A Dual Role For TGF-β1 in the Control and Persistence of Fungal Pneumonia[1]

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TGF-β1 (TGF) has been implicated in the pathogenesis of several chronic infections and is thought to promote microbial persistence by interfering with macrophage function. In rats with experimental pulmonary cryptococcosis, increased lung levels of TGF were present at 12 mo of infection. Within the lung, expression of TGF localized to epithelioid cells and foamy macrophages in areas of inflammation. Increased TGF expression was also observed in the lungs of experimentally infected mice and a patient with pulmonary cryptococcosis. TGF reduced Ab and serum-mediated phagocytosis of *Cryptococcus neoformans* by rat alveolar macrophages (AM) and peripheral blood monocytes, and this was associated with decreased chemokine production and oxidative burst. Interestingly, TGF-treated rat AM limited both intracellular and extracellular growth of *C. neoformans*. Control of *C. neoformans* growth by TGF-treated rat AM was due to increased secretion of lysozyme, a protein with potent antifungal activity. The effects of TGF on the course of infection were dependent on the timing of TGF administration relative to the time of infection. TGF treatment of chronically infected rats resulted in reduced lung fungal burden, while treatment early in the course of infection resulted in increased fungal burden. In summary, our studies suggest a dual role for TGF in persistent fungal pneumonia whereby it contributes to the local control of infection by enhancing macrophage antifungal efficacy through increased lysozyme secretion, while limiting inflammation by inhibiting macrophage/monocyte phagocytosis and reducing associated chemokine production and oxidative burst. ***The Journal of Immunology,*** **2005, 175: 6757–6763.**

One of the least understood phenomena in microbial pathogenesis is the ability of certain microbes to cause persistent infection. *Cryptococcus neoformans* is an encapsulated fungal pathogen that is remarkable for its ability to cause persistent pulmonary and CNS infections. Although immunosuppression may contribute to the chronic nature of cryptococcal disease in patients with AIDS, *C. neoformans* also causes persistent pulmonary infection in immunocompetent individuals. Affected patients can have respiratory symptoms for weeks to months before diagnosis (1, 2). Furthermore, persistent subclinical cryptococcal infections may reactivate months to years after primary infection, in a manner similar to tuberculosis (3–5).

The rat model of pulmonary infection provides a unique system to study the host and pathogen factors that contribute to persistent pulmonary cryptococcosis in the setting of an intact immune response. Experimental pulmonary infection of rats with *C. neoformans* results in a localized, subclinical granulomatous pneumonia that can persist for months to years (6). In this manner, pulmonary cryptococcosis in the rat shares many features with pulmonary cryptococcosis in immunocompetent humans (7). Persistent pulmonary infection in the rat is associated with a decrease in inflammation and macrophage activation as indicated by the following parameters: decreased NO synthase expression, decreased CD11b/c, and MHC II expression (8). Long-term infection is further characterized by decreased MCP-1 (CCL2) expression within the lung and decreased MCP-1 production by pulmonary macrophages (9). Hence, control of infection is achieved by an intense and effective granulomatous response that is attenuated by down-regulatory mechanisms that are not understood.

TGF-β1 (TGF)[3] is a multifunctional cytokine that plays a central role in the pathogenesis of several chronic infectious diseases. A range of macrophage deactivating properties for TGF has been described previously (reviewed in Ref. 10). In particular, the ability of TGF to interfere with the activation and oxidative mechanisms of macrophages (10, 11) has been implicated in promoting persistent infection by providing a survival advantage to pathogens. In this regard, the ability of pathogens to elicit TGF expression has been hypothesized as a potential virulence trait (12, 13). Little is known about the role of TGF in cryptococcal infection, although Kawakami et al. (14) demonstrated enhanced TGF message during the later stages of experimental pulmonary infection in mice. Because decreased inflammation and down-regulation of macrophage function are characteristics of persistent pulmonary cryptococcosis in the rat, we set out to determine the role of TGF in this system. To our surprise, we identified a novel role for TGF whereby this cytokine was found to decrease chemokine production by macrophages yet enhance their antifungal activity by enhancing lysozyme secretion.

Materials and Methods

C. neoformans

For in vivo experiments, *C. neoformans* ATCC strain 24067 was used. This is a D serotype strain that has been studied extensively in animal models of cryptococcosis, including the rat system (6). To study the effects of TGF on

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[3] Abbreviations used in this paper: TGF, TGF-β1; AM, alveolar macrophage; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine.

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phagocytosis, both ATCC strain 24067 and strain H99, a serotype A strain, were used.

Animals

Male Fischer rats weighing 200–250 g were obtained from Harlan Sprague Dawley. Female C57BL/6 mice ~4–6 wk of age were obtained from the National Cancer Institute.

Infection

Rats were inoculated endotracheally with 1×10^7 *C. neoformans*, strain 24067, using an otoscope as described previously (6). This inoculum produces an acute pneumonia that is controlled without dissemination and then evolves into chronic infection whereby the yeast cells are contained in well-formed granulomas. Control rats were inoculated with sterile PBS. For mice ($n = 5$/group), the trachea was surgically exposed, and animals were inoculated with ATCC strain 24067 (1×10^6) or PBS in a volume of 0.05 ml.

