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# IFN- $\gamma$ at the Site of Infection Determines Rate of Clearance of Infection in Cryptococcal Meningitis<sup>1</sup>

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In animal models, immunity to cryptococcal infection, as in many chronic fungal and bacterial infections, is associated with a granulomatous inflammatory response, intact cell-mediated immunity, and a Th1 pattern of cytokine release. To examine the correlates of human immunity to cryptococcal infection in vivo, we analyzed immune parameters at the site of infection over time and assessed the rate of clearance of infection by serial quantitative cerebrospinal fluid (CSF) fungal cultures in 62 patients in a trial of antifungal therapy for HIV-associated cryptococcal meningitis. CSF IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and IL-8 were significantly higher in survivors compared with nonsurvivors. There were negative correlations between log TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 levels and baseline cryptococcal CFU. Log IFN- $\gamma$ , G-CSF, TNF- $\alpha$ , and IL-6 were correlated positively with the rate of fall in log CFU/ml CSF/day. In a linear regression model including antifungal treatment group, baseline CFU, and these cytokines, only treatment group and log IFN- $\gamma$  remained independently associated with rate of clearance of infection. The results provide direct in vivo evidence for the importance of quantitative differences in IFN- $\gamma$  secretion in human immune control of granulomatous infections, and increase the rationale for adjunctive IFN- $\gamma$  in the treatment of refractory HIV-associated cryptococcosis. *The Journal of Immunology*, 2005, 174: 1746–1750.

Cryptococcal meningitis is a common life-threatening infection in patients with advanced HIV disease. It is a major cause of death from AIDS in Asia and accounted for 13, 17, and 44% of all deaths in three cohorts of HIV-infected patients in Africa (1–4). For comparison, tuberculosis was identified as the cause in 5, 6, and 13% of deaths in these cohorts (2–4). Even with the best currently available antifungal treatment, mortality remains unacceptably high (5–7), providing the rationale for an ongoing interest in the development of adjunctive immunotherapeutic approaches (8). Much has been learned from study of animal models and from in vitro experimentation about the immune response to cryptococcal infection. In common with a number of other chronic fungal and bacterial infections, protection is associated with an active granulomatous inflammatory response and depends on intact cell-mediated immunity and, in particular, a Th1 pattern of cytokine release (9). However, the extent to which these

correlates of protection apply in the human system in vivo is unclear, and this restricts the scientific basis for therapeutic attempts to boost immunity in patients with cryptococcosis.

In a clinical trial of combination antifungal regimens for the treatment of acute HIV-associated cryptococcal meningitis, we assessed the mycological response of individual patients accurately by means of serial quantitative cerebrospinal fluid (CSF)<sup>4</sup> fungal cultures (10). To explore the correlates of human immunity to cryptococcal infection in vivo, we analyzed the immune response at the site of infection over time in the same patients. The ability in cryptococcal meningitis, both to quantify organism load over time and to profile immune parameters at the site of infection, provides the opportunity to correlate directly immune parameters with the rate of clearance of infection. Therefore, we have examined the relationship of a wide range of immune parameters to survival, to prognostic factors known to be predictive of survival, and to the rate of clearance of infection. The results provide important new in vivo evidence linking quantitative differences in IFN- $\gamma$  secretion at the site of infection to the control of granulomatous infection, and increase the scientific basis for adjunctive immunotherapy for cryptococcal meningitis and other granulomatous infections.

## Materials and Methods

### Patients

The study was conducted at Sappasitprasong Hospital in Ubon Ratchathani in Northeast Thailand and was approved by the ethical and scientific review subcommittee of the Thai Ministry of Public Health and by the Research Ethics committee of St. George's Hospital. Between May and December 2002, with written informed consent, 64 patients with a first episode of cryptococcal meningitis, diagnosed by CSF India ink, cryptococcal Ag test, and culture, were enrolled (10). All patients received amphotericin B 0.7–0.8 mg/kg/day, alone or in combination with flucytosine

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<sup>4</sup> Abbreviations used in this paper: CSF, cerebrospinal fluid; CI, confidence interval; sTNFR II, soluble TNFR II.

