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Blockade of Endogenous TNF-α Exacerbates Primary and Secondary Pulmonary Histoplasmosis by Differential Mechanisms

Ruth Allendoerfer and George S. Deepe, Jr.²

We investigated the mechanisms by which endogenous TNF-α modulates host defenses during experimental primary and secondary pulmonary infection with *Histoplasma capsulatum* (Hc). Neutralization of TNF-α in vivo resulted in increased CFU and 100% mortality in naive and immune mice challenged with Hc intranasally. Levels of IFN-γ and granulocyte macrophage-CSF were elevated in TNF-α-neutralized naive mice, whereas IL-4, -6, -10 and TGF-β did not differ from controls. In contrast, in secondary histoplasmosis, significant elevations of IL-4 and -10 were observed in TNF-α-depleted mice. Alveolar macrophages (Mø) did not exert fungistatic activity against Hc after exposure to recombinant murine TNF-α, recombinant murine IFN-γ, or both. The increase in susceptibility to primary Hc infection was associated with diminished production of reactive nitrogen intermediates by alveolar Mφ from TNF-α-depleted mice, whereas production of nitric oxide during secondary histoplasmosis was similar in both groups. Upon secondary challenge, TNF-α-depleted mice were rescued by concomitant neutralization of IL-4 and IL-10, but not either cytokine alone. Thus, TNF-α is critical for controlling primary and secondary infection with Hc, and the mechanisms that lead mice to succumb to primary or secondary infection when endogenous TNF-α is blocked are different. *The Journal of Immunology*, 1998, 160: 6072–6082.

*Histoplasma capsulatum* (Hc) is a dimorphic intracellular pathogenic fungus endemic to the midwestern and southeastern regions of the United States (1). Although mostly an asymptomatic infection in the immunocompetent host, histoplasmosis in immunocompromised individuals is progressive and fatal if left untreated (2, 3). Microconidia are inhaled, convert into yeasts in the lung, and are phagocytosed by alveolar macrophages (Mφ), within which they multiply. Resolution of infection depends primarily on cell-mediated immunity and requires the coordinated cooperation between Mφ and T cells (4).

Activation of Mφ, the principal effector cells, are central to the immune response against Hc (4). One mechanism to achieve this state is by release of cytokines from immunocompetent cells. Mφ produce monokines that play an important part in the generation and amplification of the host response to this fungus. One monokine that affects protective immunity to Hc is IL-12, and its effects are mediated by induction of IFN-γ production (5). Although IL-12 is an absolute requisite for survival in primary infection with Hc, it does not alter mortality in secondary histoplasmosis (6).

TNF-α is involved in the immune response to Hc. This contention is supported by observations that murine Mφ infected with Hc produce abundant TNF-α, and by reports demonstrating that depletion of TNF-α in vivo increased mortality of mice infected i.v. with Hc (5, 7, 8). Although neutralization of TNF-α renders mice highly susceptible to primary infection with Hc, in vitro treatment of murine Mφ with recombinant murine (rm)TNF-α does not induce these cells to exert antihistoplasmal activity (9). Thus, the mechanism by which TNF-α modulates host defenses in histoplasmosis remains a matter of speculation.

The objective of the present report was to examine the mechanisms by which endogenous TNF-α affects host susceptibility and influences the production of cytokines, including those associated with a Th1 and Th2 type. The studies were conducted in an established murine pulmonary model, which best mimics the human condition, since the lung is the primary site of infection (1). We sought to elucidate the effects of TNF-α on both primary and secondary infection. The latter studies are particularly relevant to the human condition, because individuals residing in endemic areas are frequently reexposed to Hc (1).

The experiments revealed that TNF-α is required for host resistance to Hc in both primary and secondary pulmonary infection. TNF-α-deficient naive mice were not impaired in their ability to exhibit a Th1-dominated immune response. However, generation of reactive nitrogen intermediates (RNI) was impaired in alveolar Mφ in naive TNF-α-neutralized mice although inducible nitric oxide synthase (iNOS) expression in lung tissue was not altered. In contrast, in secondary histoplasmosis, the exaggerated susceptibility was associated with a predominant Th2-type response, and host resistance was restored by neutralization of both IL-4 and IL-10.

**Materials and Methods**

**Animals**

Male C57BL/6 mice, 6 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Athymic nude mice were purchased from the National Cancer Institute (Frederick, MD) and used to produce ascites. All
animal experiments were done in accordance with the Animal Welfare Act guidelines of the National Institutes of Health.

**Preparation of Hc and infection of mice**

Hc yeasts were prepared as described (6). To produce a sublethal infection in naive mice, animals were infected intranasally (i.n.) with 2.5 × 10^6 Hc yeasts in a 50-μl volume. For secondary histoplasmosis, mice were initially inoculated with 10^4 yeasts i.n. Six weeks later, previously exposed animals were rechallenged i.n. with 2.5 × 10^5 yeasts.

