | 6.3 ± 1.5 | NS | 4.1 | 28 |
| 5 | 2,000 | 0 | 18 | 5.3 ± 1.1 | ≤0.05 | 3.1 | **46** |
| 6 | 0 | 0.1 | 3.5 | 6.5 ± 0.6 | NS | 4.3 | 25 |
| 7 | 0 | 0.1 | 18 | 7.7 ± 1.2 | NS | 5.5 | 3 |
| 8 | 10 | 0.1 | 3.5 | 4.4 ± 0.8 | NS | 2.2 | 61 |
| 9 | 10 | 0.1 | 18 | 3.7 ± 0.1 | NS | 1.5 | 74 |
| 10 | 2,000 | 0.1 | 3.5 | 4.6 ± 0.1 | NS | 2.4 | 58 |
| 11 | 2,000 | 0.1 | 18 | 3.1 ± 0.5 | ≤0.01 | 0.9 | **84** |

[a] Time of preexposure indicates the time for which macrophage monolayers were exposed to rMuIFN-γ or LPS or both prior to the addition of *H. capsulatum*. Following exposure to yeast cells, the monolayers were incubated for an additional 18 h in medium containing the same concentration of agent(s) as that to which the monolayers were previously exposed.

[b] Results are means ± standard deviations for five experiments. The mean ± standard deviation at zero time was 2.2 ± 0.3 yeast cells per infected macrophage.

[c] Significance determined by the Student *t* test for the numbers of yeast cells per infected macrophage. The statistical significance of the following comparisons is shown: rows 2 to 5 with row 1; row 6 and row 7 with row 1; row 8 with row 2; row 9 with row 3; row 10 with row 4; and row 11 with row 5.

[d] Calculated as described in Table 1, footnote *d*.

[e] Calculated as described in Table 1, footnote *e*.

either 10 or 2,000 U/ml for 3.5 h did not activate splenic macrophages to an antihistoplasma state while peritoneal macrophages were activated under such conditions. Increasing the preexposure time to 18 h for either concentration of rMuIFN-γ resulted in enhanced antihistoplasma activity by the splenic macrophages, yet these cells were not fully activated when compared with peritoneal macrophages treated under the same conditions. These data indicate that splenic macrophages differ from peritoneal macrophages in their requirements for activation to an antihistoplasma state. A prolonged preexposure is a minimal requirement to activate these phagocytes.

**LPS cooperates with IFN-γ in activating splenic macrophages.** Numerous studies have demonstrated that rMuIFN-γ can prime macrophages to become sensitive to triggering signals such as LPS (20, 21). To investigate what was required to fully activate splenic macrophages, we used 0.1 μg of LPS per ml in combination with either 10 or 2,000 U of rMuIFN-γ per ml. The results recorded in Table 2 show that an exposure to 10 or 2,000 U of rMuIFN-γ per ml activated splenic macrophages to an antihistoplasma state only after an 18-h exposure time (Table 2, rows 2 to 5), a result that duplicated the previous observations recorded in Table 1. There was an increase in the antihistoplasma activity of splenic macrophages when both LPS and rMuIFN-γ were used but only with the higher concentration of rMuIFN-γ and only after an 18-h preexposure to that level of cytokine (Table 2, rows 8 to 11). LPS alone did not activate splenic macrophages (Table 2, rows 6 and 7). The antihistoplasma effects noted were inhibitory and not fungicidal, because all yeast cells released from splenic macrophages germinated when plated in slide cell chambers (data not shown).

**Splenic macrophages treated with IFN-γ or LPS or both IFN-γ and LPS are stimulated to release TNF-α.** It has been reported that TNF-α is released from peritoneal macrophages following exposure to rMuIFN-γ–LPS (9). To determine whether TNF-α was released as a result of the rMuIFN-γ–LPS treatment used in our study (Table 2), we collected supernatants from macrophage monolayers. Supernatants collected at 4, 8, 18, 20, and 36 h after adherence were analyzed for the presence of TNF-α. The results in Fig. 1 show that rMuIFN-γ and LPS, alone and in combination, stimulated the release of TNF-α. The combination of both IFN-γ and LPS resulted in production of TNF-α at approximately the same level as that obtained with LPS alone. These results indicate that IFN-γ and LPS, alone or in combination, stimulated splenic macrophages to release TNF-α. Such results suggested that LPS was stimulating macrophages to release TNF-α, which functioned as an autocrine in concert with rMuIFN-γ to provide the results recorded in Table 2. We set about to test this possibility.

