RAPID COMMUNICATION

# Soluble TNF and membrane TNF expressed on CD4⁺ T lymphocytes differ in their ability to activate macrophage antileishmanial defense

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**Abstract:** **In our studies of host defense against the intracellular parasite *Leishmania major*, we obtained evidence for a novel mechanism of macrophage activation for antimicrobial defense that involves direct cell contact between CD4⁺ T lymphocytes and *Leishmania*-infected macrophages. The mechanism is distinctive as it does not involve secretion of lymphokines but is apparently mediated by the membrane-anchored form of tumor necrosis factor (mTNF; ~50-60 kd) present on the surface of the effector T lymphocytes. Furthermore, it is not cytotoxic to the host cell and its expression is antigen specific and genetically restricted. We prepared a *Leishmania*-specific cloned T-T cell hybridoma line 1B6 (CD4⁺, TH1) that expresses membrane-bound TNF but does not secrete TNF or other macrophage activators. We now report that 1B6 cells can activate antileishmanial defense in inflammatory macrophages, whereas soluble recombinant murine TNF (sTNF) alone is unable to do so. On the other hand, both 1B6 cells and sTNF can act synergistically with recombinant murine interferon-γ (IFN-γ, a known soluble macrophage-activating factor) in activating antimicrobial defense and ·$NO_2^-$ release. The effects of 1B6 alone and the synergistic effects of 1B6 and IFN-γ or sTNF and IFN-γ are arginine dependent. These results suggest that mTNF may be more efficient than sTNF in macrophage activation and that contact with effector CD4⁺ lymphocytes that express mTNF may be an important mechanism of host defense. *J. Leukoc. Biol.* 51: 296-299; 1992.**

***Key Words:*** *intracellular parasites, nitric oxide · L-arginine dependence · cell-cell contact · macrophage activation*

## INTRODUCTION

Tumor necrosis factors (TNFs) are members of a family of highly homologous proteins produced by stimulated macrophages or T lymphocytes that have multiple biological functions [2]. Among their properties is the ability to participate in the activation of macrophages for defense against certain microbes [3, 12]. Two biologically active forms of TNF have been shown to exist: a secreted molecule (sTNF) and a membrane-associated protein (mTNF) identified on macrophages [9, 10] and CD8⁺ lymphocytes [13]. The only function of mTNF previously identified is tumor cytotoxicity, an effect it shares with sTNF [9, 10, 13]. We recently determined that mTNF is also expressed on selected murine CD4⁺ lymphocytes and observed that such mTNF⁺ lymphocytes could activate macrophage antimicrobial (antileishmanial) defense by cell-cell contact [23]. We therefore compared the ability of sTNF and CD4⁺ lymphocyte-associated mTNF to activate antileishmanial defense in vitro.

*Leishmania major*, NIH Seidman strain (WHO strain designation MHOM/SN/74 Seidman), was used in these investigations [14]. Female BALB/cAnNTacfBR mice (Taconic, Germantown, NY) infected subcutaneously with *L. major* were used as a source of tissue-derived amastigotes for in vitro macrophage infection [15]. Peritoneal macrophages were isolated by lavage using cold pyrogen-free saline from pathogen-free female C57BL/6NTacfBR mice (Taconic) 4-5 days after an interperitoneal injection of 2% sterile hydrolyzed starch solution in saline ("inflammatory macrophages") [15]. The 1B6 cell line (a cloned *Leishmania*-specific CD3⁺, CD4⁺, TH1, I-$A^{b/k}$ hybridoma line) was originally derived from draining popliteal lymph nodes of C57BL/6 mice infected 5 weeks with *L. major* [22]. These cells when cocultured with infected macrophages activate antileishmanial effects without secreting macrophage-activating factor (MAF) [22]. Washed peritoneal cells were cultured as a suspension in polypropylene tubes containing RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM L-glutamine. In all experiments macrophages were cultured at a concentration of $2 \times 10^5$ cells and infected with tissue-derived amastigotes at a multiplicity of 1:1 or 2:1 (parasites:macrophages).

Recombinant murine interferon γ (rIFN-γ; lot 42-96-2, specific activity $6.8 \times 10^6$ U/mg; and lot 22-71-68, specific activity $5.2 \times 10^6$ U/mg) was obtained from Genentech, South San Francisco, CA; murine rTNF-α (specific activity $4 \times 10^7$ U/mg) was purchased from Genzyme Corp., Boston, MA. Rabbit polyclonal antibody to murine TNF-α was purchased from Genzyme Corp. Cytokines or 1B6 cells were added to inflammatory macrophages infected for 24 h and the resulting antimicrobial effect was assessed as previously

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon γ; MAF, macrophage-activating factor; mTNF, membrane-associated TNF; PBS, phosphate-buffered saline; r, recombinant; sTNF, soluble TNF; TNF, tumor necrosis factor.

