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CONCISE COMMUNICATION

Human Macrophage Tumor Necrosis Factor (TNF)- α Production Induced by *Trypanosoma brucei gambiense* and the Role of TNF- α in Parasite Control

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Trypanosoma brucei gambiense, a causative agent of sleeping sickness, induced a dose-dependent production of tumor necrosis factor (TNF)- α by human macrophages in vitro. TNF- α was also induced in the Mono Mac 6 cell line, which indicates a direct effect of parasite components on macrophages. Parasite-soluble factors were also potent inducers of TNF- α . The addition of anti-TNF- α to cocultures of macrophages and parasites increased the number of trypanosomes and their life span, whereas irrelevant antibodies had no effect. TNF- α may have a direct role (i.e., direct trypanolytic activity) and/or an indirect one, such as TNF- α -mediated induction of cytotoxic molecules. A direct dose-dependent lytic effect of TNF- α on purified parasites was observed. This lytic effect was inhibited by anti-TNF- α . These data suggest that, as in experimental trypanosomiasis, TNF- α is involved in parasite growth control in human African trypanosomiasis.

Trypanosoma brucei gambiense and *Trypanosoma brucei rhodesiense*, the causative agents of human African trypanosomiasis (HAT), also called sleeping sickness, are tse-tse fly-transmitted protozoa that multiply extracellularly in the bloodstream, lymph, and interstitial fluids of their hosts [1]. There is currently a huge resurgence of HAT because of the deterioration of health facilities, war, and civil disturbances.

Blood monocytes and tissue macrophages play a key role in the control of protozoan parasites. Increases in the number of macrophages and the presence of macrophage activation markers are noted in trypanosomiasis [2]. Macrophages synthesize effector molecules with antitumoral and antimicrobial properties, including tumor necrosis factor (TNF)- α . TNF- α fulfills important functions in host-parasite interactions and plays an important role in controlling infections by various pathogens. TNF- α is also involved in the pathogenesis of septic shock and systemic inflammatory reactions [3].

Trypanosomiasis was one of the first diseases in which the involvement of TNF- α was observed [3]. Trypanosome-derived

products can induce the secretion of proinflammatory cytokines, such as interleukin (IL)-1 and TNF- α , by murine cells both in vitro and in vivo [4, 5]. TNF- α is trypanolytic for *T. b. brucei* parasites in vitro and reduces the parasite load in vivo [6, 7]. These results suggest that the presence of trypanosomes elicits TNF- α production to control the level of parasitemia. On the other hand, chronic production of this cytokine may, in turn, influence the host in terms of immunopathology. For instance, TNF- α is partially responsible for the severe immunosuppression observed in trypanosomiasis [8]. Moreover, TNF- α is a factor in inflammatory lesions of the cerebral nervous system in *T. brucei*-infected mice [9]. A correlation can be made between high serum levels of TNF- α and disease severity in HAT, and successful treatment with melarsoprol significantly reduces the circulating concentration of this cytokine in late-stage HAT patients [10, 11]. This important dual role of TNF- α in the immunopathology and control of *T. brucei* infection, as evidenced in murine models [7, 8], led us to investigate the induction of TNF- α in human macrophages cocultured with *T. b. gambiense* and the effect of TNF- α on in vitro trypanosome growth.

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Materials and Methods

Parasites and parasite-soluble factors (PSFs). *T. b. gambiense* "Féo" ("Féo"ITMAP/1893) and *T. b. gambiense* "OK" (OK/ITMAP/1841) were used. The parasites were adapted and maintained in vivo by inoculating normal mice, as described elsewhere [12]. The parasites were intraperitoneally injected (10^4 parasites/mouse) into mice and later were purified from blood by chromatography

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on DEAE cellulose. PSFs were prepared according to the protocol described by Bate et al. [13]. As reported elsewhere [13], some batch-to-batch variation occurred, so a PSF preparation (400 $\mu\text{g}/\text{mL}$) was distributed among several vials. These vials (aliquots) were stored at -80°C . One vial was used for each experiment.

Human macrophages. Blood from healthy subjects was obtained at a blood bank. Peripheral blood mononuclear cells were purified by gradient centrifugation on ficoll-hypaque and were allowed to adhere to a plastic, 96-well, flat-bottom tissue culture dish (Nunc) for 3 h. After removing nonadherent cells by washing, we cultured adherent cells (range, $6-8 \times 10^4$ cells/well) in modified culture medium (McCoy 5A; Gibco-Europe), supplemented with 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 25 mM HEPES, 0.1 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.2 mM L-cysteine, 5 $\mu\text{g}/\text{mL}$ polymyxin B, and 10% autologous human serum [12]. The human monocyte cell line Mono Mac 6 was cultured under the same conditions.