**TNF-α does not cooperate with IFN-γ to activate splenic macrophages to antihistoplasma activity.** Work in our laboratory (31) and that of others (22) has demonstrated a role for TNF-α in host defense against *H. capsulatum*. It has been shown by histological methods that splenic macrophages from mice depleted of endogenous TNF-α are not able to contain the intracellular growth of *H. capsulatum* (31). These observations suggest that endogenous TNF-α plays a role in activation of splenic macrophages. The role of TNF-α

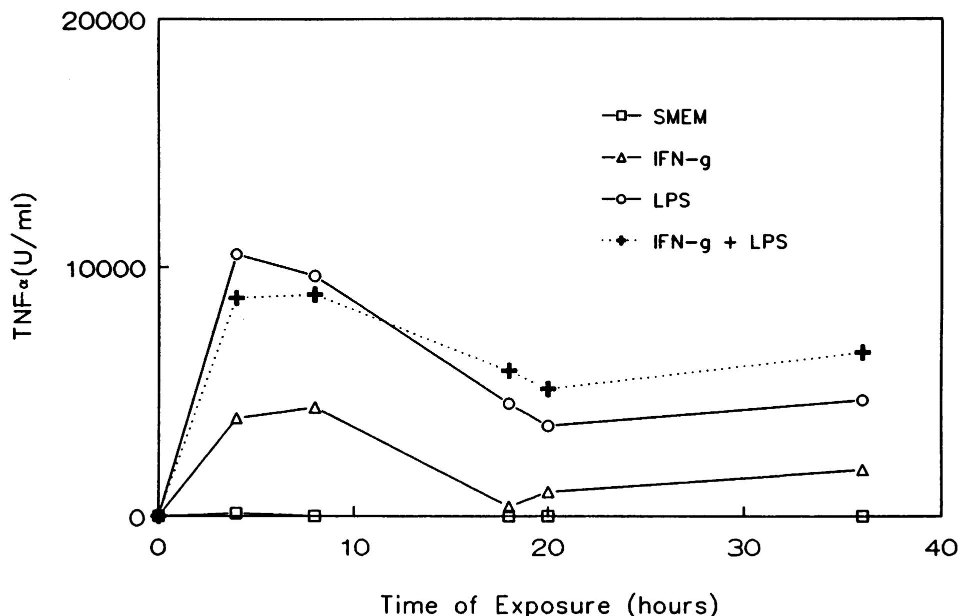


FIG. 1. Splenic macrophages treated with rMuIFN-γ, LPS, or both are stimulated to release TNF-α. Supernatants from treated wells were collected at 4, 8, 18, 20, and 36 h following adherence to coverslips and analyzed for TNF-α as described in Materials and Methods. Three experiments were performed. The data shown are from a representative experiment.

in activation of splenic macrophages was tested in vitro. rMuTNF-α alone at 2,000 U/ml did not activate splenic macrophages and combined with rMuIFN-γ did not activate splenic macrophages to any significant degree (data not shown). In further experiments the presence of 200 neutralizing units of anti-TNF-α did not significantly block activation of splenic macrophages by the combination of rMuIFN-γ and LPS (data not shown). Increasing the concentration of antibody did not alter the result.

**Iron reverses the activation of splenic macrophages by IFN-γ–LPS.** We have previously shown that iron limitation is one of the bases for the IFN-γ-induced antihistoplasma effect of mouse peritoneal macrophages (15). The results in Table 3 show that addition of 100 μM ferrous sulfate to cultures of IFN-γ–LPS-treated splenic macrophages partially abrogated activation by 2,000 U of rMuIFN-γ per ml together with 0.1 μg of LPS per ml. The addition of the reducing agent sodium thiosulfate (6) did not enhance the effect of iron on IFN-γ–LPS-treated macrophages (data not shown). In addition, increasing the concentration of ferrous sulfate did not result in a further reduction of percent intracellular growth induced by IFN-γ–LPS (data not shown). These results suggest that the mechanism by which the IFN-γ–LPS combination stimulates splenic macrophages to an antihistoplasma state involves the availability of iron (10, 15).

TABLE 3. Iron antagonizes activation of splenic macrophages

| Treatment | No. of yeast cells/infected macrophage[a] | Growth[b] | % Inhibition[c] |
|---|---|---|---|
| SMEM | 7.7 ± 1.0 | 5.6 | 0 |
| IFN-γ (2,000 U/ml) + LPS (0.1 μg/ml) | 2.8 ± 0.7 | 0.7 | 88 |
| IFN-γ (2,000 U/ml) + LPS (0.1 μg/ml) + 100 μM $FeSO_4$ | 5.0 ± 0.7 | 2.9 | 48[d] |

[a] Results are means ± standard deviations for three experiments. The mean ± standard deviation at zero time was 2.1 ± 0.1 yeast cells per infected macrophage.
[b] Calculated as described in Table 1, footnote *d*.
[c] Calculated as described in Table 1, footnote *e*.
[d] $P \leq 0.001$.