**Organ culture for Hc**

Recovery of Hc was performed as described previously (6). The fungus was centrifuged at 3,000 × g to remove insoluble material. Protein concentration of the samples was determined by the Bradford method (11). In all, 20 μg of homogenates or 5 μg of mouse Mφ lysate, prepared from the RAW 264.7 cell line, as a positive control was analyzed on 5% SDS-PAGE gels. The resolved proteins were electroblotted overnight to nitrocellulose membranes and incubated with a 1:10,000 dilution of rabbit anti-mouse TNF-α mAb and with Hc and were given 1 mg of mAb each week thereafter. In studies with anti-IL-4 and anti-IL-10 mAb, mice received 2 and 1 mg, respectively, on day 0 of infection and were boosted with 1 mg each wk. Control animals received an equal amount of rat IgG concomitantly.

**Cytokine measurement**

Lungs from mice (n = 5–6) were removed on days 3, 5, and 7 postinfection, and the tissue was homogenized in 2 ml of RPMI 1640, centrifuged at 1,500 × g, filter sterilized, and stored at −70°C until assayed. Commercially available ELISA kits were used to measure IFN-γ, IL-4, IL-10, IL-6, and granulocyte macrophage (GM)-CSF (Endogen, Cambridge, MA). The sensitivity was >100 pg/ml. TGF-β was measured by two-site ELISA (R&D Systems, Minneapolis, MN). The sensitivity was >10 pg/ml and the cross-reactivity with murine TGF-β was >90%.

**Histology**

Lungs were removed and tissues were fixed in 10% formalin and embedded in paraffin blocks. Sections (5 μm) were stained with hematoxylin and eosin or with silver for fungal elements. Inflammatory cells within lung inoculated with 10^4 yeasts i.n. Six weeks later, previously exposed animals were rechallenged i.n. with 2.5 × 10^5 yeasts.

**Results**

**Growth of Hc in infected naive and immune C57BL/6 mice depleted of TNF-α**

Naïve mice were injected i.p. with 2 mg of anti-TNF-α mAb or an equivalent amount of rat IgG 2 h before a sublethal i.n. challenge with Hc. On day 7 postinfection, mice were sacrificed, and their lungs, livers, and spleens were cultured for Hc. Lungs of TNF-α-neutralized mice demonstrated a dramatic increase (p < 0.01) in CFU (514 ± 370 × 10^3) when compared with controls (23 ± 9 × 10^3). Interestingly, CFU in either livers (12.2 ± 0.8 × 10^5) or spleens (2.4 ± 0.9 × 10^5) of TNF-α-depleted mice did not significantly exceed (p > 0.05) the number of CFU recovered from controls in livers (11 ± 0.9 × 10^5) or spleens (2.1 ± 1.1 × 10^5).

Intranasal instillation of 10^4 Hc yeasts produces a host response that manifests in accelerated clearance of the organisms upon rechallenge (data not shown). Although TNF-α is known to play an important role in primary infection with a variety of pathogens including Hc, its role in secondary immune response to Hc remains unclear (8, 12–14). Therefore, we sought to determine the effect of endogenous TNF-α in secondary histoplasmosis. Six weeks after i.n. inoculation of 10^6 Hc, mice were depleted with 2 mg of anti-TNF-α mAb on the day of rechallenge and burden determined on day 7. CFU in lungs from TNF-α-neutralized mice were more than 200-fold increased (32.0 ± 11.1 × 10^6) when compared with
that in lungs from infected controls \((0.1 \pm 0.2 \times 10^6)\) \((p < 0.001)\). Fungal burden in livers \((0.9 \pm 0.2 \times 10^5)\) and spleens \((0.3 \pm 0.1 \times 10^5)\) from TNF-\(\alpha\)-depleted mice were significantly increased \((p < 0.01)\) when compared with livers \((0.1 \pm 0.0 \times 10^6)\) and spleens \((0.01 \pm 0.0 \times 10^5)\) from infected control mice \((p < 0.01)\). The data demonstrate that TNF-\(\alpha\) is required for clearance of both primary and secondary pulmonary infection with Hc. Moreover, the findings suggest that during primary infection with Hc, the absence of TNF-\(\alpha\) results in an unopposed fungal growth in lungs, whereas spleens and livers appear to be less affected in their ability to restrict growth. On the other hand, during secondary infection, neutralization of TNF-\(\alpha\) consistently impaired clearance of Hc in each examined organ.

Neutralization of TNF-\(\alpha\) accelerates mortality in naive and immune C57BL/6 mice following i.n. Hc infection

Next, we determined whether neutralization of TNF-\(\alpha\) would alter mortality during pulmonary histoplasmosis. Naive mice were treated with 2 mg of anti-TNF-\(\alpha\) or rat IgG and infected i.n. with \(2.5 \times 10^6\) Hc. All animals depleted of TNF-\(\alpha\) died by day 12 of infection, whereas all control mice survived up to 45 days (Fig. 1A).