(100 mg/kg/day), fluconazole (400 mg/day), or both. After 2 wk, therapy was switched to fluconazole, 400 mg/day for 8 wk and 200 mg/day thereafter. Follow-up lumbar punctures were done on days 3, 7, and 14 for quantitative cultures to assess the rate of clearance of infection. One patient was excluded because HIV seronegative, and from one patient no CSF samples were available. CSF was available from 59 of the remaining 62 patients at baseline, 52 at day 3, 47 at day 7, and 47 at day 14, and was frozen at  $-80^{\circ}\text{C}$  for later analysis of inflammatory and immune parameters. Cerebral dysfunction was defined as any reduction in conscious level or seizures at presentation (10). The median (interquartile range) baseline CD4 cell count and plasma viral load of the patients were  $9\text{--}20 \times 10^9/\text{L}$  and 191,000 (104,000–320,000) copies/ml, respectively.

#### Quantitative CSF cultures and rate of clearance of infection

With a mean delay of  $<2\text{ h}$  after lumbar puncture, CSF was serially diluted 10-fold, and 100  $\mu\text{l}$  of each dilution was spotted onto each half of a Sabouraud dextrose agar plate. Counts of CFU were taken from the plate with lowest dilution, which had at least 40 CFU. The rate of decrease in log CFU/ml CSF per day was derived from the slope of the linear regression of log CFU against time for each patient, as described previously (10).

#### Multianalyte profiling and ELISA

Multiple cytokine levels were determined in each CSF sample using the Luminex 100 technology (Luminex) (11). Cytokine kits of up to 17 cytokines and chemokines were used (Bioplex kits; Bio-Rad). The cytokines measured and sensitivities of the assay in pg/ml were: IL-1 $\beta$  (4), IL-2 (4), IL-4 (1), IL-5 (1), IL-6 (4), IL-7 (1), IL-8 (1), IL-10 (1), IL-12p70 (1), IL-13 (1), IL-17 (1), G-CSF (4), GM-CSF (4), MCP-1 (10), MIP-1 $\beta$  (4), IFN- $\gamma$  (1), and TNF- $\alpha$  (1). Soluble TNFR II (sTNFR II) levels were measured in 10/1 dilutions of CSF samples using Quantikine ELISA kits (R&D Systems) with a sensitivity of 8 pg/ml.

#### Statistical analysis

Median levels of immune and inflammatory parameters were compared by the Mann-Whitney *U* test. Cytokine data were log transformed, and associations between cytokines and with baseline log CFU were examined using Pearson's correlation coefficient. Cuzick's nonparametric test for trend across ordered groups and Cox proportional hazards models were used to determine the association between baseline cytokine levels and death. In the survival analyses, TNF- $\alpha$  values were grouped into quartiles, and fitted as a linear variable. Results were similar with log-transformed values. Linear regression was used to examine the association of cytokines with rate of clearance of infection. Stata 8.0 (Stata) was used for all analyses.