## DISCUSSION

The spleen represents a site of infection upon dissemination of *H. capsulatum* within the host (1, 31). Clearance of the organism from an infected animal often correlates with clearance from the spleen, suggesting that splenic macrophages may have a profound influence on the course of the disease. Identifying the factors required for activating splenic macrophages to an antihistoplasma state would offer a better understanding of how these phagocytes cope with infection. Unlike peritoneal macrophages, treatment of splenic macrophages with rMuIFN-γ alone does not fully activate the cells to an antihistoplasma state. A second signal, LPS in this study, which can cooperate with rMuIFN-γ to activate splenic macrophages is required. Such synergism between cytokines has been reported by others. For example, Hockertz et al. (11) demonstrated that a combination of rMuIFN-γ and muramyltripeptide activated splenic macrophages in vitro to kill *Leishmania donovani* promastigotes. Treatment of splenic macrophages with IFN-γ alone resulted in limited antimicrobial activity, while exposure to muramyltripeptide alone did not activate the macrophages. These results support our observation that murine resident splenic macrophages require a second signal in addition to rMuIFN-γ for activation to an antimicrobial state.

Ingestion of erythrocytes (RBC) has been shown to inhibit macrophage-mediated cytotoxicity against tumor targets (25). The splenic macrophage isolation protocol described results in the sedimentation of RBC following passage of the spleen cells through the Percoll gradient (19). Thus, it is

unlikely that the deficient antihistoplasma response of the splenic macrophages to single biological response modifiers (i.e., rMuIFN-γ, LPS, or rMuTNF-α) is due to phagocytosis of RBC during the isolation procedure.

Our work (31) as well as that by others (22) has demonstrated a role for TNF-α in host defense against *H. capsulatum*. TNF-α is produced in spleens of mice infected with *H. capsulatum* (31). Mice infected with a sublethal dose of *H. capsulatum* and injected with a polyclonal anti-TNF-α antibody had increased mortality rates and significantly higher fungous counts in various tissues than did mice which did not receive the antibody (31). We have shown that IFN-γ and LPS alone or together induce the release of TNF-α by splenic macrophages. This suggested that TNF-α produced upon IFN-γ–LPS treatment may be acting in an autocrine manner to activate splenic macrophages (12). But evidence for this presumption was not obtained. Drapier et al. (7) have reported that anti-TNF-α antibody did not completely suppress nitrite production or the antitumor properties of IFN-γ–LPS-treated mouse peritoneal macrophages. It is possible that following IFN-γ–LPS treatment of splenic macrophages there is an increase not only of secreted TNF-α but also of plasma membrane-associated TNF-α, which may have a role in activation (2). The anti-TNF-α antibody may not completely block activation of splenic macrophages because it is unable to effectively bind membrane-associated TNF-α or has limited access to it because LPS causes rapid internalization of TNF-α receptors (4, 5, 23). Additionally, it is possible that intracellular precursors of TNF-α may have a major role in triggering the macrophage-mediated antihistoplasma mechanism. Another possibility is that monokines other than TNF-α are produced following IFN-γ–LPS treatment which are capable of triggering the macrophage response (14, 21). This suggestion is likely on the basis of some of our recent work that shows that splenic macrophages from *Histoplasma*-infected mice are fully activated (unpublished observation). Further work needs to be done in order to resolve the cytokines involved in splenic macrophage activation.

Lepay et al. (17) have demonstrated that following a sublethal infection of mice with *Listeria monocytogenes* there is an influx of immigrant macrophages to the liver. Immigrant macrophages, unlike Kupffer cells, produced substantial levels of $H_2O_2$, suggesting that these cells, not the Kupffer cells, were functioning in host defense (16, 17). A similar phenomenon occurs in the spleens of mice infected with *L. monocytogenes* (19). Resident splenic macrophages are unable to produce reactive oxygen intermediates following exposure to IFN-γ, which suggests that these cells may have an impaired antimicrobial defense system (19). However, following infection of mice with *L. monocytogenes*, splenic inflammatory macrophages actively released $H_2O_2$ (19). These observations suggest that resident tissue macrophages and immigrant macrophages have distinct functions (17). Ding and Nathan (3) have suggested that the defect in the respiratory burst by Kupffer cells is the result of frequent endocytic encounters with RBC, immune complexes, and various other ligands. These events allow the Kupffer cell to act as an effective scavenger without producing oxidative metabolites which may be damaging to bystander cells (3). A similar sequence of events may occur in the spleen. Following exposure to a sublethal infection of *H. capsulatum*, there is a massive influx of macrophages to the spleen (1, 31). Wolf et al. have demonstrated that IFN-γ-treated mouse peritoneal macrophages exhibit antifungal activity yet do not produce reactive oxygen intermediates despite extensive phagocytosis of the organism (27, 28). This fact suggests that the macrophage oxidative burst does not play a role in macrophage defense against intracellular *H. capsulatum* (27, 28). It is possible that resident splenic macrophages, like Kupffer cells, function as scavengers in removing debris from the spleen and that immigrant macrophages in the spleen may be the effector cells in host defense against *H. capsulatum*. We are currently investigating this possibility.

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