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FULL PAPER

Preliminary studies on the effects of orally-administered Transforming Growth Factor-beta on protozoan diseases in mice

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Abstract

Transforming growth factor beta-1 (TGF-β1) is a pleiotropic cytokine with both pro- and anti-inflammatory properties, depending on its environment and concentration. The present study evaluated the effects of orally-delivered TGF-β1 on mice parenterally-infected with various protozoan parasites. We report that while orally-administered TGF-β1 seems to confer partial protection against murine chronic babesiosis and acute trypanosomosis, no beneficial clinical effects were observed against acute babesiosis, malaria or toxoplasmosis. Taken together, these preliminary data suggest that the systemic effects conferred by exogenous TGF-β1 could be parasite species-specific. The variations in different parasitic infections could be due to (i) intrinsic differences between parasite species and/or strains in their ability to induce production of immunosuppressive molecules and/or (ii) differences in mechanisms governing host protection against different parasitic infections.

Key words: low-dose, mice, orally-delivered TGF-β1, protection, protozoan parasites

Introduction

Protozoan parasites such as *Plasmodium*, *Theirelia*, *Trypanosomes*, *Toxoplama* and *Babesia* cause among the most economically important diseases affecting livestock and man, particularly in developing tropical countries. Both current treatment and control regimes against these diseases are costly and not sustainable. These limitations have stimulated research into alternative control/preventive measures against protozoan diseases.

Several cytokines and their antagonists have protective potential against various human and livestock diseases[2,15]. Although the interferon (IFN) family has been used more extensively over the last two decades[2], there is need to expand the study of the protective potential. Other groups of

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cytokines may provide where the IFNs have been less effective.

Transforming growth factor beta-1 (TGF-β1), produced by a wide range of cells, has both pro- and anti-inflammatory properties, depending on its environment and concentration[1,17]. TGF-β1 therapy has been shown to exert enhancing effects on interleukin (IL)-12 production and natural killer (NK) cell activities in the spleen and liver[4,12]. Consequently, TGF-β1 may potentially enhance innate/acquired immunity against protozoan parasites[3,10]. In agreement with that notion, parenterally-delivered TGF-β1 has been documented to confer beneficial protective effects during the early phase of chronic murine trypanosomosis[6].

The evidence that maternal transfer of cytokines via milk plays a role in neonatal development may provide a rationale for oral administration of cytokines for the control of protozoan diseases. To date, most *in vivo* studies with cytokines have involved routes of parenteral administration to achieve desired biological effects[2]. This is mainly because of speculations that oral administration may result in proteolytic digestion and unavailability of cytokines. However, parenteral administration may deliver excessive levels of cytokines in the peripheral tissues and may result in systemic toxicity[5]. Under normal physiological conditions, cytokines are biologically active in minute fractions of the doses commonly administered parenterally. As such, the oral route may provide a more rational use of cytokines in biologically appropriate concentrations and frequencies. For instance, orally administered TGF-β has been shown to enhance the antigen-specific antibody responses against parenteral infections[4]. Orally delivered TGF-β has also been reported to increase the number of protective IgA antibodies, which are responsible for the immediate defense of the intestinal mucous membranes against pathogens[4]. The present study thus aims to evaluate the effects of orally-delivered TGF-β1 on mice parenterally-infected with various protozoan parasites.

Materials and Methods

Animals and Parasites: Seven-weeks-old female BALB/c mice (Nihon CLEA Inc., Tokyo, Japan) were inoculated intraperitoneally (ip) with either (i) the highly virulent IL3000 strain of *Trypanosoma congolense*, (ii) RH strain of *Toxoplasma gondii*, (iii) PLK strain of *T. gondii*, and USDA strains of (iv) *Plasmodium berghei,* (v) *Babesia rodhaini,* (vi) *B. microti,* maintained in our institute. All animal experiments were conducted in accordance with the Standards relating to the Care and Management of Experimental Animals of Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan).