We also ascertained the effect of depletion of TNF-\(\alpha\) on survival during secondary histoplasmosis. Neutralization of endogenous TNF-\(\alpha\) resulted in an increased mortality; all anti-TNF-\(\alpha\)-treated mice succumbed by day 10 whereas control animals survived throughout the observation period of 45 days (Fig. 1B). Thus, TNF-\(\alpha\) is required for resolution of primary and secondary pulmonary histoplasmosis, and death is associated with elevated CFU in lungs in naive and lungs, livers, and spleens in immune mice.

**Lung cytokine production during primary and secondary infection with Hc in TNF-\(\alpha\)-depleted compared with control mice**

We determined whether neutralization of TNF-\(\alpha\) altered protein levels of cytokines known to be involved in the immune response to Hc \((5, 6, 8, 10)\). Previously unexposed or pre-exposed mice were treated with either 2 mg of anti-TNF-\(\alpha\) mAb or rat IgG 2 h before i.n. infection with \(2.5 \times 10^6\) Hc yeasts. On days 3, 5, and 7 postinfection, lungs were removed and cytokine production assayed in total lung homogenates. We chose to measure the cytokines during the acute infection and before the onset of the moribund state in mice given anti-TNF-\(\alpha\) mAb.

The production of IFN-\(\gamma\) in lungs of naive and immune control mice demonstrated a sharp increase by day 5 of infection followed by a decline at day 7. Naive mice depleted of TNF-\(\alpha\) exhibited a continuous rise of IFN-\(\gamma\)-levels reaching a sixfold difference compared with controls by day 7 of infection \((p < 0.001)\) (Fig. 2A), whereas IFN-\(\gamma\) in immune mice depleted of TNF-\(\alpha\) declined steadily (Fig. 2B). While lungs of immune control mice displayed higher IFN-\(\gamma\)-levels on day 5 \((p < 0.05)\), anti-TNF-\(\alpha\)-treated animals exhibited statistically significant higher levels on day 7 of infection \((p < 0.001)\).

As shown in Figure 2C, IL-12 levels in naive infected controls reached a maximum on day 3 and declined thereafter. In contrast, levels in TNF-\(\alpha\)-depleted mice rose between days 3 and 5 and diminished on day 7. The levels of IL-12 in infected control mice were three- to fourfold higher compared with anti-TNF-\(\alpha\)-treated mice on day 3 of infection \((p < 0.005)\); this time point was the only one in which there were significant differences (Fig. 2C). During secondary infection, measurement of IL-12 (Fig. 2D) revealed increased levels in lungs of control mice at day 3 compared with TNF-\(\alpha\)-depleted animals \((p < 0.01)\). On day 5, amounts of IL-12 were elevated in TNF-\(\alpha\)-neutralized animals compared with control mice \((p < 0.02)\). No differences were present at day 7 of secondary infection.

During primary infection, IL-4 and IL-10 levels (Fig. 2, E and G) did not differ between controls and TNF-\(\alpha\)-deficient mice at each time point. Conversely, in secondary histoplasmosis, amounts of IL-4 in TNF-\(\alpha\)-depleted mice were considerably increased \((p < 0.001)\) on days 5 and 7 of infection when compared with controls (Fig. 2F). Likewise, the concentrations of IL-10 in lungs of TNF-\(\alpha\)-neutralized mice increased steadily and exceeded those of controls \((p < 0.01)\) on days 5 and 7 postinfection (Fig. 2H).

TNF-\(\alpha\) induces the production of GM-CSF, which in turn has been implicated in the protective immune response against Hc by enhancing the fungistic activity of Mφ (10, 15). We reasoned that depletion of TNF-\(\alpha\) may alter GM-CSF levels and thus diminish the ability of Mφ to exert antifungal activity. As demonstrated in Figure 3A, protein levels of GM-CSF in naive control mice were highest on day 3 of infection, and declined modestly on days 5 and 7. In TNF-\(\alpha\)-deficient mice, GM-CSF levels were elevated on day 3; they decreased by day 5 and rose on day 7 postinfection. At this time point, the amount of GM-CSF was significantly elevated when compared with infected controls \((p < 0.05)\). In secondary infection, assessment of GM-CSF in lungs of infected controls revealed a modest decline over 7 days, whereas levels in TNF-\(\alpha\)-depleted mice rose by day 5 of reinfection and were significantly higher than those of control mice \((p < 0.001)\) Fig. 3B). Although levels in anti-TNF-\(\alpha\) mAb-treated mice declined by day
FIGURE 2. IFN-γ, IL-12, IL-4, and IL-10 production in lung tissue of naive (left) and immune (right) C57BL/6 mice infected i.n. with 2.5 × 10^6 viable Hc. Lungs were removed on days 3, 5, and 7 of infection and homogenized in RPMI 1640. Cytokine levels were determined by ELISA. Five to six lungs/group were analyzed. Bars are mean ± SEM. One representative of three experiments is shown. An asterisk (*) denotes statistical significance; specific p values are provided in the text. Uninfected normal lungs (n = 3) contained 180 ± 9 pg/ml of TNF-α. Levels of TNF-α in lungs of naive infected controls ranged from 1450 ± 248 (day 3) to 863 ± 284 (day 7) pg/ml, whereas immune infected controls demonstrated amounts of 645 ± 49 pg/ml on day 3 to 1280 ± 50 pg/ml. Lungs from naive and immune TNF-α-depleted mice exhibited levels of <100 pg/ml at all time points measured.
the amounts remained significantly elevated compared with control mice ($p < 0.001$).