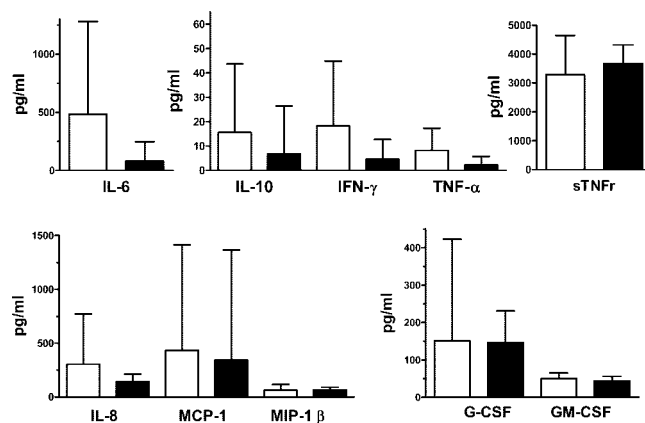
## Results

#### Survival is associated with increased concentrations of proinflammatory cytokines at the site of infection

In initial univariate analyses, cytokine levels in baseline CSF from survivors and nonsurvivors at 2 wk were compared (Fig. 1). Median levels of IL-6 and IFN- $\gamma$  were 5- and 4-fold higher, respectively, in survivors compared with nonsurvivors ( $p = 0.016$  and  $0.019$ , respectively; Mann-Whitney *U* test). Median levels of TNF- $\alpha$  and IL-8 were greater than 3- and 2-fold higher, respectively, in survivors compared with nonsurvivors ( $p = 0.046$  and  $0.044$ ). In contrast, levels of IL-10, MCP-1, MIP-1 $\beta$ , G-CSF, and GM-CSF were not significantly different in survivors and nonsurvivors. Levels of IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-7, IL-12p70, IL-13, and IL-17 were not consistently detectable in the CSF. We also measured sTNFR II levels in CSF, given a prior report that levels were higher in survivors than nonsurvivors of HIV-associated cryptococcal meningitis (12). However, sTNFR II levels did not differ between survivors and nonsurvivors (Fig. 1). When divided into quartile categories, higher baseline levels of TNF- $\alpha$ , IL-6, and IFN- $\gamma$  were associated with reduced risk of death by 10 wk ( $p = 0.005$ ,  $0.02$ , and  $0.03$ , respectively; Table I).

#### The coordination and time course of the immune response

The coordination of the immune response to cryptococcosis in vivo was investigated by exploring the correlation between different cytokines. Log<sub>10</sub> levels of IFN- $\gamma$ , IL-6, and TNF- $\alpha$  were



**FIGURE 1.** Baseline cytokine levels in CSF in survivors ( $\square$ ,  $n = 50$ ) and fatal cases ( $\blacksquare$ ,  $n = 9$ ) at 2 wk. Results shown are median and 75% quartile. Median levels of IL-6 and IFN- $\gamma$  were 5- and 4-fold higher in survivors compared with nonsurvivors ( $p = 0.016$  and  $0.019$ , respectively; Mann-Whitney *U* test). Median levels of TNF- $\alpha$  and IL-8 were greater than 3- and 2-fold higher, respectively, in survivors compared with nonsurvivors ( $p = 0.046$  and  $0.044$ , respectively; Mann-Whitney *U* test).

strongly positively correlated ( $r = 0.94$ ,  $0.89$ , and  $0.86$  for IFN- $\gamma$ /IL-6, IFN- $\gamma$ /TNF- $\alpha$ , and IL-6/TNF- $\alpha$ , respectively;  $p < 0.0001$  for all pairings; Fig. 2). There was also significant correlation ( $r > 0.7$ ) between these three cytokines and G-CSF and between TNF- $\alpha$  and MIP-1 $\beta$ . All other pairwise correlation coefficients were  $<0.7$ .

In patients who survived, median cytokine levels peaked at day 3 of therapy (IL-6, IL-8, IFN- $\gamma$ , TNF- $\alpha$ , MIP-1 $\beta$ ), or decreased progressively over the first 2 wk of therapy. By day 14, levels of all cytokines were significantly reduced compared with baseline (comparing baseline and day 14 values,  $p < 0.0001$  for all cytokines shown except MIP-1 $\beta$ ,  $p = 0.005$ ; Fig. 3).

#### Proinflammatory cytokine concentrations are not related to altered mental status, but are negatively correlated with baseline organism load