Experimental design: Initial experiments showed that when BALB/c mice were treated orally with 1 ng to 100 ng TGF-β1, maximal beneficial effects against some protozoan diseases were observed at lower doses of 5 ng. Consequently, 5 ng of TGF-β1 was used in the rest of the experiments performed in this study. Mice were divided into groups of 20. For each group, 20 BALB/c mice were subdivided into two equal subgroups (n = 10). One subgroup was treated orally with 200 μl of 5 ng of recombinant human TGF-β1 (Sigma, Saint Louis, Missouri; dissolved in phosphate buffered saline containing 2 mg/ml bovine serum albumin [PBS/BSA]) for five days, by gastric intubation using a syringe fitted with a ball-type feeding needle. The bioactivity of TGF-β1 was confirmed by the growth inhibition assay of Mv. 1. Lu mink lung epithelial cells as previously described[9]. Control mice (n = 10) were treated with 200 μl PBS/BSA.

After 5 days of TGF-β1 treatment, each mouse was inoculated ip with (i) 500 *P. berghei*-infected erythrocytes; (ii) 500 *B. rodhaini*-infected erythrocytes; (iii) 500 *B. microti*-infected erythrocytes; (iv) 2000 bloodstream form of *T. congolense* parasites; (v) 500 RH strain of *T. gondii* tachyzoites; and (vi) 500 PLK strain of *T. gondii* tachyzoites. Parasitemia, morbidity development in mice and survival rates were monitored. On day 6 post-infection (pi) with *T. congolense*, cyto-

kine levels were quantified in sera of 3 TGF-β1 pre-treated mice and 3 control animals.

In vivo neutralization of TGF-β1: To test the role of endogenous TGF-β1 during various protozoan infections, 10 BALB/c mice were each initially inoculated ip with 50 μg of anti-TGF-β1, 2, 3 (R & D Systsems, Abingdon, UK) monoclonal antibody (mAb) that specifically neutralizes TGF-β1, -β2 and -β3 activities, in a total volume of 200 μl, 24 hr prior to challenge and every 2 days thereafter for a week. That dose and treatment regime of anti-TGF-β1, 2, 3 mAb was previously shown to completely neutralise endogenous anti-TGF-β1 activity in mice[9]. On day 0 pi, the mice were challenged ip with protozoan parasites and the course and outcome of infection monitored. Control mice received 50 μg of an isotype purified mouse IgG1 mAb (CHEMICON International, Germany).

Sera collection: On day 6 following infection with *T. congolense*, blood collected by heart puncture was centrifuged (10,000 × g, at 4°C for 10 min), and serum was stored at −80°C until use.

Measurement of soluble cytokines: Cytokines were quantified in sera by specific ELISA kits from Endogen (Rockford, IL) for IFN-γ or R&D Systems (Minneapolis, MN) for IL-10, following the manufacturers' protocols.

Statistical Analysis: For each parameter, results were expressed as the mean response of the 10 TGF-β1 pretreated animals tested individually (± SE) and compared to the same parameters assessed in 10 control mice. Results are representative of at least 3 similar experiments performed. Statistical analyses were assessed using a PRISM computer program, Version 3.0c (GraphPad software Inc., San Diego, CA) to validate the data. *P*-values of < 0.05 were considered statistically significant.

Results

Blocking endogenous TGF-β by neutralizing antibodies increases parasitemia in T. congolense-infected mice

Since exogenous TGF-β1 was recently reported to confer protection against early stage of chronic murine trypanosomosis[6], we examined the possible protective role of endogenous TGF-β1 naturally produced following infection of mice with *T. congolense* parasites through neutralization of the cytokine with an anti-TGF-β1, 2, 3 mAb. Fig. 1 shows that although not statistically significant, neutralization of endogenous TGF-β1 exhibited a tendency towards an increase in the levels of circulating *T. congolense* in acutely infected mice compared to mice pretreated with the isotype control IgG1 mAb. However, anti-TGF-β1, 2, 3 mAb pretreated and control mice died around the same time (Fig. 1).