TGF tissue levels

At various times following pulmonary inoculation, rats were injected i.p. with a lethal dose of pentobarbital. The chest cavity was opened, and a needle was placed in the left ventricle, the inferior cava cut, and animals were then perfused for 7 min at a constant pressure of 220 mm Hg. TGF concentrations were measured using a commercially available ELISA (Promega) that detects biologically active TGF.

Immunohistochemistry

Rats were killed by asphyxiation with CO_2 and lungs inflated with OCT (Sakura Finetek). For frozen sections, lungs were removed, placed in OCT, and then frozen at −80°C. Tissue sections (5 μm) were fixed with ice-cold methanol and blocked sequentially in 3% H_2O_2 and goat serum. Polyclonal rabbit anti-TGF (Santa Cruz Biotechnology) was applied at a dilution of 1/50 and detected with biotinylated goat anti-rabbit Ab and avidin-labeled HRP (Vector Laboratories). TGF staining was also done on formalin-fixed, paraffin-embedded tissues. For these studies an additional 6 min pronase incubation was done (15). Staining for cryptococcal polysaccharide was done using a murine IgG1 mAb to cryptococcal polysaccharide (18B7) as the primary Ab and an alkaline phosphatase labeled goat-anti-murine IgG1 as the secondary Ab (16). Color was developed with Fast Blue (Sigma-Aldrich). Double staining for TGF and cryptococcal polysaccharide was done sequentially on paraffin sections.

Human tissue

Paraffin-embedded tissue from a child with a pulmonary nodule secondary to cryptococcosis was generously donated by M. Caserta (Strong Children's Hospital, Rochester, NY) (17). This tissue was stained for TGF using the same protocol described for rat tissue.

Cells

The rat alveolar macrophage (AM) cell line NR8383 was obtained from the American Type Culture Collection. These cells are derived from normal rat AM and display characteristics of macrophages (18). Primary rat AM were obtained by lung lavage as described previously (19). Briefly, a 16-gauge angiocatheter placed in the trachea and the airway washed with 5 volumes of cold PBS (10 ml each). Cells were collected by centrifugation. Following lysis of RBC by incubation in cold ammonium chloride solution (0.17 M), cells were washed with PBS, centrifuged, and resuspended in medium. Peripheral rat blood monocytes were obtained using Optiprep (Axis-Shield) as per manufacturer's instructions. Briefly, PBMC were obtained by centrifugation of heparinized blood in a solution of Optiprep. The layer of mononuclear cells was removed, washed with PBS, centrifuged, and placed in ammonium chloride to lyse RBC. The remaining pellet was washed and resuspended in medium. Cells were then placed in 96-well plates and allowed to adhere for 2 h.

Opsonins

18B7 is a murine IgG1 mAb that recognizes glucuronoxylomannan, the main component of the cryptococcal polysaccharide capsule (16). Serum was obtained from uninfected, control rats and kept frozen at −20°C before use. All serum was used within 1 wk of being obtained.

Phagocytosis studies

Cells were plated in 96-well tissue culture plates (Costar) at a density of 4×10^4 cells/well in 0.1 ml of DMEM-10% FCS and allowed to attach at 37°C for 2 h. Medium was then replaced with medium containing recombinant human TGF (BioSource International) at a concentration ranging from 1 to 100 ng/ml. Twenty hours later, medium was replaced with medium (0.1 ml) containing 1×10^6 yeast/ml and either 10% rat serum or mAb 18B7 (10 μg/ml). Monocytes/macrophages and *C. neoformans* (E:T 1:1) were incubated for 2 h at 37°C. Cells were gently washed with HBSS, fixed with ice-cold absolute methanol, and stained with Giemsa (Sigma-Aldrich). For some experiments, TGF was preincubated with a murine mAb specific for human TGF (R&D Systems) at a concentration of 1 μg/ml. The phagocytic index was defined as the number of attached and ingested cryptococci divided by the number of macrophages per microscope field.

Chemokine and TNF-α induction

For these studies, primary AM and N3838 were plated in 96-well tissue plates and treated with TGF in the same manner as in phagocytosis experiments. Twenty-four hours after TGF treatment, cells were incubated with *C. neoformans*, and serum or Ab was allowed to incubate. Supernatants were removed at 4 or 24 h and stored at −20°C. MCP-1 and MIP-2 levels were measured using commercially available ELISA kits from BD Biosciences and BD Pharmingen, respectively, and BioSource International. To ensure that treatment with TGF had no effect on macrophage viability, cells were treated with varying concentrations of TGF (1–100 ng/ml). For TNF-α studies, primary rat AM were obtained and treated with TGF for 24 h at a concentration of 10 ng/ml and then incubated with mAb-opsonized *C. neoformans*. TNF-α levels were measured at 24 h using a commercially available ELISA (BD Pharmingen). At various times, cells were detached and stained with trypan blue. No increase in cell death was observed in association with TGF treatment.