In this study, the two most important prognostic factors for survival were cerebral dysfunction at presentation and baseline *Cryptococcus neoformans* organism load as defined by the baseline CSF cryptococcal CFU count (10). We therefore assessed whether CSF immune parameters were associated with either of these other variables. There was no difference in the levels of IL-6, IFN- $\gamma$ , or TNF- $\alpha$  between patients with and without cerebral dysfunction. However, of the 12 patients with cerebral dysfunction, levels of these cytokines in the six who survived were 13-, 11-, and 8-fold higher, respectively, than in the six who died ( $p = 0.04$ ,  $0.03$ ,  $0.02$ , respectively; data not shown), again suggesting a possible role for these cytokines in control of infection. There was a significant negative correlation between log TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 levels and baseline log CFU ( $r = -0.48$ ,  $-0.45$ ,  $-0.36$ ;  $p = 0.0001$ ,  $0.0004$ ,  $0.005$ , respectively) such that patients with high baseline CFU counts tended to have lower levels of these cytokines (Fig. 4). Log TNF- $\alpha$  and IFN- $\gamma$  were much less strongly associated with baseline CSF cryptococcal Ag titer ( $r = -0.27$ ,  $-0.26$ ;  $p = 0.04$ ,  $0.05$ , respectively). Of other factors potentially influencing the CSF immune response, there was no correlation between CSF white cell count and cytokine levels, and no significant correlation between peripheral CD4 cell counts or plasma or CSF viral loads and CSF cytokine levels.

#### Multivariate analysis of survival

As cytokine levels correlated with survival and baseline CFU, we used Cox proportional hazards models to assess whether cytokine

Table I. Association of baseline CSF IL-6, IFN- $\gamma$ , and TNF- $\alpha$  levels, grouped into quartiles, and death by 10 wk

	IL-6 Deaths (%)	IFN- $\gamma$ Deaths (%)	TNF- $\alpha$ Deaths (%)
1 <sup>st</sup> quartile	6/15 (40)	8/17 (47)	8/15 (53)
2 <sup>nd</sup> quartile	6/15 (40)	2/13 (15)	3/15 (20)
3 <sup>rd</sup> quartile	0/14 (0)	2/15 (13)	2/15 (13)
4 <sup>th</sup> quartile	2/15 (13)	2/14 (14)	1/14 (7)
$p^a$	0.02	0.03	0.005

<sup>a</sup> Cuzick's nonparametric test for trend.

levels constituted an additional predictor of outcome, independent of cerebral dysfunction and baseline quantitative CFU count. In a model containing cerebral dysfunction, baseline log CFU, and TNF- $\alpha$ , higher TNF- $\alpha$  levels showed a trend toward protection from death by 10 wk with a hazard ratio for each increment in quartile group of 0.51, 95% confidence interval (CI) 0.25–1.04,  $p = 0.055$ . The hazard ratios for the association of cerebral dysfunction and baseline log CFU with death by 10 wk were 7 and 3, respectively, little changed from the previous analysis without addition of TNF- $\alpha$  (10). In an exploratory analysis, when TNF- $\alpha$  levels were split into two groups with a cutoff at the 25th centile (2.2 pg/ml), there was a significant independent association of higher TNF- $\alpha$  levels with survival (hazard ratio 0.23, 95% CI 0.07–0.72,  $p = 0.012$ ).

#### IFN- $\gamma$ concentrations are independently associated with rate of clearance of infection

We took advantage of the precision of the rate of fall in cryptococcal CSF CFU as a measure of the rate of resolution of infection to determine directly whether, and if so which, immune parameters at the site of infection were associated with more rapid clearance of infection. Log IFN- $\gamma$ , G-CSF, TNF- $\alpha$ , and IL-6 were all significantly correlated with the rate of fall in log CFU/ml CSF/day, such that higher levels of these cytokines were associated with a more rapid fall in CFU ( $r = -0.52, -0.43, -0.42, -0.42$ ;  $p = 0.0002, 0.0023, 0.0028, 0.003$ , respectively). Other cytokines were not significantly associated with rate of fall in CFU. In a linear regression model, we have previously shown that the rate of fall in CFU is strongly associated with antifungal treatment group and associated with baseline log CFU (10). In a linear regression model including these factors and log IFN- $\gamma$ , G-CSF, TNF- $\alpha$ , and IL-6, only treatment group and log IFN- $\gamma$  remained independently associated with rate of clearance of infection. In a final model including only treatment group and log IFN- $\gamma$ , calculated differences in rate of fall in CFU due to treatment group were little changed compared with analysis without addition of cytokine levels (10). As previously reported, the mean rate of fall in CSF log CFU counts was significantly greater for amphotericin B plus flucytosine compared with amphotericin B alone (difference = 0.19 log CFU/day, 95% CI 0.08–0.31,  $p = 0.002$ ), amphotericin B plus fluconazole (difference = 0.17, 95% CI 0.04–0.30,  $p = 0.011$ ), or triple therapy with amphotericin B, flucytosine, and fluconazole (difference = 0.17, 95% CI 0.05–0.29,  $p = 0.007$ ). Log IFN- $\gamma$  was significantly associated with more rapid fall in CFU (increase in rate of fall in CFU for each increment in log IFN- $\gamma = 0.15$  log CFU/ml CSF/day, 95% CI 0.08–0.22,  $p < 0.001$ ; Fig. 5).