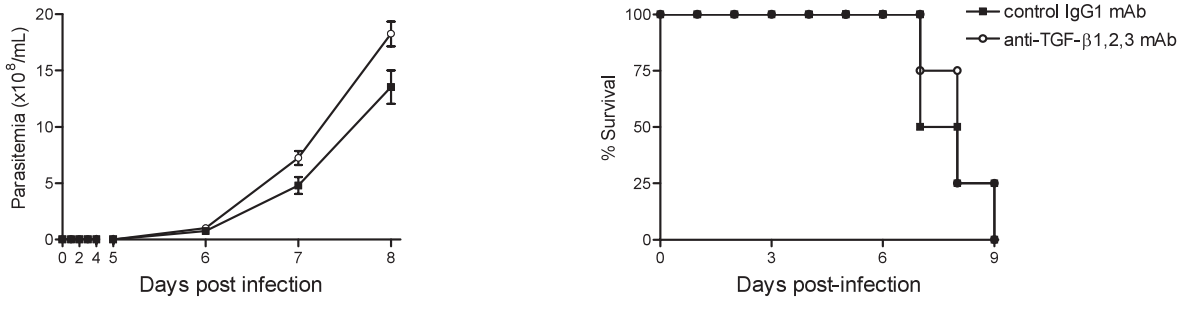

Fig. 1. Effects of neutralization monoclonal antibodies to TGF-β1, 2, 3 on parasitemia and survival rate of *T. congolense* infected mice. Data (mean ± SE; n = 10) are representative of 5 independent experiments.

Orally-delivered TGF-β1 has different effects against different protozoan diseases in mice

In order to test whether orally-administered TGF-β1 had any beneficial effects against murine babesiosis, toxoplasmosis, malaria or trypanosomosis, BALB/c mice were initially treated orally with 5 ng TGF-β1 daily for five days after which mice were challenged ip with respective protozoan parasites. As shown in Fig. 2A–B, orally-administered TGF-β1 had no beneficial effects against acute babesiosis (*B. rodhaini*) or malaria (*P. berghei*) in mice. Such mice developed similar