Intracellular growth

TGF was added to primary monocytes/macrophages that had ingested *C. neoformans* in vitro. Experiments were done in this manner because TGF alters the ability of monocytes/macrophages to take up *C. neoformans*. Approximately 3×10^4 cells/well were seeded in 96-well plates. Cells were incubated for 2 h at 37°C, then incubated with *C. neoformans* (3×10^4 organisms/well) for an additional 2 h, at which time the wells were washed and medium removed. Medium containing various concentrations of TGF was then added, and plates were returned to the incubator for an additional 20 h. For some experiments, TGF was preincubated with a murine mAb specific for human TGF (R&D Systems) at a concentration of 1 μg/ml. In other experiments, TGF was added together with 10 ng/ml recombinant rat TNF-α (BioSource International). Supernatants were removed and plated. The remaining attached cells were lysed by incubation in distilled water for 30 min at room temperature. To ensure that TGF had no direct effect on *C. neoformans* growth, fungal cells were incubated in varying concentrations (1–100 ng/ml) of TGF in medium.

Extracellular growth

AM were seeded in 96-well plates at a density of 1×10^5/well and allowed to adhere for 2 h. *C. neoformans* were then added at density of 1×10^4/well without opsonin. TGF was added to wells at different concentrations. At various times, culture supernatants were removed and fungal burden determined by growth on Sabouraud's Dextrose agar.

Oxidative burst

AM were seeded at a density of 4×10^5/well into 6-well plates. Following 2 h of incubation, TGF at various concentrations was added to cultures that were returned to the incubator for an additional 20 h. Cells were detached

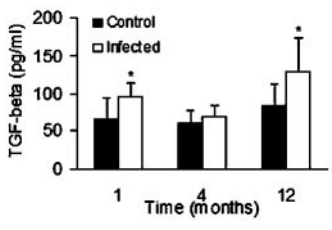

FIGURE 1. Lung levels of TGF at various times of infection in the rat. Levels determined by ELISA on lung homogenates ($n = 5$ rats/group). Bars represent 1 SD. *, $p < 0.05$ compared with control lung from same time of infection.

by treatment with trypsin EDTA and resuspended at 2×10^5/ml in HBSS containing 50 μM Luminol (Sigma-Aldrich). Ab (18B7)-opsonized *C. neoformans* (1×10^6) were then added to AM suspensions. Luminescence was measured in a luminometer (Moonlight 2010; Analytical Luminescence Laboratory) at 10-min intervals for 1 h.

Inhibition of oxidative burst and nitrogen free radicals

To determine the role of oxygen and nitrogen free radicals in the control of intracellular growth mediated by TGF, intracellular growth studies were done in the presence of oxidative burst and NO synthase inhibitors, 0.4 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine (Tempol; Sigma-Aldrich) and 1 mM aminoguanidine (Sigma-Aldrich), respectively (20). For these studies, AM were isolated and allowed to adhere for 2 h. Cultures were then treated with *C. neoformans* in the presence of 18B7 at an E:T of 1:1. Two hours later, cultures were washed to remove nonphagocytozed organisms. Medium was replaced with medium containing TGF with or without Tempol or aminoguanidine. Twenty-four hours later fungal burden in the supernatant and lysates was determined.

Lysozyme determinations

Approximately 1×10^5 AM were seeded into 96-well plates and allowed to adhere. Medium was then replaced with medium containing TGF. For some cultures, supernatants were removed at 4 h and for others at 24 h. Supernatants were tested for lysozyme using *Micrococcus lysodeikticus* as described previously (21). Briefly, 40 μl of a 0.03% suspension of bacteria in 0.1 M potassium phosphate buffer (pH 6.2) was added to 200 μl of supernatant or lysozyme standard (Roche) and incubated 37°C for 30 min. Absorbance at 450 nm was then determined.

Inhibition of lysozyme

Approximately 1×10^5 AM were plated in 96-well plates and incubated for 2 h. *C. neoformans* (1×10^4/well) were added to each well in the presence or in the absence of TGF (10 ng/ml). To some wells, *N,N,N*-triacetyl chitotriose (1 mM; Sigma-Aldrich) was added along with TGF and *C. neoformans*. *N,N,N*-triacetyl chitotriose is a potent inhibitor of the enzymatic activity of lysozyme (22). At various times, supernatants were removed and plated on Sabouraud's Dextrose agar.

TGF treatment studies

TGF treatment studies were done in the contest of established infection at the time of *C. neoformans* inoculation. To determine the effects of TGF on established infection, rats were infected with 1×10^7 *C. neoformans* by intratracheal inoculation. One month later, rats were given 1 μg of rTGF by tail vein injection on 4 successive days. On the fifth day, rats were killed and lungs removed and analyzed for fungal burden. To determine the effects of TGF on incipient infection, rats were i.v. injected with 1 μg of TGF beginning the day before infection for 4 successive days. On the fifth day, rats were killed and tissues removed for analysis. Five to six rats were used per group in both experiments. Each experiment was repeated with similar results.