## Discussion

By using multianalyte profiling to determine multiple protein concentrations in the small volume of CSF available, this study significantly extends understanding of the immune response to HIV-

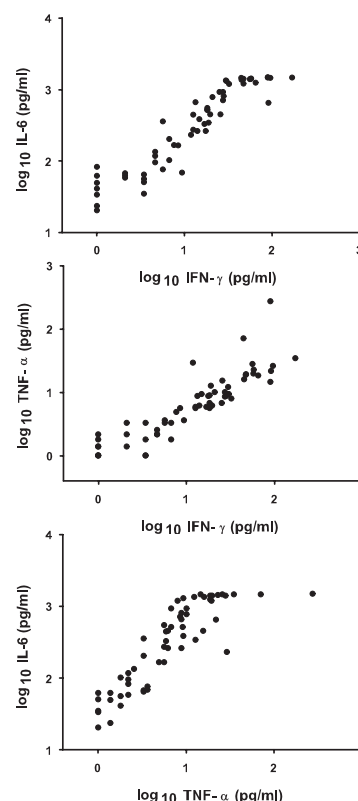


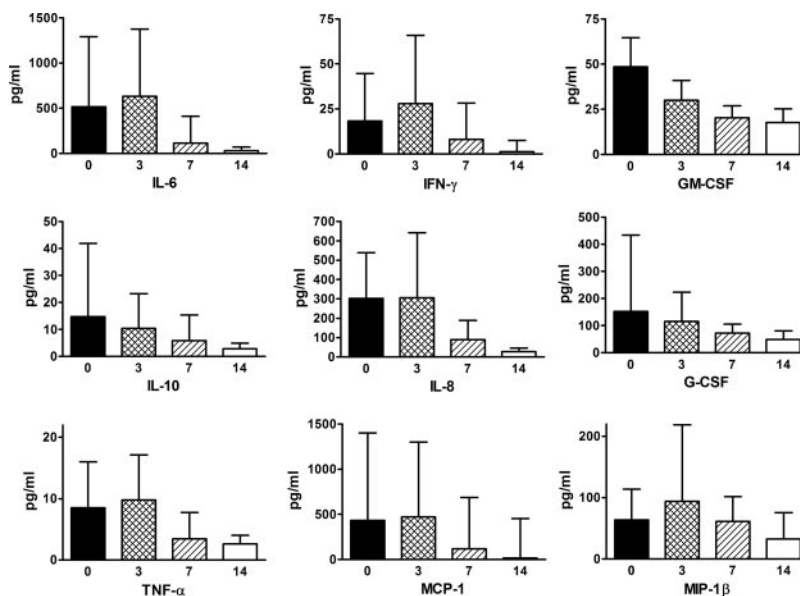
FIGURE 2. Correlation between CSF levels of IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 at baseline. Log<sub>10</sub> levels of IFN- $\gamma$ , IL-6, and TNF- $\alpha$  were highly significantly positively correlated (Pearson's correlation coefficient,  $r = 0.94, 0.89$ , and  $0.86$  for IFN- $\gamma$ /IL-6, IFN- $\gamma$ /TNF- $\alpha$ , and IL-6/TNF- $\alpha$ , respectively;  $p < 0.0001$  for all pairings).