TGF-β and TNF-α exert opposing effects on Mφ functions: the former inhibits Mφ activation, and the latter increases their state of activation (16). We hypothesized that upon depletion of TNF-α, TGF-β protein levels might be elevated in lungs of mice, and therefore contribute to the loss of host resistance apparent in the lungs. A significant difference between TNF-α-depleted and infected control mice during primary infection was not detected at any time point (Fig. 3C). In contrast, by day 5 postinfection of secondary histoplasmosis, TGF-β levels had increased sharply and were approximately fivefold higher in TNF-α-depleted animals compared with controls ($p < 0.001$), but levels had declined in these mice by day 7 and were similar to those observed in control mice (Fig. 3D).

In primary infection, IL-6 levels in lungs of controls and TNF-α-depleted animals increased from days 3 to 5 and declined by day 7. No significant differences between the controls and TNF-α-depleted mice were detected (Fig. 3E). In secondary infection, protein concentrations of IL-6 in control lungs did not differ from those measured in TNF-α-neutralized mice on day 3. Although amounts of IL-6 did not change dramatically in control animals on days 5 and 7 postinfection, levels in mice depleted of TNF-α had risen by day 5 and were significantly
different when compared with controls on days 5 and 7 ($p < 0.01$, Fig. 3F).

Thus, primary Hc infection in TNF-α-neutralized mice is associated with increased IFN-γ and GM-CSF levels, but not IL-4, IL-6, IL-10, or TGF-β. In contrast, administration of anti-TNF-α altered levels of several cytokines in lungs of mice with secondary Hc infection, including those that are associated with a Th2-type response (IL-4 and IL-10).

Histopathologic changes in lungs of TNF-α-neutralized naive and immune mice

Histopathologic changes in lungs following treatment with anti-TNF-α mAb were examined on day 7 of infection. TNF-α-neutralized and control mice exhibited a diffuse granulomatous pneumonia with destruction of the lung parenchyma (Fig. 4, A and B, respectively). There was moderate to severe peribronchiolar and perivascular lymphoid cuffing with moderate debris in the bronchiolar lumen. In TNF-α-neutralized mice, inflammatory regions were composed of 48 ± 3% PMN, 21 ± 3% lymphocytes, and 32 ± 3% Mφ, whereas in infected controls 52 ± 1% PMN, 18 ± 2% lymphocytes, and 32 ± 3% Mφ were detected.

Histopathologic examination of lungs from re-infected TNF-α-deficient mice revealed a severe, diffuse granulomatous pneumonia with severe perivascular lymphoid cuffing and marked destruction of alveoli (Fig. 4C). In contrast, lungs of control mice exhibited a mild focal granulomatous infiltration of the alveoli with mild to moderate perivascular lymphoid cuffing (Fig. 4D). In areas of inflammation, the percentage of lymphocytes in infected controls was 79 ± 9%; Mφ, 14 ± 3%; and PMN, 7 ± 4%. In the tissue of TNF-α-depleted mice, lymphocytes composed 81 ± 11%, Mφ-12 ± 5, and PMN-8 ± 3%.

Depletion of endogenous TNF-α does not dramatically change the inflammatory character in infected lungs in primary and secondary histoplasmosis. However, the extent of the inflammatory response in secondary histoplasmosis was substantially greater in TNF-α-depleted mice, whereas it did not differ from controls during primary infection with Hc, despite the fact that there was increased fungal burden.

FACS analysis of BAL cells from TNF-α-deficient mice vs controls following primary or secondary infection with Hc