Statistics

Comparisons of normally distributed data were done using a Student's *t* test. Multiple comparisons were done using ANOVA, and subsequent analysis of differences between groups was done using either a Dunnett or a Student-Newman-Keuls test. For growth studies, fungal burden was first logarithmically transformed before analysis. A value of $p \leq 0.05$ was considered significant.

Results

TGF expression

TGF tissue concentrations were elevated in the lungs of rats with persistent pulmonary cryptococcal infection (Fig. 1). Higher TGF levels in the lungs of infected rats relative to sham-infected animals were measured as early as 1 mo of infection (earliest time studied). Elevated levels of TGF persisted up to 12 mo.

At 1 mo of infection in the rat, TGF immunoreactivity was present within epithelioid cells of some granulomas but not others (Fig. 2*A*). TGF expression was also present in scattered inflammatory cells surrounding some granulomas. Respiratory epithelium (data not shown) also expressed TGF. At the later times of infection, fewer epithelioid cells were TGF immunoreactive, but prominent TGF immunoreactivity was present within foamy macrophages (Fig. 2, *B* and *C*). Double staining for TGF and cryptococcal polysaccharide revealed that foamy macrophages containing polysaccharide were often TGF immunoreactive (Fig. 2*D*). We also examined expression of TGF in lungs of infected mice and in

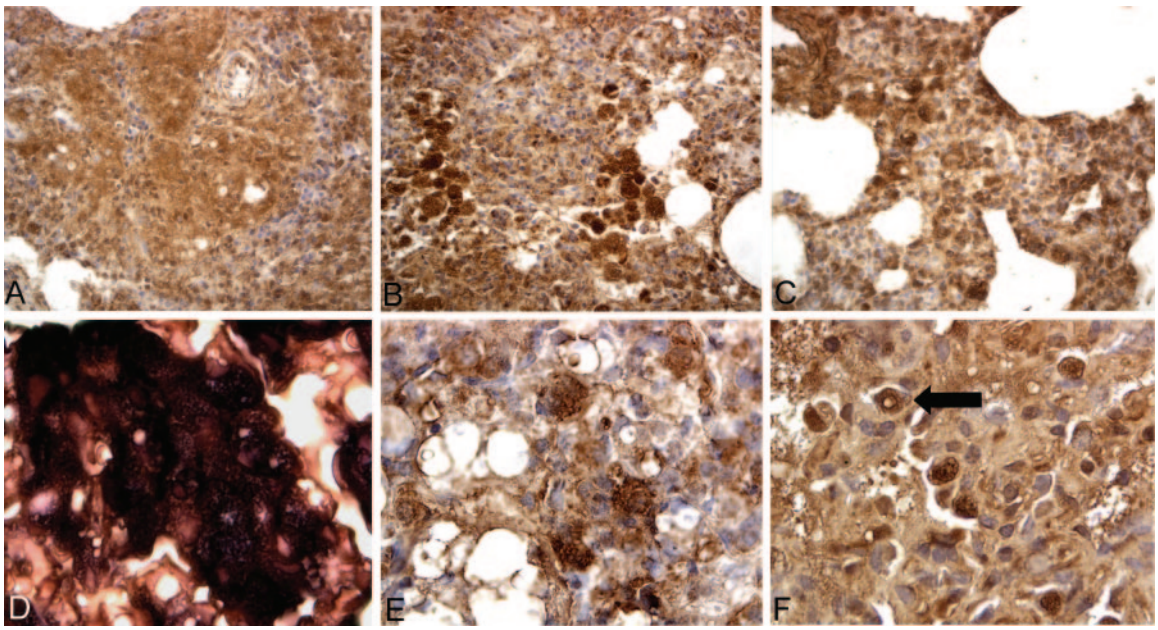

FIGURE 2. Immunohistochemistry for TGF rat (*A–D*), mouse (*E*), and human (*F*) lung tissue. Rat lung tissue from 1 mo of infection (*A*) shows granuloma with extensive with TGF reactivity (brown) (original magnification, ×200). At 4 mo of infection in the rat (*B*), TGF reactivity is prominent within macrophages (original magnification, ×200). At 12 mo of infection (*C*), persistent TGF reactivity is present within macrophages (original magnification, ×200). Collection of macrophages (*D*) from rat lung shows colocalization of TGF (brown) and cryptococcal polysaccharide (blue) from 4 mo of infection (original magnification, ×400). Lung tissue from mouse (*E*) at 14 days of infection shows TGF immunoreactive macrophages (original magnification, ×400). Lung tissue from child with pulmonary cryptococcosis (*F*) shows TGF immunoreactive macrophages and epithelioid cells (original magnification, ×400). Arrow points to a *C. neoformans* cell that has been detected using a cryptococcal polysaccharide-specific mAb.

a patient with pulmonary cryptococcosis. For mice, TGF expression was noted within the lungs on day 14 of infection with *C. neoformans*. Expression was present in focal collections of mononuclear cells, including lymphocytes and macrophages within areas of inflammation (Fig. 2*E*). TGF expression was also identified within the lung of a patient with a pulmonary nodule secondary to cryptococcosis (17). This was present in scattered mononuclear cells within areas of inflammation (Fig. 2*F*).