PSFs and trypanosome-macrophage cocultures. Purified trypanosomes or PSFs were added to adherent cells. Parasite counts were determined at the times indicated by use of a hemocytometer. Anti-human TNF- α (CellTech) or antibodies of irrelevant specificities (control antibodies) were added to some cocultures. As assessed by the limulus assay, parasites, PSFs, and supernatants of macrophage cultures contained <0.1 ng/mL lipopolysaccharide (LPS).

Trypanolysis assay. The lysis assay was done by use of light microscopy, as described elsewhere [7]. A 96-well plate was preincubated at 37°C for 1 h. Each well contained PBS (pH 8.1) supplemented with 1% glucose (PSG), dilutions of TNF- α , and/or anti-human TNF- α or control antibodies in a volume of 100 μL . Blood-purified parasites ($2 \times 10^6/\text{mL}$) were resuspended in PSG. Parasite suspension (100 $\mu\text{L}/\text{well}$) was added to each well. The percentage of lysis was calculated 5 h later.

TNF- α determination. TNF- α levels were determined in duplicate for each sample by use of a sensitive sandwich RIA (Medgenix). Values of TNF- α in samples were calculated from a curve obtained from standards.

Results

Trypanosomes induce the production of TNF- α by human macrophages. CD14, a marker of the monocyte/macrophage lineage, was expressed on 85% (range, 78%–95%) of the adherent mononuclear cells from healthy subjects (data not shown). These cells were cultured with *T. b. gambiense* “Féo” and “OK” (10^4 cells/well). Supernatants were collected at various times. An increase in TNF- α was measured in these supernatants, compared with supernatants from uninfected cultures. Results in figure 1A were obtained with 2 parasite preparations (“Féo” and “OK”) and cells from 6 donors, all of which were prepared the same day and used for cocultures. This increase was greater after 24 h than after 48 h. No striking difference was noted when the “Féo” or the “OK” strains of *T. b. gambiense* were cocultured with cells from the same donors. There was a direct relationship between TNF- α levels and the number of trypanosomes added to the cocultures (figure 1B).

Mono Mac 6 cells (5.10^4 cells/well) were cocultured with trypanosomes (10^4 cells/well). There was a higher concentration

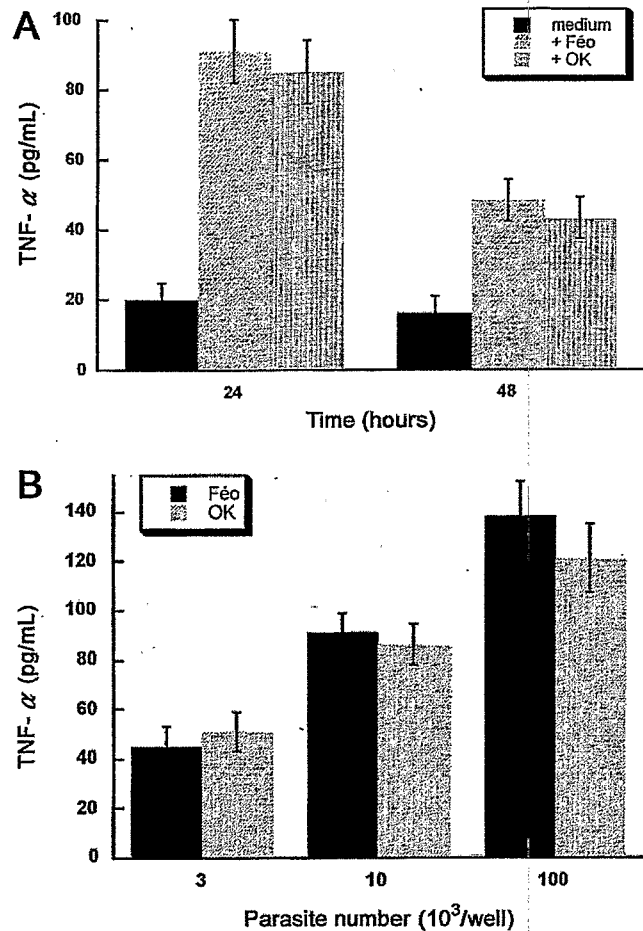


Figure 1. Production of tumor necrosis factor (TNF)- α by human macrophages cocultured with *Trypanosoma brucei gambiense*. **A**, Human macrophages ($6-8 \times 10^4$ cells/well) were cocultured with *T. b. gambiense* “Féo” or “OK” (10^4 parasites/well), and supernatants were collected 24 and 48 h later for TNF- α determination. Each result represents the mean \pm SD for 6 donors. **B**, Parasites (3×10^3 , 10^4 , and 10^5 parasites/well) were cocultured with macrophages (8×10^4 cells/well), and TNF- α was measured in culture supernatants 24 h later. The TNF- α content of supernatants from uninfected cultures was 18 ± 3 pg/mL. Each result is the mean \pm SD for 4 experiments.

of TNF- α in supernatants from 24-h cocultures (180 ± 15 pg/mL) than from control supernatants (35 ± 7 pg/mL; mean \pm SD from 5 experiments).

