Modulation of Tumor Necrosis Factor Production by Macrophages in *Entamoeba histolytica* Infection

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The macrophage-derived mediator tumor necrosis factor alpha (TNF) is a cytokine with pleiotropic effects. TNF exhibits potent immunologic and inflammatory properties in parasitic diseases. The present study examined the production of TNF by macrophages isolated from gerbils infected with *Entamoeba histolytica* and by naive macrophages in response to amoebae in vitro. Amebic liver abscess-derived macrophages produced low constitutive basal levels of TNF; in response to lipopolysaccharide (LPS) stimulation, TNF production was enhanced by 14-, 11-, and 6-fold at 10, 20, and 30 days postinfection, respectively. Amebic liver abscess-derived macrophages pretreated with either recombinant gamma interferon (IFN-γ) or the cyclooxygenase inhibitor indomethacin augmented TNF production in response to soluble amoebic proteins and LPS. Kupffer cells and peritoneal and spleen macrophages from infected animals did not release TNF constitutively in vitro. However, TNF production in response to LPS stimulation was significantly higher at 10 and 20 days postinfection. Macrophages from infected and naive animals pretreated with recombinant IFN-γ or indomethacin produced increased amounts of TNF in response to LPS but not in response to soluble amoebic protein stimulation. Pretreatment of naive macrophages with amoebic proteins inhibited LPS-induced TNF production by 69 to 79%; the effect of the amoebic proteins was partially reversed by indomethacin pretreatment. In contrast, IFN-γ and LPS-activated naive macrophages produced enhanced levels of TNF in response to live amoebae and soluble amoebic proteins. Our results demonstrate that TNF production by macrophages is altered during *E. histolytica* infection and in response to amoebae and suggest a role for IFN-γ and prostaglandin E2 in regulating TNF production during the infection.

Amoebiasis caused by the parasitic protozoan *Entamoeba histolytica* results in substantial morbidity and mortality, with over 40 million cases of invasive disease per year and 40,000 deaths per year worldwide (35). Macrophages have been shown to be important in cell-mediated immunity and resistance to re-infection with *E. histolytica* (14, 15, 30).

Tumor necrosis factor alpha (TNF), a cytokine produced by macrophages and monocytes, is a potent mediator of inflammatory and immunological reactions (2, 3). TNF plays an important role in the pathogenesis of a variety of parasitic diseases (11, 28). TNF has been implicated in a number of pathological processes, including inflammatory responses (both local and generalized) like fever, wasting, diarrhea, disseminated intravascular coagulation, and hypotension (2). Abnormally high levels of circulating TNF are seen in patients with visceral leishmaniasis or severe *Plasmodium falciparum* infection and have been implicated in endotoxicity and death of the host (1, 31). However, TNF was not found in the serum of patients or animals with invasive amoebiasis (17), despite the intense inflammatory reaction seen in acute stages of the disease.

Several studies have demonstrated that TNF has a protective effect in parasitic infections. Administration of recombinant human TNF released from intraperitoneal osmotic pumps was shown to effectively suppress *Plasmodium chabaudi* subsp. *adami* infection in CBA mice (12). In experimental *Trypanosoma cruzi* infections, treatment of macrophages with recombinant TNF plus lipopolysaccharide (LPS) resulted in a significant reduction in the number of intracellular organisms compared with those in mock-treated macrophages (37). In combination with gamma interferon (IFN-γ), TNF has been shown to endow murine macrophages and human neutrophils with the capacity to kill *E. histolytica* in vitro (15, 16).

Amebic liver abscess (or granuloma) is important clinically in amoebiasis because of its high frequency of occurrence and serious clinical consequences (21). Granuloma macrophages secrete TNF, which plays a role in the development of granulomas during *Mycobacterium bovis* BCG infection (23). However, little is known about the production of TNF in *E. histolytica* infections and the role of TNF in amoebic liver granuloma formation.