Phagocytosis

TGF treatment of the rat AM cell line NR8383 resulted in a dose-dependent decrease in mAb and serum-mediated phagocytosis. At doses of 10 and 100 ng/ml TGF, phagocytic activity in the presence of mAb was reduced by 29 and 40%, respectively. A similar decrease in Ab-mediated phagocytosis was also seen when primary rat AM (Fig. 3*A*), and rat peripheral blood monocytes (data not shown) were used as the effector cells. Coincubation of TGF with a murine Ab to TGF prevented the decrease in Ab-mediated phagocytosis produced by TGF treatment (Fig. 3*B*). TGF treatment also resulted in a decrease in serum-mediated phagocytosis by primary rat AM (Fig. 3*A*). Similar results were obtained for ATCC strain 24067 and H99, although serum-mediated phagocytosis of *C. neoformans* was greater for H99. All experiments were done at least twice with similar results.

Chemokine and TNF-α production

TGF treatment of macrophages decreased MCP-1 and MIP-2 induction associated with both Ab and serum-mediated phagocytosis (Fig. 4). For MCP-1, this decrease was dependent on the dose of TGF used, with a maximal reduction of 80% for mAb-mediated phagocytosis and 60% for serum-mediated phagocytosis. For MIP-2, decreased production in the context of serum-mediated phagocytosis was observed only at the highest dose of TGF. Similar results were observed for both the NR8383 cell line and primary AM (data shown). TGF treatment also resulted in a reduction in both spontaneous and LPS-mediated production of MCP-1 (data not shown). Experiments were done at least twice with similar results. TGF treatment of AM also reduced the amount of TNF-α released into supernatant following Ab-mediated phagocytosis of *C. neoformans* (1006 ± 25 vs 1783 ± 161 pg/ml, $p < 0.01$).

Intracellular and extracellular growth

Treatment of infected rat monocytes and AM with TGF decreased intracellular growth of *C. neoformans* as manifested by dose-dependent decrease in the number or organisms isolated from the lysates of infected cells (Fig. 5*A*). Ab to TGF prevented the reduction in lysate fungal burden mediated by TGF (Fig. 5*B*). TNF-α treatment resulted in a reduction of lysate fungal burden, which was similar in magnitude to the reduction observed with TGF treatment. Interestingly, TGF did not prevent the reduction of lysate fungal burden mediated by TNF-α (Fig. 5*C*). No direct effects of TGF on *C. neoformans* growth were observed (data not shown). Experiments were done twice with similar results.

TGF treatment of macrophages decreased extracellular growth of *C. neoformans* at both 4 and 12 h. At 12 h, 33 and 41% reductions were seen in the extracellular fungal burden for macrophage cultures treated with 10 and 100 ng/ml TGF (Fig. 6) relative to medium alone. Experiments were done twice with similar results.

Oxidative burst

Baseline chemiluminescence levels were higher for primary AM treated with 10 and 100 ng/ml TGF ($p < 0.05$). Nevertheless, TGF-treated cells failed to show an increase in chemiluminescence over the subsequent 60 min that control cells exhibited in association with Ab-mediated phagocytosis of *C. neoformans* (Fig. 7*A*). These results are consistent with the decrease in Ab-mediated phagocytosis observed in association with TGF treatment.

To determine the mechanisms by which TGF mediates control of intracellular *C. neoformans* growth, *C. neoformans*-infected macrophages were treated with a superoxide dismutase mimetic (Tempol) or a competitive inhibitor of inducible NO synthase (aminoguanidine) in the presence of TGF. Primary AM infected with *C. neoformans* and then treated with Tempol showed an increase in lysate fungal burden consistent with the importance of oxidative burst in limiting *C. neoformans* growth. Incubation of infected cells with TGF and Tempol showed a decrease in lysate fungal burden relative to infected cells treated with Tempol alone