associated cryptococcal infection in the CNS. The size of the study enabled us to demonstrate for the first time that survival is significantly associated with higher levels of proinflammatory IL-6, IFN- $\gamma$ , TNF- $\alpha$ , and IL-8. In contrast, the chemokines MIP-1 $\beta$  and MCP-1; colony-stimulating factors G-CSF and GM-CSF; and sTNF- $\alpha$  were secreted in significant concentration in the CSF, but were not associated with clinical outcome. The reasons for the discrepancy in findings with regard to sTNF- $\alpha$  between this and a prior report (12) are unclear, although a number of patients in the latter study were already on antiretroviral medication.

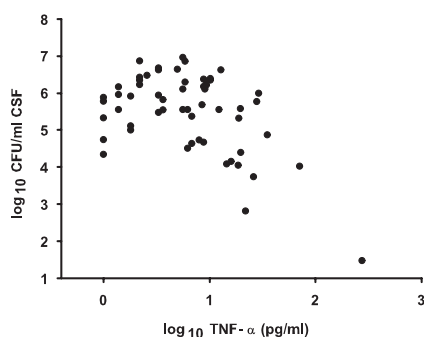
The results confirm the low TNF- $\alpha$ , IFN- $\gamma$ , and IL-10, and high IL-6 and IL-8 baseline CSF concentrations seen in previous work (12–14). In agreement with a study by Lortholary et al. (12), there was no correlation between CSF white cell count and levels of any cytokine, suggesting CSF cytokines are derived from activated resident cells (15), and the limited infiltrate of macrophages and lymphocytes seen in the brain parenchyma in HIV-associated cryptococcal meningitis (16). The uniformly low CD4 cell counts of the patients (median  $9 \times 10^9/L$ , interquartile range 6–20) may have limited our ability to demonstrate an association between cytokine levels and CD4 cell count. Secretion of IL-6, IFN- $\gamma$ , and TNF- $\alpha$  was shown to be highly coordinated, as might be expected from the known enhancement of IL-6 production by TNF- $\alpha$ , and of TNF- $\alpha$  and IL-6 production by IFN- $\gamma$  (15, 17). Secretion of many cytokines peaked at day 3 after initiation of treatment, suggestive of increased immune stimulation on commencement of antifungal therapy. This could result from the release of Ags from dying fungal cells, or the known immunostimulatory properties of amphotericin B (18). Levels of all measured cytokines were significantly reduced, many markedly so, by day 14.



**FIGURE 3.** Time course (days) of selected cytokines in CSF of patients surviving at 2 wk with cryptococcal meningitis. Results shown are median and 75% quartile.  $n = 50, 52, 47$ , and  $47$ , at baseline, days 3, 7, and 14, respectively. By day 14, levels of all cytokines were significantly reduced compared with baseline (comparing baseline and day 14 values,  $p < 0.0001$  for all cytokines shown, except MIP-1 $\beta$   $p = 0.005$ , Mann-Whitney  $U$  test).



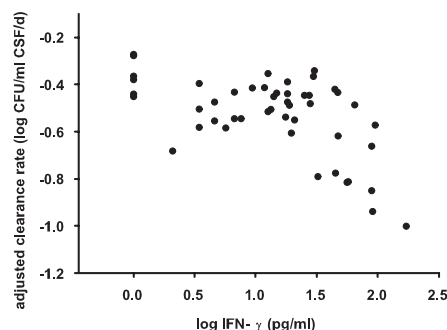
Of critical importance in understanding the pathophysiology of cryptococcal meningitis is understanding the relationship of immune parameters to factors of known importance in prognosis, notably altered mental status at presentation, and high organism load, as determined by quantitative CSF cultures (10). We found no association between any of the measured cytokines and altered mental status. Of note, altered mental status was also independent of organism load in this study. The pathophysiological basis of this key determinant of outcome remains unclear. In contrast, the important trio of proinflammatory cytokines, TNF- $\alpha$ , IFN- $\gamma$ , and IL-6, were all significantly correlated with baseline organism load, such that patients with high CSF CFU counts had lower levels of these cytokines. The failure to mount a vigorous inflammatory response in the presence of large organism numbers could be cause or effect. The immune response may be overwhelmed in the face of a large Ag load. Alternatively, patient variation in the ability to mount a proinflammatory response to cryptococcal infection may contribute to differences in organism load. In which case, it is not surprising that baseline organism load, rather than cytokine levels, remained the dominant factor in survival, given that baseline organism load, in addition to host immune factors, will also be affected by time to presentation and initiation of treatment. The role of genetic factors in susceptibility and outcome of cryptococcal meningitis will be tested by examination of case and control DNA