In the present study, we used the gerbil model of amoebic liver abscess (8) to determine whether the levels of TNF produced by abscess macrophages and macrophages distant from the abscess site are altered during the course of an infection. In addition, we investigated whether *E. histolytica* can modulate TNF production by naive and activated macrophages. These studies are important in our understanding of the immunopathology of amoebiasis and the pathogenesis of amoebic granuloma formation. In this report, we demonstrate that inflammatory macrophages from animals with amoebic liver abscess produced high levels of TNF in response to LPS early in the infection and decreased at late stages of the disease. Soluble amoebic proteins inhibited LPS-induced TNF production by naive macrophages but stimulated TNF release by recombinant IFN-γ- and LPS-activated macrophages.

**MATERIALS AND METHODS**

Cultivation of *E. histolytica* and preparation of amoebic proteins. The pathogenic strain *E. histolytica* HM1-IMSS,
grown axenically in TYI-S-33 medium (18), was used for the experiments. Amoebic trophozoites in the midlog phase were harvested, washed three times in ice-cold sterile Dulbecco phosphate-buffered saline (GIBCO Laboratories, Grand Island, N.Y.) (pH 7.1), and suspended in phosphate-buffered saline. The amoebae were lysed by three freeze-thaw cycles. The amoebic suspensions were centrifuged at 15,000 × g at 4°C for 15 min to remove cellular debris. The supernatant was used as soluble amoebic proteins. The protein concentration was determined by the method of Bradford (5) with bovine serum albumin as a standard. Fresh amoebic proteins were made daily for the experiments.

**Animals and infection procedures.** Male gerbils (Meriones unguiculatus; Tumblebrook Farms, West Brookfield, Mass.) 50 to 60 days old and weighing between 55 and 60 g and female BALB/c mice (Charles River, St. Constant, Québec, Canada) weighing 18 to 20 g were used for the experiments. Gerbils were infected intrahecphatically with 5 × 10⁸ amoebic trophozoites in the midlog phase (72 h of growth) to generate amoebic liver abscesses as described previously (7).

**Isolation and preparation of macrophages.** Macrophages were isolated from amoebic liver abscesses as previously described (14). In brief, at 10, 20, or 30 days postinfection (p.i.), gerbils were sacrificed; the abscesses were aseptically harvested and excised in culture dishes containing complete medium (RPMI 1640 medium [GIBCO], with 5% heat-inactivated fetal calf serum [Hyclose Laboratories, Logan, Utah], 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [Sigma, St. Louis, Mo.], 100 U of penicillin [Sigma] per ml, 100 μg of streptomycin sulfate [Sigma] per ml). Abscess tissues were cut into small pieces with scalpels, washed three times with medium, suspended in a warm (37°C) digestion medium (Hanks balanced salt solution with 0.5% collagenase [type 1 from Clostridium histolyticum; Sigma], 100 μg of DNase [type 1; Sigma] per ml), and incubated in a water bath at 37°C for 40 min with frequent agitation. Erythrocytes were lysed by osmotic shock with 0.17 M NH₄Cl (Sigma), and the cell suspension was washed five times in complete medium. Cells (8 × 10⁶/ml per well) were incubated in 24-well plates at 37°C for 4 h, washed twice gently with warm medium, and then incubated overnight. The cells were then washed 10 times with warm medium to remove nonadherent cells. This procedure allowed the recovery of >97% macrophage cells, as determined by Giemsa staining (Sigma) and differential counting of cells prepared with a cytocentrifuge. Macrophage viability as determined by the trypan blue exclusion assay was >98%, regardless of the day p.i.

Resident peritoneal macrophages from infected gerbils, naïve age-matched gerbils, or mice were obtained by rinsing the peritoneal cavity with 10 ml of cold RPMI 1640 containing 5% heat-inactivated fetal calf serum. The cells were washed twice and adjusted to 3 × 10⁶/ml, and 1 ml of this cell suspension was added to each well of 24-well plates. The plates were then incubated for 2 h at 37°C in 5% CO₂. Nonadherent cells were removed by washing the cells with warm medium.

Spleen macrophages from infected and naïve gerbils were prepared as described previously (14). In brief, the spleens of gerbils were removed aseptically and a cell suspension was made. Cells were washed three times in medium and freed of erythrocytes with 0.17 M NH₄Cl. Cells were incubated at 37°C in 5% CO₂ for 4 h and then washed with warm medium to remove nonadherent cells.