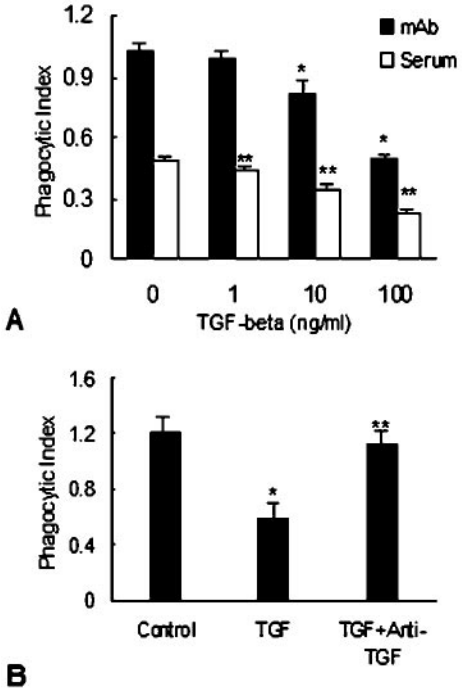

FIGURE 3. Phagocytic indices for rat AM treated with TGF. *A*, The effects of different concentrations of TGF on serum and Ab-mediated phagocytosis of *C. neoformans*. Bars represent 1 SD. *, $p < 0.05$ for comparison with mAb-mediated phagocytosis in the absence TGF. **, $p < 0.05$ for comparison with serum-mediated phagocytosis in the absence of TGF. *B*, Phagocytic indices for rat AM using the 18B7 mAb as the opsonin. Cells were treated with medium alone (control), TGF (10 ng/ml), or TGF that was preincubated with a mAb against TGF. Bars represent 1 SD. *, $p < 0.05$ for comparison with control cells. **, $p < 0.05$ for comparison with TGF treated cells.

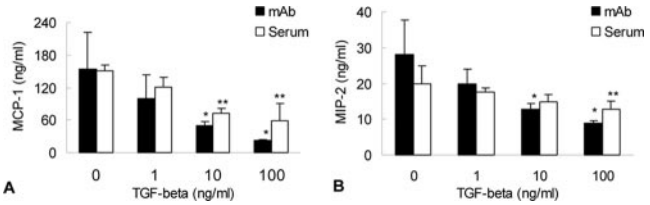

FIGURE 4. Effects of TGF on MCP-1 (*A*) and MIP-2 (*B*) production. Macrophages were pretreated with various concentrations of TGF and allowed to ingest *C. neoformans*. Chemokine levels were determined in culture supernatants 24 h following serum or Ab-mediated phagocytosis. Bars represent 1 SD. *, $p < 0.05$ for comparison with no TGF and mAb. **, $p < 0.05$ for comparison with no TGF and serum.

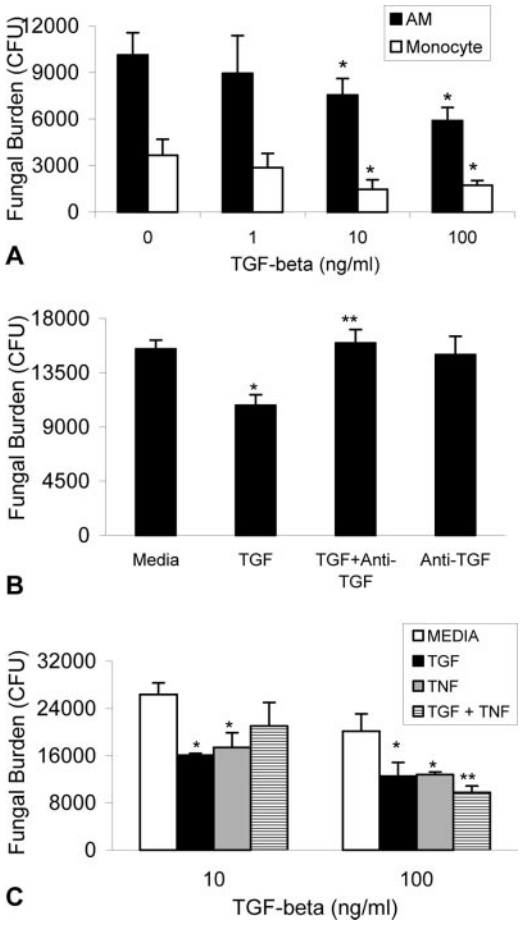

FIGURE 5. Effects of TGF on intracellular *C. neoformans* growth. *A*, Lysate fungal burden following treatment of infected primary AM (■) and monocytes (□) with different concentrations of TGF. *, $p < 0.05$ for comparison against no TGF. *B*, Incubation of TGF with anti-TGF prevented the reduction in lysate fungal burden observed with TGF treatment. *, $p < 0.05$ compared with no TGF. **, $p < 0.05$ compared with TGF. *C*, Lysate fungal burden for infected cells treated with TGF (■), TNF-α (▨), or TGF together with TNF-α (▤). The concentration of TNF-α was 10 ng/ml. *, $p < 0.05$ for comparison with medium alone; **, $p < 0.05$ for comparison with TNF-α. Bars represent 1 SD.

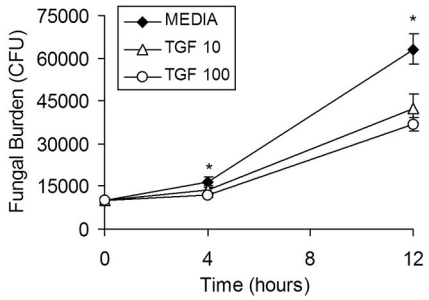

FIGURE 6. Effects of TGF on extracellular *C. neoformans* growth. Equal numbers of organisms were added to AM in the presence or absence of TGF. Two different concentrations of TGF were used 10 ng/ml (TGF 10) and 100 ng/ml (TGF 100). No opsonin was added. Fungal burden in supernatant was then determined at 4 and 12 h. *, $p < 0.05$ for TGF-treated AM compared with AM grown in media alone. Error bars represent 1 SD.