Induction of TNF- α by PSFs. PSFs, like trypanosomes, could induce dose-dependent TNF- α production when added to cultures of monocyte-derived macrophages. Electrophoresis revealed that the main component of PSFs preparation was the variable surface glycoprotein (data not shown). We used a 400- $\mu\text{g}/\text{mL}$ PSFs preparation, which had been aliquoted and stored at -80°C , to stimulate cultures of monocyte-derived macrophages (8×10^4 cells/well) or Mono Mac 6 cells (5×10^4 cells/well) at a final concentration of 20 $\mu\text{g}/\text{mL}$.

The range of TNF- α levels in 24-h culture supernatants from healthy donors in the presence of this PSF preparation was

65–158 pg/mL; levels of TNF- α in the absence of PSFs were 10–27 pg/mL). The increase in TNF- α production above background was \sim 5 for each donor. PSFs alone and cell-free trypanosome supernatants did not contain TNF- α .

Role of TNF- α on parasite growth. When trypanosomes were added to macrophage cultures, there was an increase, followed by a rapid decrease in trypanosome number. The addition of anti-TNF- α to cocultures resulted in an increase in trypanosome number and prolonged survival (figure 2A). The addition of anti-TNF- α (2 μ g/mL) was effective in the first 4 h of cocultures. The subsequent addition of anti-TNF- α had little or no effect. The addition of control antibodies had no noticeable effect on trypanosome numbers. The results clearly show that TNF- α plays a role in the elimination of parasites *in vitro*. This role might be direct (i.e., direct trypanolytic activity as substantiated in figure 2B) and/or indirect, such as TNF- α -mediated induction of nitric oxide.

Trypanolysis assay. Trypanosomes "OK" purified from the blood of infected mice were incubated at 37°C in PSG containing different concentrations of recombinant human TNF- α . A dose-dependent lysis of the parasites was recorded after a 3–4-h incubation, reaching maximal levels after 5 h (figure 2B). No significant lysis was recorded in the absence of TNF- α .

Preincubation of TNF- α with anti-TNF- α (2 μ g/mL) for 1 h suppressed the lytic effect of TNF- α , whereas preincubation with control antibodies had no effect. In the absence of TNF- α , these antibodies alone had no effect on trypanosomes.

Discussion

Our results show that African trypanosomes and trypanosome-derived components induced the production of TNF- α in human macrophages and that TNF- α is involved in controlling the growth of trypanosomes.

Most of the studies related to the induction of TNF- α and its antimicrobial role have been done in murine models. Since human and murine macrophages may respond differently, as evidenced for another macrophage effector molecule, nitric oxide, the induction and role of TNF- α were investigated in cocultures of human cells and trypanosome species pathogenic to humans. Moreover, serum sensitivity of African trypanosomes varies according to serum host origin, and the transfer of trypanosomes from nonhuman animals to man might be accompanied by a modification of trypanosome sensitivity to effector molecules, in particular TNF- α .

The direct induction of TNF- α by trypanosomes and trypanosome-derived products on Mono Mac 6 cells, which is considered to match the parasite number in HAT [1], shows that T lymphocytes and cytokines, such as interferon (IFN)- γ , are not required for macrophage activation. Indeed, this direct induction is probably due to glycosylphosphatidylinositol (GPI)-anchored trypanosome components. Macrophages express GPI-anchored surface proteins, and crosslinking of GPI-