Kupffer cells of gerbils were prepared by perfusing uninfected liver lobes with sterile Hanks balanced salt solution containing 0.05% collagenase and then teasing the liver tissues apart with forceps. The cell suspension with remnants of tissues was incubated in Hanks balanced salt solution with 0.05% collagenase and 10 μg of DNase per ml at 37°C in a water bath and agitated for 40 min. Cell suspensions were then washed five times, and erythrocytes were lysed by osmotic shock. An adherent Kupffer cell monolayer was prepared as described above for the preparation of abscess macrophages.

**Generation of macrophage-conditioned supernatant and TNF assay.** Monolayers of macrophages in 24-well tissue culture dishes were incubated at 37°C for 4 h with complete RPMI 1640 medium containing 10% fetal bovine serum. To exclude the possibility that traces of contaminating endotoxin might contribute to TNF production, macrophages were incubated with complete medium containing 1 μg of polymyxin B (Sigma) per ml except when LPS was used as the stimulus. Macrophages were incubated as indicated in the figure legends and Table 1. Cell-free conditioned supernatants were harvested at various times as indicated and tested for TNF activity either fresh or after storage at −70°C.

The levels of TNF were determined by cytotoxicity of macrophage-conditioned supernatants with TNF-sensitive L929 cells as previously described (22). Briefly, L929 cells (4 × 10⁴ in 0.1 ml) were incubated with serially diluted macrophage supernatants in 96-well microtiter plates at 37°C in the presence of actinomycin D (1 μg/ml; Sigma). After 18 h of incubation, the cells were fixed with 2.5% glutaraldehyde (Sigma) and stained with 0.1% methylene blue (Sigma). The dye was extracted with 0.1 N HCl. The A₆₅₀₅ of the cell supernatants were photometrically measured with an enzyme-linked immunosorbent assay reader (Bio-Tek Instruments, Mandel Scientific, Ontario, Canada). TNF activity in the supernatants of mouse peritoneal macrophages was completely neutralized with anti-mouse TNF antibody (Genzyme, Boston, Mass.). Recombinant TNF (Genzyme) was used as an internal standard for the cytotoxicity assay. TNF units were calculated from a standard curve generated with serially diluted recombinant TNF.

**Statistics.** The Student t test was used to determine differences between control and experimental groups. P values of >0.05 were considered not significant.

**RESULTS**

TNF production by macrophages from animals with amoebic liver abscess. (i) AMO. Amoebic liver abscess-derived macrophages (AMO) isolated from gerbils at 10, 20, and 30 days p.i. were assayed for TNF production after stimulation with amoebic proteins or LPS. The basal release of TNF after 2, 4, and 12 h was not significantly different. Similarly, TNF release by AMO after stimulation with LPS after 4 or 12 h varied less than 10%; therefore, a 4-h stimulation with LPS or amoebic proteins was chosen for all subsequent studies. Regardless of the day p.i., AMO produced low constitutive basal levels of TNF (Fig. 1). AMO did not produce TNF levels higher than basal levels in response to amoebic proteins. In contrast, after stimulation with LPS, TNF production by AMO at 10, 20, and 30 days p.i. was increased by 14-, 11-, and 6-fold, respectively, as compared with basal TNF release. However, as the infection progressed (20 and 30 days p.i.), TNF production by AMO was significantly lower (P < 0.01) than that at 10 days p.i. in response to LPS stimulation.