(Fig. 7*B*). Infected cells treated with aminoguanidine showed no increase in lysate fungal burden compared with control infected cells. Furthermore, a reduction in lysate fungal burden was observed following treatment with aminoguanidine and TGF (100 ng/ml) relative to non-TGF-treated cells (Fig. 7*C*). Taken together, these findings suggest that the inhibition of fungal intracellular growth by TGF was not related to either nitrogen or oxygen free radical production.

Lysozyme

TGF treatment of macrophages produced a dose-dependent increase in lysozyme levels in culture supernatants at 4 h (Fig. 8*A*). An ~50% increase in lysozyme supernatant levels was seen for cells treated with the highest concentration of TGF. No difference in lysozyme levels in culture supernatants between TGF and non-treated cells was observed at 24 h.

To determine the role of lysozyme in TGF-mediated control of *C. neoformans* replication, experiments were done with an inhibitor of lysozyme, chitotriose. The addition of chitotriose to macrophage cultures prevented the reduction in extracellular growth mediated by TGF (Fig. 8*B*). Chitotriose alone had no effect on *C. neoformans* growth (data not shown). Experiments were done twice with similar results.

TGF treatment

To determine the relevance of in vitro findings to active infection, TGF was given to rats with experimental pulmonary cryptococcosis. TGF treatment was done both in the context of established infection and at the time of infection was induced. Intravenous TGF treatment of rats that had been infected for 1 mo with *C. neoformans* produced an average 0.5 $\log_{10}$ decrease in lung fungal burden (5.42 ± 0.29 vs 5.95 ± 0.23, $p = 0.01$). In contrast, early treatment (i.e., at the time of *C. neoformans* inoculation) with TGF resulted in an ~0.5 $\log_{10}$ increase in lung fungal burden (5.41 ± 0.36 vs 4.95 ± 0.4, $p = 0.04$). No differences in lung chemokine (MCP-1 and MIP-2) levels were observed in association with TGF treatment for either early or late treatment (data not shown). Both experiments were done twice with similar results.

Discussion

The association of TGF expression with *Mycobacterium tuberculosis*, *Leishmania major*, and *Trypanosoma cruzii* infections suggests that this cytokine contributes to the pathogenesis of chronic bacterial and parasitic infections (23–25). In the present study, we extend this phenomenon to fungal infections by demonstrating TGF expression in the lungs of mice, rats, and a patient with pulmonary cryptococcosis.

Macrophages play a central role in both producing TGF and as targets of TGF action in chronic infection. Previous studies have shown that TGF can act as a chemoattractant for monocytes and neutrophils (26, 27). Our results suggest a mechanism by which TGF can limit chemokine and TNF-α production by macrophages within granulomas by impeding serum and Ab-mediated phagocytosis of *C. neoformans*. Our findings are consistent with those of Tridandapani et al. (28) who found that TGF inhibits Ab-mediated phagocytosis of sheep RBC by decreasing expression of the γ subunit of the FcγR of monocytes. The ability of TGF to limit phagocytosis by macrophages could serve to limit inflammation and ongoing tissue damage by decreasing chemokine production and oxidative damage during chronic infection.

It has been hypothesized that TGF contributes to the persistence of certain infections by impeding the antimicrobial activity of macrophages (29, 30). In this regard, TGF is a potent inhibitor of macrophage killing for several pathogens, including *T. cruzii*, *M. tuberculosis*, and *L. major* (24, 31, 32). For these pathogens, increased intracellular growth produced by TGF is associated with