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described [15]. The antileishmanial effect was calculated using the following formula:

$$1 - \frac{\text{mean number of amastigotes per 100 treated macrophages}}{\text{mean number of amastigotes per 100 untreated macrophages}} \times 100$$

Nitrite concentrations were measured spectrophotometrically ($OD_{550}$) by published methods after treating culture supernatants with Greiss reagent [4]. The TNF activity in culture supernatants was determined using a modification of the actinomycin D-sensitized L929 cell cytotoxicity assay [5] or by enzyme-linked immunosorbent assay (ELISA) (Genzyme Corp.) employing a monoclonal antibody specific for murine TNF-α that has been previously described [19].

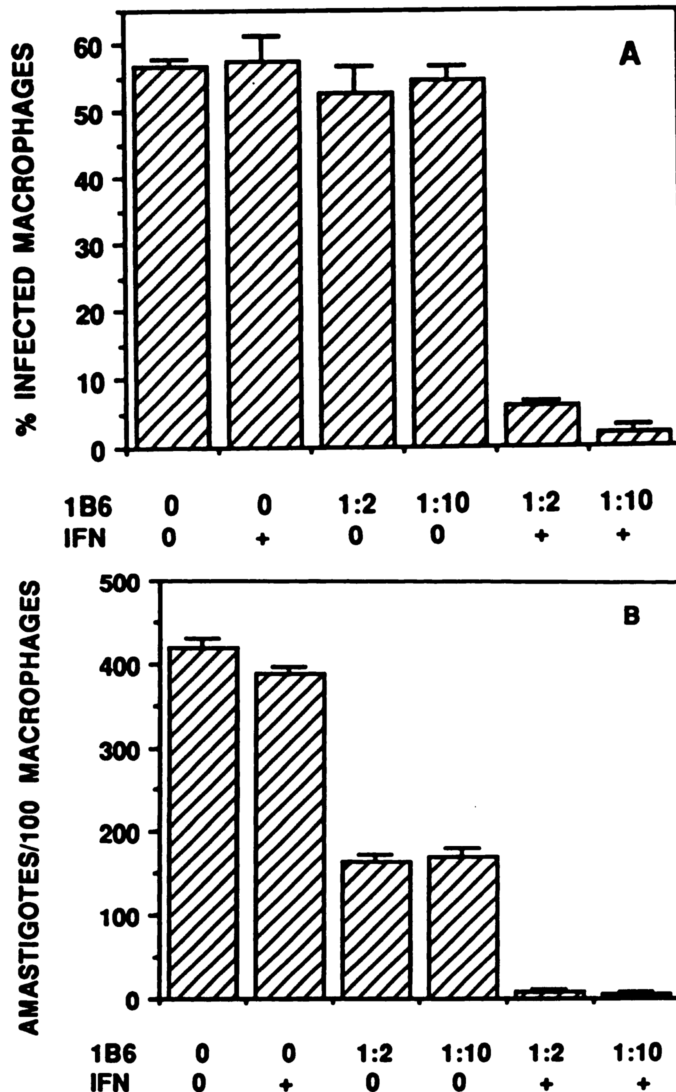


**Fig. 1.** Synergistic interaction of recombinant IFN-γ and the T cell hybridoma 1B6 for the induction of antileishmanial activity in starch-elicited macrophages. Twelve-hour infected macrophages were incubated concurrently for 60 h with or without rIFN-γ (100 U/ml) and 1B6 cells at an effector/target ratio of 0 (no 1B6 cells added), 1:2, or 1:10. Effects of rIFN-γ and 1B6 cells on (A) the percentage of *Leishmania*-infected macrophages remaining in culture at 72 h and (B) the number of amastigotes per 100 macrophages at 72 h are shown for one of three representative experiments. The data shown are the mean ± SEM of triplicate determinations.