To gain insight into the mechanisms involved in the
modulation of TNF production in amoebiasis, we determined whether IFN-γ priming could enhance TNF production by AMO in response to a secondary stimulus. IFN-γ has been shown to upregulate TNF synthesis under certain conditions (4). After stimulation with LPS, TNF production by IFN-γ-treated AMO at 10, 20, and 30 days p.i. was increased by 17-, 15-, and 8-fold, respectively, as compared with basal TNF release by IFN-γ-treated or untreated controls (*P < 0.01*) (Fig. 1). In response to amoebic protein stimulation, TNF production was increased by twofold from day 10 and 20 AMO (*P < 0.01*) and by onefold from day 30 AMO. We recently showed that AMO produced a high constitutive basal release of prostaglandin E₂ (PGE₂) and that live *E. histolytica* and soluble amoebic proteins can stimulate naïve macrophages to produce PGE₂ (36). PGE₂ downregulates TNF production by macrophages (27). Inhibition of PGE₂ biosynthesis with the cyclooxygenase inhibitor indomethacin significantly (*P < 0.01*) enhanced the levels of TNF produced by AMO in response to LPS stimulation by 19-, 16-, and 8-fold at 10, 20, and 30 days p.i., respectively, as compared with basal TNF release from homologous unstimulated controls (Fig. 1). Amoebic protein stimulation caused a twofold increase in TNF levels from AMO at days 10 and 20 but not day 30 p.i. In this experiment, the cells were washed free of indomethacin to prevent any possible interference with the stimulants and indomethacin in combination. TNF levels measured with or without indomethacin present throughout the experiment did not vary significantly. Taken together, these results strongly suggest a role for PGE₂ in modulating macrophage TNF production by AMO during the course of the infection.

(ii) Kupffer cells and peritoneal and spleen macrophages. Gerbils with amoebic liver abscess produce an acute inflammatory response with large numbers of neutrophils and macrophages in the peritoneal cavity and spleen and Kupffer cell hyperplasia in the uninfected liver lobes (9). We were interested in determining whether macrophage populations distant from the amoebic liver abscess were altered in their ability to produce TNF during the course of the infection.

In contrast to AMO, these macrophage populations (Fig. 2) did not release TNF constitutively. However, after stimulation with LPS, these macrophages produced enhanced levels of TNF. Kupffer cells from infected animals at 10, 20,
and 30 days p.i. released levels (P < 0.01) of TNF that were enhanced by 3-, 1.7-, and 0.6-fold, respectively, relative to those from uninfected control animals (Fig. 2). Peritoneal and spleen macrophages at 10 and 20 days p.i., but not those at 30 days p.i., also produced levels of TNF after LPS stimulation that were significantly (P < 0.01) enhanced relative to those of macrophages from naive age-matched animals. Pretreatment of the macrophages from naive and infected animals with IFN-γ or indomethacin resulted in levels of TNF produced in response to LPS stimulation that were significantly (P < 0.01) higher than those of untreated controls or macrophages from infected animals stimulated with LPS alone. Macrophages at day 10 p.i. produced the highest amount of TNF, whereas those at 30 days p.i. either were unresponsive or produced low amounts of TNF. Amoebic proteins did not evoke TNF production by inflammatory macrophages regardless of the experimental conditions.

Modulating effects of amoebic proteins on LPS-induced TNF production by macrophages. The results from the above experiment show that amoebic proteins alone do not enhance TNF production by macrophages. To determine whether amoebae can modulate TNF production directly, resting peritoneal macrophages from naive gerbils and mice were pretreated with amoebic proteins and/or indomethacin and then stimulated with LPS. Pretreatment of macrophages with amoebic proteins significantly (P < 0.01) decreased the levels of TNF produced in response to LPS stimulation by 69 to 79% of the levels produced by homologous controls (Table 1). These results suggest that amoebic proteins have a suppressing effect on TNF production by macrophages, possibly by stimulating PGE₂ release (36). Pretreatment of macrophages with indomethacin and amoebic proteins together resulted in TNF production that was 33 to 40% lower than that in untreated LPS-stimulated macrophages. However, indomethacin pretreatment alone significantly enhanced the LPS-induced production of TNF by 30 to 50%. These results suggest that amoebic proteins can downregulate macrophage TNF production in response to LPS and that PGE₂ may be one of the modulators involved.