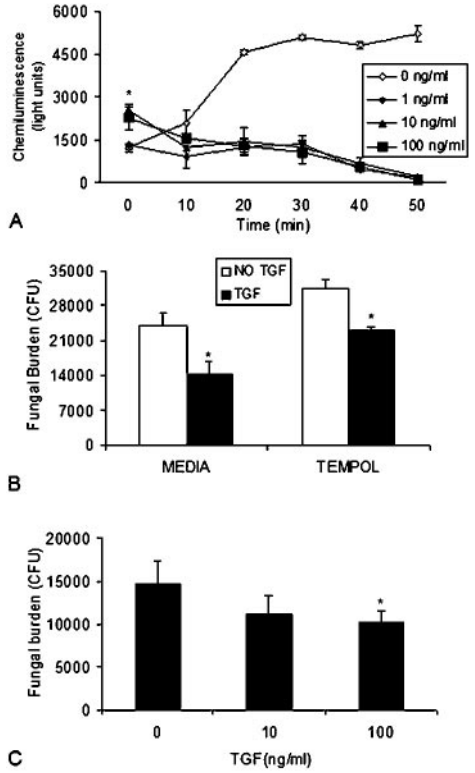

FIGURE 7. *A*, Chemiluminescence as an indicator of oxidative burst for AM that were pretreated with TGF and then incubated with mAb opsonized *C. neoformans*. Baseline chemiluminescence was greater for AM treated with TGF (10 and 100 ng/ml) compared with cells treated with medium alone (*, $p < 0.05$). Nevertheless, macrophages treated with TGF failed to exhibit an increase in chemiluminescence in association with phagocytosis. *B*, The decrease in lysate fungal burden observed in association with TGF treatment of infected AM was not abrogated by treatment with Tempol, a superoxide dismutase mimetic. *C*, Treatment with TGF in the presence of aminoguanidine (1 mM), a competitive inhibitor of inducible NO synthase, resulted in a decrease in lysate fungal burden. *, $p < 0.05$ relative to no TGF. Error bars represent 1 SD.

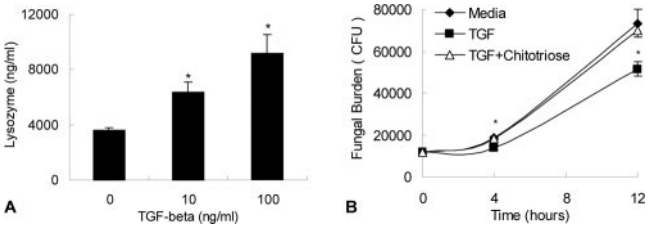

FIGURE 8. *A*, TGF increases supernatant lysozyme levels. Supernatant lysate levels were measured 4 h after treatment with TGF. *, $p < 0.05$ compared with no TGF. Bars represent 1 SD. *B*, Inhibition of lysozyme activity with chitotriose 1 mM prevented the reduction in extracellular *C. neoformans* growth mediated by TGF (10 ng/ml). This effect was more prominent at 12 h. Error bars represent 1 SD.

inhibition of macrophage activation by IFN-γ or TNF-α stimulation (13, 33) and subsequent free radical production (34, 35). Interestingly, we found that TGF reduced both intracellular and extracellular growth of *C. neoformans*. The basis for this discrepancy may relate to the effects of TGF on nonoxidative pathways and the susceptibility of various pathogens to nonoxidative killing mechanisms.

We found that TGF stimulation of macrophages was associated with increased levels of lysozyme in culture supernatants. Furthermore, inhibition of lysozyme activity prevented the reduction in extracellular fungal burden mediated by TGF. The rapid increase in lysozyme levels following TGF treatment of macrophages suggests that TGF increases release of preformed protein. Lysozyme is produced by macrophages and is found in granulomas induced by a variety of agents (36, 37). Lysozyme has broad antimicrobial activity that includes enzymatic lysing of bacteria and the inhibition of fungal growth, including *C. neoformans* (38–40). In addition to its enzymatic activity, lysozyme may exert antimicrobial activity as a result of its cationic properties. Our results help explain the findings of previous studies, which demonstrate a protective effect of TGF in murine models of *Candida albicans* and *Listeria monocytogenes* infection (41, 42). Both of these pathogens are susceptible to the antimicrobial effects of lysozyme (39, 43). Lysozyme has been associated with a variety of immunosuppressive effects (44–46). This includes the depression of oxidative metabolism of neutrophils and the inhibition of neutrophil migration (47). We hypothesize that lysozyme may contribute to the down-regulation of granulomatous inflammation in persistent pulmonary cryptococcosis. We note that enhanced secretion of this lysozyme by TGF is consistent with the immune down-regulating functions of TGF.

Interestingly, the effects of TGF treatment on infected animals were dependent on the timing of administration relative to infection. In the context of established infection, TGF resulted in a reduction in lung fungal burden, while early TGF treatment resulted in increased lung fungal burden. This differential effect may relate to the importance of different aspects of macrophage biology at different stages of infection. Early treatment with TGF may impede phagocytosis of *C. neoformans* and subsequent chemokine and proinflammatory cytokine induction, which is needed to generate an effective inflammatory response. In this regard, Huffnagle et al. (48) have demonstrated the importance of early TNF-α expression in the generation of a protective Th1 response in mice.

In summary, our studies demonstrate conserved expression of TGF in rats, mice and humans in response to pulmonary cryptococcal infection. TGF appears to contribute to the persistence of infection by limiting inflammation during the chronic stages of infection by interfering with phagocytosis of *C. neoformans* and subsequent chemokine induction and oxidative burst by macrophages. However, TGF also promotes lysozyme release, which has potent antifungal effects. The ability of TGF to promote lysozyme release by macrophages appears to be a previously unrecognized function of this cytokine. Thus, TGF may limit inflammation in the chronic stages of infection but also help limit cryptococcal growth. The damage-response framework of microbial pathogenesis views microbial virulence in the context of host damage, which is a function of the immune response (49). Accordingly, host damage can result either from direct microbial processes or overexuberant host responses. The regulation of inflammation by TGF may be a critical parameter is determining the outcome of microbial infection whereby the host can trade persistence of infection for the benefit of decreased inflammation.

Disclosures

The authors have no financial conflict of interest.

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