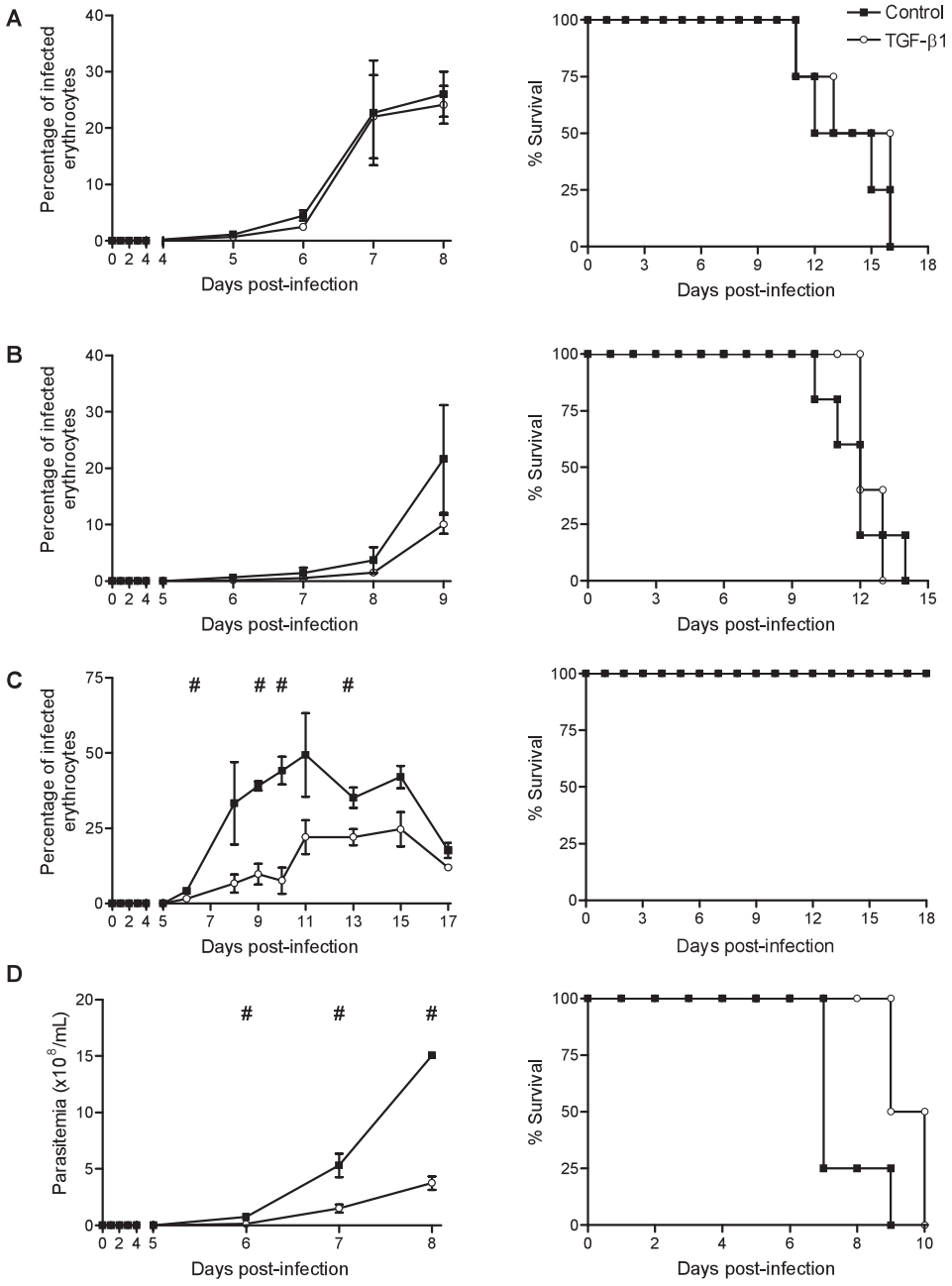

Fig. 2. Effects of orally-delivered TGF-β1 on different protozoan diseases in mice. Parasitemia and survival rates of TGF-β1 *vs* PBS/BSA pretreated mice infected with *P. berghei* (A); *B. rodhaini* (B); *B. microti* (C) or *T. congolense* (D) parasites. Data (mean ± SE; n = 10) are representative of 5 independent experiments. # statistically significant difference between infected TGF-β1 and PBS/BSA pre-treated mice.

high parasitemia, morbidity and further died about the same time as control animals. Similarly, oral TGF-β1 treatment had no clinical beneficial effects against acute and highly virulent (RH strain of *T. gondii*) or chronic and low virulent (PLK strain of *T. gondii*) murine toxoplasmosis (data not shown). In the latter case, TGF-β1-treated and control mice simultaneously developed similar clinical signs including piloerection, huddling, reduced activity and emaciation. However, about 4 days thereafter, all groups of *T. gondii* (PLK strain)-infected mice recovered and appeared clinically well, without any recorded deaths (data not shown).

In contrast, 5 ng oral TGF-β1 therapy significantly reduced the first parasitemic peak ($p < 0.05$) of *B. microti*- and *T. congolense*-infected mice respectively, compared to controls (Fig. 2C–D). Furthermore, as shown in Fig. 2D, a tendency towards delayed mortality was observed in *T. congolense*-infected and TGF-β1-treated (with average survival time of 9.5 days pi) as compared to control mice (with average survival time of 7.5 days pi). However, neither TGF-β1-treated nor control *B. microti*-infected animal died during the entire experimental period (Fig. 2C).

Orally-delivered TGF-β1 induces a type-I-skewed cytokine response in sera of T. congolense-infected mice

Following recent reports that ip TGF-β1-pretreatment of C57BL/6 mice resulted in a type-I cytokine environment that was associated with partial protection against trypanosomosis[6], we examined the amount of IFN-γ (type-I cytokine) and IL-10 (type-II cytokine) in sera from oral-TGF-β1-pretreated BALB/c mice, infected (day 6 pi) or not with *T. congolense* parasites. As shown in Fig. 3A–B, orally-delivered TGF-β1 induced increased serum IFN-γ and to a lesser extent, IL-10 levels in uninfected BALB/c mice compared to PBS/BSA pretreated uninfected controls. Following infection with *T. congolense*, the serum levels of both IFN-γ and IL-10 greatly increased. Whereas both *T. congolense*-infected TGF-β1-pretreated and PBS/BSA-pretreated control mice had similar increased serum IFN-γ levels, the latter group of mice exhibited significantly higher serum IL-10 levels ($p < 0.05$) (Fig. 3A–B). As shown in Fig. 3C, a net type-I-skewed cytokine response, characterized by a higher IFN-γ/IL-10 ratio, was observed in sera from TGF-β1-pretreated non-infected mice and to a lesser extent ($p < 0.05$), in sera from TGF-β1-pretreated *T. congolense*-infected mice. On the other hand, *T. congolense*-infected PBS/BSA-pretreated control mice showed increased levels of both IFN-γ and IL-10 and hence exhibited a significantly lower IFN-γ/IL-10 ratio ($p < 0.01$).