**FIGURE 4.** Correlation between CSF levels of TNF- $\alpha$ , and cryptococcal CSF CFU at baseline. There was a significant negative correlation between log TNF- $\alpha$  and baseline log CFU (Pearson's correlation coefficient,  $r = -0.48$ ,  $p = 0.0001$ ). Similar significant negative correlations were seen between log IFN- $\gamma$  and IL-6 levels, and baseline log CFU.

for imbalances in polymorphisms in immune response genes, in particular differences in TNF- $\alpha$  receptor polymorphisms (19–21). Our data would suggest alleles associated with enhanced TNF- $\alpha$  production might protect individuals from severe cryptococcal meningitis.

In univariate analysis, higher IFN- $\gamma$ , G-CSF, TNF- $\alpha$ , and IL-6 levels were associated with a more rapid decline in CSF cryptococcal CFU. In multivariate analysis, only antifungal drug regime and IFN- $\gamma$  remained independently associated with the rate of clearance of infection. The importance of IFN- $\gamma$  levels in clearance of cryptococcal infection is supported by recent data showing the proportion of patients with HIV-associated cryptococcal meningitis with a negative CSF culture at 2 wk was higher in those receiving adjunctive IFN- $\gamma$  compared with patients on chemotherapy alone (36 vs 13%,  $p = 0.07$  (8)). Previous studies have documented the susceptibility of patients with impaired production of or response to IFN- $\gamma$ , through mutations in the genes encoding IL-12p40, IL-12R $\beta$ 1, or the IFN- $\gamma$  receptor, to a variety of mycobacterial and bacterial infections (22). Regarding fungal infections, only one case of disseminated histoplasmosis (23) and one case of



**FIGURE 5.** Association between log IFN- $\gamma$  levels and rate of clearance of infection. The rate of decrease in log CFU/ml CSF/day has been adjusted for the effect of treatment group, according to the results of a linear regression model, including treatment group and log IFN- $\gamma$ . Thus, differences due to treatment group have been added to rates of decline in CFU for patients in the three less rapidly fungicidal treatment groups. In the model, log IFN- $\gamma$  was independently associated with a more rapid fall in CFU (increase in rate of fall in CFU for each increment in log IFN- $\gamma$  =  $0.15$  log CFU/ml CSF/day, 95% CI  $0.08$ – $0.22$ ,  $p < 0.001$ ).

mucosal candidiasis (24) have been seen to date in 100 individuals with genetic defects in this pathway. Thus, our results provide further direct evidence for the importance of IFN- $\gamma$  in human immune control of granulomatous infection in vivo, extending this role to fungal infection, and demonstrating that more subtle quantitative differences in IFN- $\gamma$  expression, in individuals without major mutations in the IFN- $\gamma$  pathway, may still be critical to the outcome of such infections.

The study illustrates the power of quantitative assessment of pathogen load over time not only to address therapeutic questions, but also to determine host and pathogen factors influencing the outcome of infection. Trends suggesting benefit have been seen in trials of adjunctive IFN- $\gamma$  therapy in a number of granulomatous infections (8, 25–27). However, lack of powerful surrogate markers of response has restricted progress in defining optimal dosing schedules. In the setting of cryptococcal meningitis, quantitative assessment of organism load over time also provides the means by which the effects of alternative dosages and schedules for the administration of immunotherapeutic agents, in particular IFN- $\gamma$ , could be tested.

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