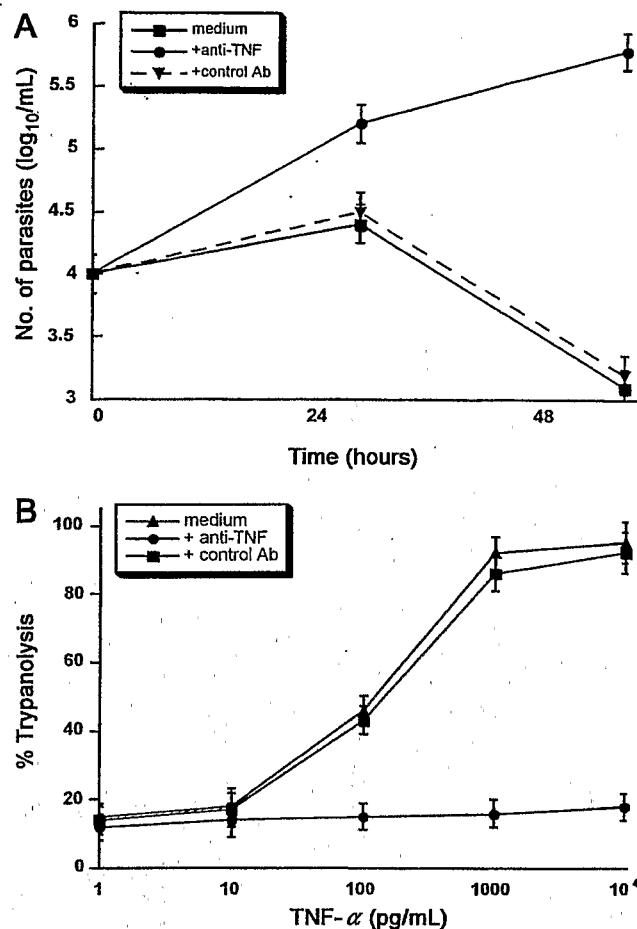


Figure 2. Effect of tumor necrosis factor (TNF)- α on *Trypanosoma brucei gambiense*. **A**, Human macrophages ($6\text{--}8 \times 10^4$ cells/well) were cocultured with *T. b. gambiense* "OK" (10^4 parasites/well) in medium alone, in medium containing anti-human TNF- α , or in control antibody. Parasite counts were determined 24 and 48 h later. Each result is the mean \pm SD for 6 donors. **B**, Trypanosomes were incubated with serial concentrations of TNF- α in medium alone, in medium containing anti-human TNF- α , or in control antibody. The percentage of trypanolysis was calculated 5 h later. Each result is the mean \pm SD for 5 assays. Ab, antibody.

anchored surface proteins generates regulatory signals of the immune system within cells. GPI from African trypanosomes was reported to be a potent inducer of cytokine expression, analogous to the GPI from the malaria parasite [4]. *In vitro*, soluble and membrane-bound variable surface glycoproteins possess TNF- α -inducing properties, which may be increased in the presence of IFN- γ and LPS. We do not exclude the possibility that the presence of increased serum levels of LPS and IFN- γ in HAT may further up-regulate TNF- α production [14, 15]. Since parasites and parasite products might induce TNF- α production in various cell types, the analysis of TNF- α -producing cells and the recruitment and location of these cells during the evolution of the disease will provide useful

information on the physiologic and pathophysiologic mechanisms and deserves further investigation.

TNF- α is involved in host resistance to several pathogens. TNF- α can act indirectly, through immune cells, or directly as an effector molecule. Indeed, anti-TNF- α was documented to increase the number of parasites in the first wave of parasitemia in *T. brucei*-infected mice [8]. Antibodies play a protective role in trypanosomiasis [2]; however, the phenomenon of antigenic variation limits the efficiency of antibodies. In such instances, TNF- α may facilitate the antibody-dependent clearance of parasites by reducing the number of parasite-dividing forms. A recent study reported the dose-dependent lysis of *T. brucei* by TNF- α and the action of TNF- α on bloodstream parasites that were isolated at the peak of parasitemia [7]. In vitro, we observed a lytic effect of human TNF- α on *T. b. gambiense*. This suggests that TNF- α participates in the elimination of parasites and facilitates the control of HAT by the host, despite the antigenic variation phenomenon.

At the late stage of HAT, the number of bloodstream trypanosomes is low, but levels of TNF- α are high. This may indicate the efficiency of immune mechanisms and an induction of TNF- α not linked to parasitic elements but instead to a cytokine network dysregulation. The role of TNF- α production in HAT may largely depend on timing, rate, and site of production. In the early phase of HAT, TNF- α may be involved in the elimination of blood parasites and also in inflammatory reactions, immunosuppression, and autoantibody production. In the late stage of the disease, TNF- α may contribute to the alteration of the blood-brain barrier and to the infiltration of the brain by cellular and humoral elements of the immune response that participate in the pathophysiology of the disease. An association of overproduction of TNF- α with mortality has been reported in various diseases. TNF- α blockers have been beneficial with regard to certain diseases. Their use at the late stage of HAT deserves further investigation. Moreover, the roles of other cytokines and, more particularly, anti-inflammatory cytokines, such as IL-10, which also increases in HAT [7], probably modulate the effects of TNF- α and should be taken into account in the pathophysiology of the disease.

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