Discussion

This study has shown that treatment of

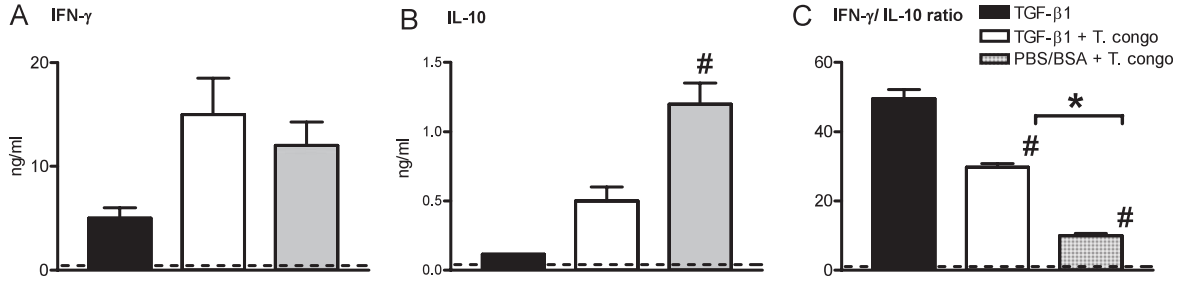

Fig. 3. Effects of orally-delivered TGF-β1 on serum cytokine responses in *T. congolense*-infected mice. Data (mean ± SE; n = 10) are representative of 3 independent experiments. Dashed horizontal lines represent average cytokine levels in non-infected PBS/BSA pre-treated mice. # statistically significant difference between non-infected TGF-β1 pre-treated (*Black bar*) and *T. congolense*-infected mice; ✻ statistically significant difference between *T. congolense*-infected TGF-β1 (*White bar*) and PBS/BSA (*Dotted bar*) pre-treated mice.

BALB/c mice orally with TGF-β1 can confer protection against parenteral infection with protozoan parasites. Indeed, lower-dose (5 ng), but not higher-dose ($\geq$ 50 ng), orally-delivered TGF-β1 partially protected mice against *B. microti* and *T. congolense* infections as evidenced by significant reduction in parasitemia in the pretreated animals. Although TGF-β1-pretreated *B. microti* exhibited reduced parasitemia compared to controls, both groups later managed to control the infection and remained chronically infected but without any clinical manifestation of the disease. In contrast, a slight tendency towards increased survival rate was observed in TGF-β1-pretreated *T. congolense*-infected mice compared to controls, supporting the notion that survival of *T. congolense*-infected mice correlates with their ability to control the first parasitemic peak[7]. Interestingly, in both cases, orally-delivered TGF-β1 seems to play a role in controlling parasite growth mainly during the early phase of infection as described for other infection models[6,9,10,13]. That notion was further highlighted by observations that anti-TGF-β1 mAb tended to enhance parasite growth during the early stage of trypanosomosis.

Low-dose TGF-β1 has been documented to induce pro-inflammatory responses that confer protective anti-protozoan effects during the early stage of the disease[6,8,9,11]. Indeed, Omer and Riley[9] demonstrated that lower TGF-β1 doses (5 ng) produced maximal benefits against murine malaria while higher doses (20 ng) were less effective. Yet, in another study[16], very high TGF-β1 doses (10 μg) exacerbated murine malaria infection. Moreover, Omer *et al.*[8] reported that while mice infected with the nonlethal strain of *P. yoelii* produced lower levels of endogenous TGF-β1 only detected 5 days pi, animals infected with the lethal *P. yoelii* strain produced high levels of the cytokine within 24 hr pi. Production of TGF-β1 above the physiological levels has also been reported to reduce resistance to leishmaniasis, toxoplasmosis and Chagas' disease[16]. Taken together our present data and those of others[6,9,16] stress the pleiotropic nature of TGF-β1.