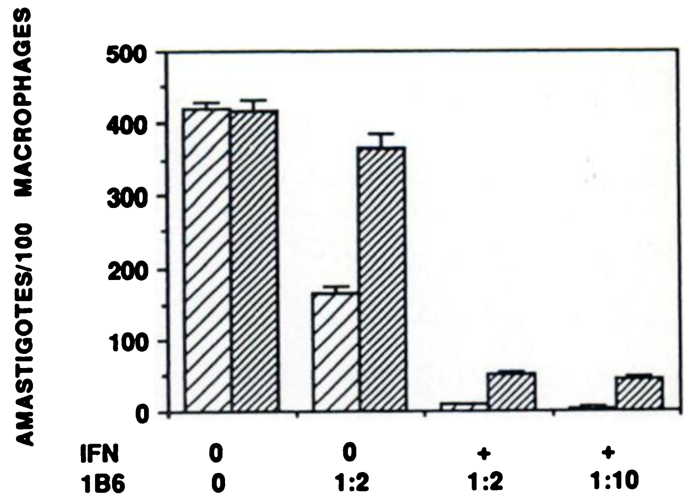


**Fig. 2.** Antileishmanial activity of the T cell hybridoma 1B6 on infected starch-elicited macrophages is inhibited by anti-TNF antibody. Infected macrophages were incubated with or without recombinant IFN-γ (100 U/ml) and 1B6 cells at an effector/target of 0 (no 1B6 cells added), 1:2, or 1:10, and the effect on the number of amastigotes per 100 macrophages at 72 h was determined in the absence (□) or presence (▨) of anti-TNF antiserum (10,000 neutralizing U/ml). The data shown are the mean ± SEM of triplicate determinations for one of three representative experiments.

The *Leishmania*-specific $CD4^+$T hybridoma clone 1B6, which does not secrete MAFs [22] and can express mTNF [23], was examined for its ability to induce antileishmanial activity in infected macrophages. The 1B6 line can be separated into a subpopulation that expresses mTNF ($mTNF^+$) and one that does not ($mTNF^-$). The $mTNF^-$ subpopulation has no antimicrobial activity [23]. When cultured in vitro (3–7 days), however, $mTNF^-$ 1B6 cells become $mTNF^+$ (40–60%) and acquire antileishmanial capabilities [23]. The 1B6 clone is not cytotoxic to infected host cells, however [22]. Addition of the 1B6 cells to infected macrophage cultures results in a marked decrease (>50% reduction) in the number of amastigotes per 100 macrophages (Fig. 1A and B). The antimicrobial effects induced by the 1B6 clone have been demonstrated to be antigen specific and genetically restricted [22, 24].

When fixed 1B6 cells (1% paraformaldehyde and extensively washed in PBS [23]) were added to infected macrophages, the magnitude of antileishmanial effect they induced was similar to that expressed by unfixed effector cells. In cultures containing untreated macrophages, the number of amastigotes per 100 macrophages was greater than 600 ± 50 (mean ± SE, three determinations), whereas infected macrophages treated with fixed 1B6 cells (effector/target ratio 1:1) had less than 200 ± 25 amastigotes per 100 macrophages. These observations suggest that the effects we observed were not attributable to any soluble MAFs the T hybridoma may have produced, but rather were due to mTNF present on the surface of these effector cells. The antimicrobial effects induced by the 1B6 clone (Fig. 2) (using either live or fixed cells) can also be inhibited by the addition of anti-TNF antibody (>50% increase in the number of amastigotes per 100 macrophages).

We noted that when soluble TNF alone was added to suspension cultures of infected macrophages, minimal or no antileishmanial activity was observed (data not shown). Over a range of 10 to 1000 U/ml, sTNF consistently failed to induce any antimicrobial effects in infected macrophages. These observations suggest that although the soluble form of TNF

lacks the capacity to induce antimicrobial effects in macrophages, the mTNF present on the 1B6 cells does appear to have this capacity.

When both sTNF and IFN-γ were used in combination, marked antileishmanial effects were observed (Fig. 3A), as has been noted by others [6, 11]. We also observed that when both 1B6 and IFN-γ were used in combination, a marked decrease (>95% reduction) in the number of amastigotes per 100 macrophages (Fig. 1B) as well as a decrease (>90% reduction) in the percentage of infected macrophages (Fig. 1A) was seen. These effects are of greater magnitude than the effects when 1B6 cells are used alone. Incubation of *L. major*-infected macrophages in medium supplemented with IFN-γ alone produced only a minor decrease in either the percentage of infected macrophages (Fig. 1A) or the number of amastigotes per 100 macrophages (Fig. 1B). These observations suggest that both forms of TNF, sTNF and mTNF, have the capacity to synergize with IFN-γ to exert antileishmanial effects in infected macrophages.

The antileishmanial activity induced in infected macrophages by the action of IFN-γ alone or together with sTNF is correlated with nitrite production (Fig. 3A), as previously demonstrated [6, 11]. When we examined the contact-mediated effector mechanism using the 1B6 T hybridoma clone alone (Fig. 3A) or in combination with IFN-γ (Fig. 3A), we observed that its antimicrobial activity could also be correlated with the production of nitrite. Both nitrite production and the antileishmanial effects induced by either IFN-γ itself or IFN-γ combined with sTNF are markedly diminished (>85% reduction in antileishmanial activity and nitrite levels) when L-arginine is not present in the macrophage suspension cultures (Fig. 3B), as observed by others [6, 11]. The effects of 1B6 cells alone (Fig. 3B) or in synergy with IFN-γ (Fig. 3B) are also dependent on the presence of L-arginine (>70% reduction in antileishmanial activity and >85% reduction in nitrite levels).