CD4+ cells are pivotal in controlling infection with Hc, and lymphocytes constitute a major immune effector cell found in lung alveoli (1, 4). To determine the effect of neutralization of TNF-α on inflammatory cells, lungs of infected, naive mice were lavaged on day 7 of infection and cells analyzed by flow cytometry. PMN (GR-1+) constituted the majority of cells in BAL in both groups, composing up to 67 ± 11% (mean ± SD) of the total cell population in TNF-α-depleted mice compared with 60 ± 7% of infected controls. The absolute numbers of PMN in BAL fluid from TNF-α-depleted mice (3.4 ± 2.3 × 106) differed ($p = 0.02$) from that of infected controls (0.5 ± 0.2 × 106). The percentage of both CD4+ (2 ± 1%) and CD8+ (1 ± 0.5%) cells in BAL fluid from TNF-α-depleted mice did not differ from that of infected controls (CD4+, 2 ± 0.5% and CD8+, 2 ± 0.5%). However, the absolute number of CD4+ and CD8+ cells was increased in anti-TNF-α-treated mice, since there was an increased number of inflammatory cells present in BAL. In TNF-α-depleted mice, 4.8 ± 4 × 103
BAL cells were CD4$^+$ and 3.8 ± 3 × 10³ were CD8$^+$ compared with 2.3 ± 2 × 10³, CD4$^+$, and 2.1 ± 2 × 10³ CD8$^+$. These data demonstrate that administration of anti-TNF-α mAb does not significantly perturb the numbers of CD4$^+$ or CD8$^+$ cells in BAL fluid of mice during primary infection with Hc.

We next analyzed the composition of BAL cells in immune mice to determine whether blockade of TNF-α altered the numbers of inflammatory cells during secondary histoplasmosis. On day 7 postinfection, lungs were lavaged and cells stained for GR1, CD4, and CD8 and analyzed by FACS. The percentage of GR-1$^+$ cells in TNF-α-depleted mice was 17 ± 6% compared with 18 ± 6% in infected controls ($p = 0.03$). The absolute numbers in mice given anti-TNF-α (2.7 ± 1.6 × 10⁵) was significantly greater ($p = 0.03$) than that of controls (0.9 ± 0.5 × 10⁵). The percentage of CD4$^+$ cells was higher (60 ± 12%) in TNF-α-depleted mice compared with infected controls (42 ± 17%), whereas the percentage of CD8$^+$ cells did not differ between the groups (8 ± 4% in TNF-α-neutralized mice vs 10 ± 4% in infected controls). The absolute number of both T cell subsets was increased approximately two- to threefold ($p < 0.05$) between anti-TNF-α-treated mice (6.2 ± 3.0 × 10⁵ for CD4$^+$, and 0.8 ± 0.5 × 10⁵ for CD8$^+$) and rat IgG-treated mice (2.5 ± 2.0 × 10⁵ for CD4$^+$ and 0.45 ± 0.1 × 10⁵ for CD8$^+$).

In vitro growth inhibition of Hc following activation of alveolar or peritoneal MΦ with rmTNF-α and rmIFN-γ
Neutralization of TNF-α inhibits the microbicidal activity of MΦ against Listeria monocytogenes (17). In some models, cooperation between IFN-γ and TNF-α is essential to enhance MΦ function in a synergistic fashion (18, 19).

In vitro experiments were conducted to determine whether TNF-α and IFN-γ would act synergistically to augment the anti-Hc activity of alveolar MΦ. Cells from normal mice were cultured in the presence of either LPS, TNF-α, IFN-γ alone, or combinations thereof and assayed for fungistatic activity against Hc yeasts. None of the factors activated alveolar MΦ to inhibit the intracellular growth of the yeasts. As an example, in one of four experiments, exposure to IFN-γ, LPS, or TNF-α at 10 ng/ml increased $[^{3}H]$leucine incorporation of Hc by 23, 14, and 38%, respectively, rather than diminish intracellular growth. Moreover, the admixture of IFN-γ plus TNF-α at 10 ng/ml each did not stimulate inhibition of Hc growth within alveolar MΦ and enhanced $[^{3}H]$leucine incorporation of Hc by 7%. IFN-γ (10 ng/ml) plus LPS (10 ng/ml) caused a 15% reduction in Hc growth.

In contrast, resident peritoneal MΦ from normal mice cocultured with IFN-γ at a concentration of 10 ng/ml exhibited 78% inhibition of Hc growth. Addition of TNF-α or LPS did not increase the

### FIGURE 5
RNI production by alveolar MΦ of naive (A, B) and immune (C, D) TNF-α-depleted and rat IgG-treated controls. Mice were infected with 2.5 × 10⁶ Hc yeasts and BAL cells obtained on days 3, 5, and 7 postinfection. Alveolar MΦ were either unstimulated in vitro (spontaneous release), or stimulated with 1 μg/ml of LPS and 100 ng/ml of rmIFN-γ, or 100 ng/ml of rmTNF-α and 100 ng/ml of rmIFN-γ. Data are expressed as Δ NO₂ (NO₂ stimulated − NO₂ unstimulated). Two experiments are shown; n = 6 mice/group.
inhibitory activity of peritoneal Mφ. Fungistatic activity remained unchanged with 68% inhibition for IFN-γ and LPS at 10 ng/ml and 51% for IFN-γ and TNF-α at 10 ng/ml. In contrast, culture of peritoneal Mφ with either LPS or TNF-α (10 ng/ml) alone promoted growth of Hc by 63 and 14%, respectively.