TNF production by activated macrophages in response to amoebic stimulation. In experiments carried out to determine whether *E. histolytica* can evoke the production of TNF by recombiant IFN-γ- and LPS-activated macrophages, rest-
exposed to a variety of inflammatory mediators locally were in a heightened state of activation throughout the infection, since the TNF levels produced in response to amoebae were constant throughout the infection. A common finding in animals with amoebic liver abscess is diffuse liver inflammation in the uninfected liver lobes characterized by Kupffer cell hyperplasia, lymphocyte infiltrate, granuloma formation, and degenerative and necrotic reactions (7–9). Perhaps TNF produced by Kupffer cells in response to lymphokines and other mediators during the infection is involved in these pathologic reactions. Even though abscess macrophages and macrophages distant from the abscess produced TNF in response to LPS stimulation, optimum TNF levels were only obtained by pretreating the macrophages with IFN-γ. Regardless of the day p.i., TNF production by macrophages increased by 25 to 50% in IFN-γ-treated cells. Two mechanisms may be involved in IFN-γ activation for enhanced TNF production by macrophages. First, IFN-γ has been shown to augment TNF secretion of human mononuclear phagocytes by increasing the duration of transcription of gene-encoding TNF and enhancing TNF mRNA stability (6). Second, PGE₂ inhibits macrophage TNF production and gene expression (25, 26), whereas IFN-γ downregulates monocyte PGE₂ synthesis (4). Inflammatory macrophages from the abscesses and peritoneal cavities of gerbils produced constitutively high basal levels of PGE₂ and leukotriene C₄ (36). However, after pretreatment of macrophages with indomethacin to impair PGE₂ synthesis, TNF levels were increased by up to 25%. Collectively, these data strongly suggest that IFN-γ can upregulate, whereas PGE₂ can downregulate, TNF production by macrophages during *E. histolytica* infection.

An unexpected finding in our study was the ability of amoebic proteins to downregulate LPS-induced macrophage TNF production in naive macrophages. The mechanism appears to be related, in part, to stimulation of the arachidonic acid metabolism in response to amoebic proteins. Concentrations of amoebic proteins as low as 10 μg/ml stimulated naive peritoneal macrophages to produce significant amounts of PGE₂; maximum release occurred with 50 μg of amoebic protein per ml (300 pg of PGE₂ per 5 x 10⁶ cells after 2 h) (36). Whereas pretreatment of macrophages with amoebic proteins inhibited LPS-induced TNF by 79%, simultaneous pretreatment of macrophages with amoebic proteins and indomethacin inhibited LPS-induced TNF levels by 33%. Interestingly, indomethacin pretreatment alone augmented TNF production by 30 to 50% in response to LPS, suggesting that LPS may also cause the release of PGE₂ or other mediators that can regulate TNF production directly or indirectly. We have previously (36) shown that LPS (100 ng/ml) stimulates PGE₂ release (200 pg per 5 x 10⁶ cells after 2 h) from naive peritoneal macrophages. PGE₂ regulates macrophage TNF production by increasing intracellular levels of cyclic AMP, which inhibits LPS-induced TNF production (22) and downregulates LPS receptors (20). Inhibition of macrophage TNF production by amoebae may be important in the suppression of cell-mediated immunity to ensure the survival of the amoebae in the host. Unlike the case with *P. falciparum* and several murine malaria species that directly stimulate macrophages to produce TNF (1, 29), soluble amoebic proteins did not stimulate TNF release from naive or inflammatory macrophages that were distant from the amoebic liver abscess. However, when macrophages were fully activated, TNF secretion was induced in response to live amoebae and amoebic proteins, suggesting that the effects of amoebic proteins on TNF production may be dependent on the state of activation of the macrophages. Compared with LPS, amoebic proteins stimulated a 30% increase in TNF gene transcription (32) in naive macrophages after 1 h. Taken together, these results suggest that amoebae may provide a better signal for the release of TNF by activated macrophages.

In conclusion, our study clearly demonstrates that TNF production by inflammatory macrophages is altered locally and in systemic sites during *E. histolytica* infections and in response to amoebic protein stimulation in vitro. TNF released by macrophages locally in amoebic liver abscesses may play an important role in the pathogenesis of granulomas and in the activation of macrophages in an autocrine or paracrine fashion to inhibit the dissemination of amoebae to other tissues.

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