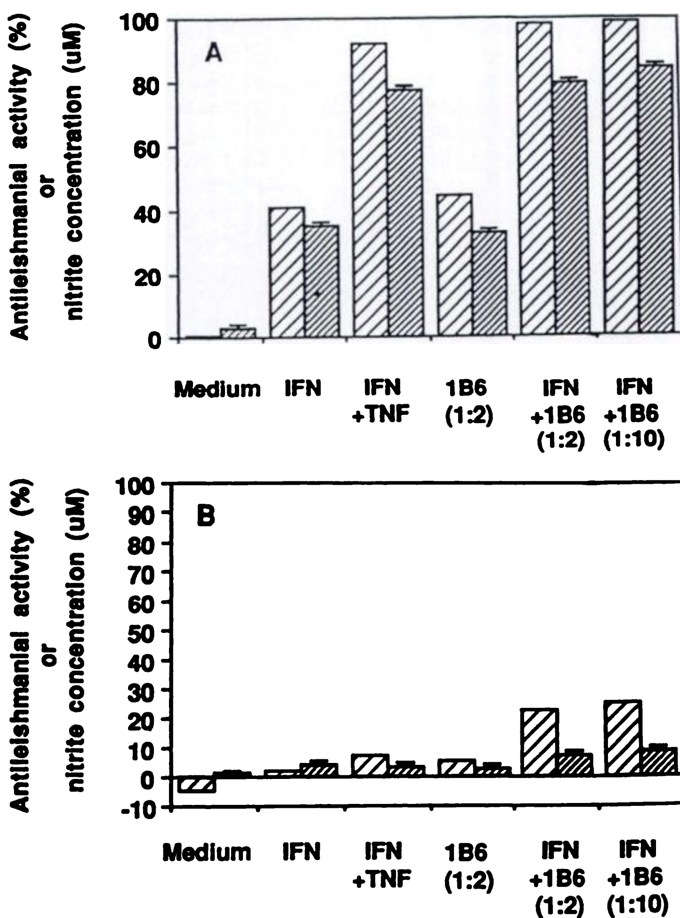


**Fig. 3.** Antileishmanial activity induced by the T cell hybridoma 1B6 is correlated with nitrite production and dependent on the presence of arginine. Infected macrophages were incubated with or without recombinant IFN-γ (10 U/ml) and 1B6 cells at an effector/target ratio of 0 (no 1B6 cells added), 1:2, or 1:10, and the effects on antileishmanial activity (%) (◪) and supernatant nitrite concentration (▨) were determined at 72 h. Determinations were conducted in the presence (A) or absence (B) of exogenous arginine. Results of the synergistic effect of rIFN-γ and rTNF-α (1000 U/ml) are included for comparison. The data shown are the mean ± SEM of triplicate determinations for one of three experiments.

The contact mechanism mediated by mTNF present on CD4⁺ T cells (such as that represented by 1B6 cells) can activate macrophages to inhibit the growth of intracellular *Leishmania*, whereas sTNF is unable to do so. These findings suggest that the mTNF present on CD4⁺ T cells may provide a more efficient activation signal to infected macrophages than is provided by the soluble form of this cytokine. One explanation for our observations may be that TNF in its membrane-anchored form presents a multimeric signal that might provide for receptor-ligand interactions that differ qualitatively or quantitatively from those with sTNF [8, 9]. Alternatively, TNF on the T cell plasma membrane, by virtue of its polar presentation to receptors on macrophages in the region of cell-cell contact, could influence macrophage responses in a unique manner [16]. Finally, as a consequence of the antigen-specific and genetically restricted nature of this activation mechanism [22, 24], the concomitant interaction of CD4⁺ T cell receptor with leishmanial antigen and Ia and other possible ligand-receptor systems (i.e., CD4, LFA-1, ICAM, etc.) may influence the delivery of the TNF signal [17, 20].

These findings could have relevance for defense in vivo. mTNF found on the 1B6 cell and mTNF⁺ CD4⁺ T cells from infected mice can activate antileishmanial defense in both resident and elicited (inflammatory) macrophages for antileishmanial effects [15, 22], whereas neither sTNF nor IFN-γ is able to do so [6, 7, 11]. These findings suggest that mTNF may provide a more efficient signal than a soluble cytokine(s) alone to heterogeneous macrophage populations that are present during different stages of lesion development in the course of the disease. Because elicited macrophage populations include many recently recruited and relatively immature mononuclear phagocytes [1], our observations suggest that mTNF may be an effective and sufficient activation signal to macrophages being recruited to cutaneous areas where infections initially occur.

In addition, 1B6 cells and mTNF present on CD4⁺ cells can induce antileishmanial effects at 34°C and 37°C effectively [21, 22], whereas soluble MAFs do so only at 37°C [18]. Again, this observation may be of particular relevance to antileishmanial defense in the skin, where temperatures are lower and where inflammatory macrophages may be present.

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