**Effect of TNF-α neutralization on iNOS expression in lungs and RNI production by alveolar Mφ during primary and secondary infection with Hc**

A major mechanism for the killing of intracellular pathogens is the formation of nitric oxide (NO) (20). TNF-α synergizes with IFN-γ to stimulate Mφ and enhance nitrogen intermediates that are essential in the destruction of *Leishmania major* (18, 21). Expression of iNOS protein can be correlated with generation of NO (22). We sought to determine whether neutralization of TNF-α altered expression of iNOS in lungs of naive and immune mice infected with Hc. The amount of iNOS protein in lungs was determined on day 7 postinfection. iNOS expression in lungs of rat IgG-treated mice (174 ± 7.3 pixel density) did not differ (*p* > 0.05) from that found in TNF-α-depleted (165.7 ± 7.3) animals in primary histoplasmosis. Moreover, the amount of iNOS was similar in TNF-α-neutralized (164.3 ± 8.6; pixel density ± SD) and rat IgG-treated mice (164.5 ± 11.4) in secondary infection with Hc.

Although Western blot analysis indicated that lungs of TNF-α-deficient mice produced comparable quantities of iNOS, we sought to measure RNI by alveolar Mφ, since a recent report suggested that expression of iNOS in lungs does not necessarily correlate with production of NO (23). Alveolar Mφ were obtained on days 3, 5, and 7 of primary and secondary Hc infection and cultured in endotoxin-free media alone or stimulated in vitro with IFN-γ plus LPS or IFN-γ plus TNF-α. After 48 h, the supernatants were tested for the presence of nitrite (Fig. 5, A–D). Unstimulated Mφ from rat IgG-treated and TNF-α-depleted mice during primary infection demonstrated the release of RNI, which ranged from 24 to 42 and 23 to 36 μM, respectively, in one experiment (Fig. 5A), and 42 to 85 (controls) and 41 to 90 μM (TNF-α-depleted) in the second (Fig. 5B). In two experiments, RNI production by in vitro-stimulated alveolar Mφ from TNF-α-depleted mice was strikingly less than that of control cells on days 5 and 7 postinfection. In fact, generation of RNI by these Mφ declined over time. In contrast, Mφ from controls exhibited either a progressive rise in RNI production with maximum levels at day 7 postinfection (Fig. 5A) or plateaued at days 5 and 7 of infection (Fig. 5B). To determine whether the weak generation of RNI by Mφ from TNF-α-depleted mice was caused by a lower number of cells, we enumerated the percentage of Mφ in BAL. The percentage of Mφ recovered from control and anti-TNF-α-treated mice ranged from 23 to 38% and 23 to 32%, respectively, and thus cannot account for the difference measured in RNI production.

In secondary infection, RNI production by Mφ did not differ between TNF-α-neutralized and control mice upon in vitro stimulation with LPS and IFN-γ or IFN-γ and TNF-α in two experiments (Fig. 5, C and D). Interestingly, both groups demonstrated a decline in RNI release at day 5 followed by an increase at day 7 postinfection. The release of RNI in unstimulated alveolar Mφ from control and TNF-α-depleted mice ranged from 75 to 138 μM and 79 to 140 μM, respectively. Mφ constituted 21 to 34% in control and 16 to 27% in anti-TNF-α-treated mice.

**Concomitant neutralization of IL-4 and IL-10 abrogates mortality in secondary histoplasmosis in mice lacking endogenous TNF-α**

The elevated protein concentrations of IL-4 and IL-10 during secondary histoplasmosis prompted us to examine whether these cytokines contributed in part to the increase in susceptibility of TNF-α-depleted mice. Six weeks after i.n. infection with 10⁵ yeast cells, groups of mice were depleted on the day of secondary challenge with either 2 mg of anti-TNF-α, or treated with the same amount of anti-TNF-α plus 2 mg of anti-IL-4 and 1 mg anti-IL-10 mAb. Additional groups received either TNF-α mAb plus anti-IL-4 or anti-IL-10 mAb. As demonstrated in Figure 6, all control mice and mice depleted of IL-4 and IL-10 alone survived secondary challenge over an observation period of 45 days. In contrast, all mice depleted of TNF-α alone or combined with either anti-IL-4 or anti-IL-10 mAb succumbed to infection by day 11 postchallenge. Concomitant neutralization of IL-4 and IL-10 rescued 75% of mice from the inimical effects of anti-TNF-α mAb treatment. Thus, depletion of IL-4 and IL-10 reverses the deleterious effects of treatment with anti-TNF-α mAb and restores host resistance.

**Discussion**

The present study demonstrates the crucial role of TNF-α during primary and secondary pulmonary histoplasmosis. Depletion of
TNF-α resulted in a high mortality rate of naive mice infected with Hc and was associated with increased CFU in lungs at 1 wk postinfection. Interestingly, fungus burden in livers and spleens of TNF-α-depleted mice was not elevated when compared with infected controls at day 7. These results contrast with the findings in mice administered anti-IL-12 mAb or in IFN-γ knockout mice in which CFU in lungs, livers, and spleens were considerably elevated at wk 1 of Hc infection. These mice were challenged with the same inoculum size and the same strain of Hc (6, 24). Thus, the influence of endogenous TNF-α in experimental Hc infection appears to be compartmentalized. Disparate effects of endogenous TNF-α in individual organs also have been observed in experimental tuberculosis. In TNF-α-depleted mice infected i.v. with Mycobacterium tuberculosis, pronounced tissue necrosis was detected only in lungs and not in liver or spleen (12). Taken together, these findings suggest the existence of a differential requirement among visceral organs for endogenous TNF-α in inflammation and host resistance mechanisms.