According to a recent report[6], TGF-β1 does not seem to have direct anti-protozoan effects. Instead, this cytokine appears to be an immunostimulant, acting indirectly through induction of pro-inflammatory mediators and macrophage activation that leads to parasites clearance[6,9,11,14,18]. In agreement with that notion, evidence has been provided in the present study that orally-delivered TGF-β1 increased serum levels of IFN-γ (a pro-inflammatory mediator) which was associated with partial protection against acute lethal infection of mice with *T. congolense*. In particular, the balance between type-I and type-II cytokines seems to be critical in determining the course and outcome of infection with protozoan parasite infections[6,9,18]. In the trypanosome model, a skewed type-I cytokine environment, characterized by increased IFN-γ with a concomitant reduced IL-10 levels, seems to be protective, at least during the early phase of the disease[6].

Interestingly, the same dose and treatment regime of TGF-β1 that conferred partial systemic beneficial effects against murine trypanosomosis and nonlethal babesiosis had no clinical beneficial effects against acute lethal babesiosis (*B. rodhaini*), toxoplasmosis (RH strain of *T. gondii*) or malaria (*P. berghei*) in BALB/c mice as evidenced by comparable development of morbidity, parasitemia and mortality between TGF-β1-treated and control animals. Similarly, oral TGF-β1-treatment failed to protect mice against infection with the relatively lower virulent PLK strain of *T. gondii*. It seems the systemic effects of TGF-β1 therapy cannot easily be predicted as each parasite species or strain may have its own specific circumstances (including specific immunological environment) that may lead to clinical beneficial effects following the cytokine treatment. Indeed, it is intriguing to note that while the treatment regime used in the present study led to partial protection against *B. microti* infection, no effect against the closely related *B. rhodhaini* was observed. On the other hand, orally-administered TGF-β1 did not confer any protective effects against both the mild, self-limiting infection of

mice with PLK strain of *T. gondii* and the acute lethal infection with RH strain of *T. gondii*. Taken together, these data suggest that the systemic effects conferred by exogenous TGF-β1 could be parasite species- and indeed strain-specific. Thus the variations in different parasitic infections could be due to intrinsic differences between parasite species and/or strains in their ability to induce the production of immunosuppressive molecules that probably determine their survival in the host. Differences may occur at species or strain level in the individual parasite's ability to induce the production of such immunosuppressive substances. In agreement with that notion, we recently reported that *T. congolense* induced increased production of endogenous bioactive TGF-β1 and IL-10 which seem to suppress the type-I cytokine environment required for the control of the initial and most aggressive parasitic waves during the early stage of the disease[6]. Hyperproduction of suppressive molecules, possibly a parasite survival strategy, has also been described in other parasitic infection models including malaria[8], leishmaniais and toxoplasmosis[16]. It is also possible that the mode of action of TGF-β1 may be different in each infection model. Indeed, while TGF-β1 therapy seems to protect mice against trypanosomosis through induction of proinflammatory molecules[6] and enhancement of antigen-specific antibody responses in other infections[4], exogenous TGF-β1 has been documented to confer protection against murine malaria through reduction of tumor necrosis factor alpha and interleukin 4 with a concomitant increase in IFN-γ and IL-10[9]. Moreover, exogenous TGF-β1 pretreatment may further exacerbate the disease condition (through increased tissue pathology and early mortalities) in models that induce exaggerated pro-inflammatory immune responses since low-dose TGF-β1 pretreatment induces a skewed type-I cytokine response[8,9]. It is also interesting to note that although no clinical beneficial effects of TGF-β1 against *P. berghei* infection in mice was observed in the present study, Omer and Riley[9] reported that treatment of *P. berghei*-infected mice with TGF-β1 reduced the rate of parasite proliferation and prolonged their survival. The discrepancy between the two studies could be due to the different routes used for TGF-β1 administration.

In conclusion, this preliminary study shows the potential role of orally-administered TGF-β1 against protozoan infections. To obtain maximum and reliable beneficial clinical effects, the dose; dosing schedule and route; type of disease conditions and circumstances when beneficial effects may be observed, need to be optimized. Considering the pleiotropic nature of TGF-β1 and its potent immunomodulatory role, further efforts should be made to determine appropriate circumstances when it can confer beneficial clinical effects against various protozoan diseases and to optimize such circumstances for individual conditions. Furthermore, the precise mechanisms of protection conferred by exogenous TGF-β1 against different protozoan diseases should be investigated.

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