Histopathologically, the components of the inflammatory response in lung parenchyma did not differ between TNF-α-deficient and control mice. Both groups exhibited granulomatous infiltration composed of lymphocytes, Mϕ, and PMN. However, mice depleted of TNF-α manifested more extensive inflammation, most likely a consequence of increased fungal burden. Prior work has suggested the necessity of endogenous TNF-α for the generation of granulomatous inflammation in response to bacillus Calmette-Guérin (25) and to Schistosoma mansoni eggs (26). Other reports, however, have provided evidence that this cytokine is not universally required for this type of inflammatory response. Mice infected with Brucella abortus and depleted of TNF-α form granulomas (27). Likewise, mice lacking the TNF receptor p55 develop granulomas in response to M. tuberculosis (12). Therefore, it is reasonable to conclude that the necessity of this cytokine for the development of granulomatous inflammation varies with the pathogen.

In experimental cryptococcosis, administration of anti-TNF-α mAb results in a marked perturbation in the inflammatory response (14). CD4+ cells, PMN, and Mϕ were sharply decreased in lungs. Our findings contrast with those of cryptococcosis. In experimental Hc, the percentage of CD4+ and CD8+ cells, PMN, and Mϕ in BAL fluid did not differ significantly between TNF-α-neutralized and infected control mice. However, the absolute numbers of CD4+, CD8+, and PMN were elevated over controls. This finding is surprising, since TNF-α is a prominent proinflammatory cytokine (28). In immune mice, the elevated levels of the proinflammatory cytokine IL-6 may have contributed to the increased recruitment of cells to the lungs. However, this observation does not explain the findings in naive mice. It is possible, therefore, that other cytokines stimulated egress of inflammatory cells into the lungs of TNF-α-depleted mice. Thus, in experimental cryptococcosis, TNF-α regulates recruitment of inflammatory cells, whereas in Hc infection, the contribution of this cytokine as a stimulus for cellular infiltration appears to be less prominent.

Depletion of TNF-α was not associated with an impairment of IFN-γ generation. In fact, lung levels of IFN-γ were markedly increased in TNF-α-deficient mice. Hence, the presence of TNF-α during pulmonary histoplasmosis was not obligatory for the in vivo production of IFN-γ. Although this cytokine is important for clearance of Hc, its presence, even in elevated levels compared with controls, was not sufficient for resolution of infection. We have demonstrated previously that IFN-γ knockout mice succumb to pulmonary Hc infection although high levels of TNF-α are present in their lungs (24). Thus, it appears that production IFN-γ and TNF-α are independently regulated in Hc infection and that both must be active in vivo for optimal clearance.

Based on the in vivo observations, we endeavored to demonstrate that exposure of alveolar Mϕ to TNF-α and/or IFN-γ would activate these cells to express anti-Hc activity. Mϕ incubated with either cytokine alone or an admixture failed to manifest anti-Hc activity although peritoneal Mϕ exhibited pronounced anti-Hc activity when exposed to IFN-γ alone. The inability to demonstrate convincingly that alveolar Mϕ exert anti-Hc activity in vitro when exposed to both IFN-γ and TNF-α suggests that yet another cytokine or cytokines must be synthesized in vivo that lead to their activation. It is unlikely to be GM-CSF, IL-6, TGF-β, IL-4, or IL-10, since they were produced in amounts equal to or greater than those found in controls while mice succumbed to overwhelming histoplasmosis. Thus, these cytokines failed to compensate for inadequate host resistance mechanisms.

Another salient point is that much of the prior data has indicated that cytokine activation of human or murine Mϕ results in fungal, not fungicidal, activity (10, 29, 30). However, since mice with sufficient amounts of both TNF-α and IFN-γ sterilize tissues, actual killing must be mediated by a cytokine whose production requires engagement of both TNF-α and IFN-γ.

Accumulated data indicate that NO is a potent killing mechanism for several intracellular pathogens (20). Moreover, this nitrogen intermediate has been implicated in the elimination of Hc (9). Since TNF-α contributes to the production of NO (31), we compared iNOS expression in lungs of TNF-α-depleted mice to that of infected controls. We hypothesized that the progression of infection in mice receiving anti-cytokine mAb could be explained by alterations in iNOS expression and, thus, NO production. However, we did not detect any differences in iNOS expression in TNF-α-depleted and control mice, during both primary and secondary histoplasmosis.

A recent report has suggested that iNOS expression does not necessarily correlate with NO production (23). Therefore, we measured RNI production by alveolar Mϕ in response to LPS plus IFN-γ or to IFN-γ plus TNF-α, both potent stimuli. The inability to control infection was associated with a marked decrement in the production of RNI by stimulated alveolar Mϕ from TNF-α-neutralized naive mice. In addition, the failure of alveolar Mϕ from TNF-α-depleted mice to produce RNI upon stimulation with IFN-γ plus TNF-α ex vivo indicates that the cells manifest a persistent defect in cytokine responsiveness. These results suggest that the receptors for these cytokines are dysfunctional or that signal transduction has been perturbed. In contrast, RNI production was not altered during secondary histoplasmosis, and these findings indicate that accelerated mortality is not accompanied by decreased NO production.

Analysis of the cytokines detected in lungs of naive and immune mice exposed to Hc revealed pronounced differences, particularly in the production of IL-4 and IL-10. Whereas levels of these Th2-associated cytokines in TNF-α-depleted mice were no different from controls during primary infection, significant differences between immune mice treated with anti-TNF-α mAb and controls were noted. Up-regulation in IL-4 and IL-10 production was not associated with decrements in IFN-γ by immune mice depleted of endogenous TNF-α. In fact, IFN-γ levels in lungs of TNF-α-depleted mice re-infected with Hc were higher than controls.

In some models of infectious diseases, high levels of IL-4 and/or IL-10 have been associated with progressive disease (32, 33). As an example, unrestricted growth of L. major in susceptible mice is accompanied by high production of IL-4 and low to nonexistent levels of IFN-γ (34). As a corollary, neutralization of IL-4 cures mice infected with L. major or ameliorates listeriosis in mice lacking the IFN-γ receptor gene (35, 36). Furthermore, neutralization
of IL-4 during primary pulmonary infection with Hc abrogates mortality in mice depleted of IL-12 (6).

Since the levels of IL-4 and IL-10 were elevated in immune animals given anti-TNF-α mAb, we sought to determine the in vivo influence of these two cytokines on host resistance mechanisms in these mice. Administration of either anti-IL-4 or anti-IL-10 mAb in TNF-α-depleted mice did not restore protective immunity. However, 75% of TNF-α-neutralized mice given both mAb survived for 45 days. Thus, blockade of both Th2-associated cytokines was necessary to restore host resistance in mice treated with anti-TNF-α mAb. These results indicate that both of these cytokines contribute to the down-regulation of protective immunity in immune mice deficient in endogenous TNF-α.

In secondary histoplasmosis, restoration of protective immunity by treatment with anti-IL-4 and anti-IL-10 mAb in TNF-α-depleted mice may seem surprising since the IFN-γ levels were high. However, it can be more informative to examine the balance between Th1 and Th2 cytokines than absolute values. In this regard, the ratio of IFN-γ to IL-4 or to IL-10 was 1000 and 350, respectively, in TNF-α-neutralized mice on day 7 of secondary infection. These values were greater in TNF-α-depleted naïve animals (IFN-γ to IL-4 = 1600 and IFN-γ to IL-10 = 1000). This finding also was true for day 5 of infection. Although the relative increase in Th2 cytokines between primary and secondary histoplasmosis may not appear markedly dissimilar, it is possible that even slight differences in the ratio of Th2 to Th1 cytokines impair protective immunity to Hc. Nevertheless, IL-4 and IL-10 contribute to disease exacerbation in secondary infection if TNF-α is deficient. The importance of the concerted action of IL-4 and IL-10 in disease exacerbation also has been reported in an experimental model of L. donovani (37), although the precise mechanisms by which these cytokines act to impair host defenses are unknown.

Recently, it has been shown that administration of anti-TNF-α does not alter the course of a secondary systemic infection with Hc in immunocompetent C57BL/6 mice (38). These results differ considerably from the findings herein. Although the precise reasons for the differences have not been determined, one of the most likely reasons is that in our study Hc was delivered i.n. and in the other study it was inoculated i.v. Thus, the route of exposure may have a significant impact on the constituents that are required for a protective immune response. It is also possible that Hc strain differences may be involved, since two different isolates were employed in these studies.

In summary, TNF-α is pivotal in controlling primary and secondary pulmonary histoplasmosis. Based on accumulated data, it is reasonable to conclude that host resistance to Hc during primary infection requires the presence of both IFN-γ and TNF-α. Furthermore, in primary infection, the mounting evidence is that at least one other soluble mediator, if not more, must be involved in the activation of Mφ to express fungicidal activity. Alternatively, the lack of TNF-α during secondary infection with Hc biases the host to a Th2-type response. The elevated levels of both IL-4 and IL-10 contribute to the increased susceptibility of animals depleted of endogenous TNF-α. Thus, the mechanisms that underlie host susceptibility to Hc in the absence of TNF-α appear disparate and remain to be completely elucidated.

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References


