

# Antileishmanial Defense in Macrophages Triggered by Tumor Necrosis Factor Expressed on CD4<sup>+</sup> T Lymphocyte Plasma Membrane

By Joseph P. Sypek and David J. Wyler

From the Division of Geographic Medicine and Infectious Diseases, Department of Medicine, New England Medical Center Hospitals and Tufts University School of Medicine, Boston, Massachusetts 02111

## Summary

In our studies of host defense to the intracellular protozoan *Leishmania major*, we uncovered a novel mechanism of antileishmanial defense that involves direct cell contact between effector CD4<sup>+</sup> lymphocytes and *Leishmania*-infected macrophages. The mechanism is distinctive because it does not involve lymphokine secretion and induces no cytotoxic effects in the host cells; its expression is antigen-specific and genetically restricted. We now demonstrate that these effector CD4<sup>+</sup> cells display tumor necrosis factor (TNF) on their surface and provide evidence that the membrane-associated TNF is involved in the activation of the antileishmanial defense. Using a *Leishmania*-specific cloned T-T cell hybridoma line (1B6; CD4<sup>+</sup>, T helper type 1) that activates antileishmanial defense in macrophages through cell contact and does not secrete TNF, we noted that only cells bearing surface TNF (TNF<sup>+</sup>), but not ones lacking surface TNF (TNF<sup>-</sup>), exerted these effects. Moreover, the antileishmanial effects exerted by TNF<sup>+</sup> 1B6 cells as well as by lymph node CD4<sup>+</sup> TNF<sup>+</sup> lymphocytes could be blocked with anti-TNF antibody. We propose that membrane-associated TNF on CD4<sup>+</sup> T cells may provide a mechanism of targeting activation signals to macrophages in an antigen-specific and genetically restricted manner.

Antimicrobial effects can be induced in infected macrophages by lymphocytes that recognize specific microbial antigens and are syngeneic with the macrophages (1). Soluble macrophage-activating lymphokines (MAFs)<sup>1</sup> released by these lymphocytes are known to be instrumental in such defense (2, 3). Lymphocyte-mediated activation of antimicrobial defenses in macrophages also may occur via interactions that do not involve lymphokine secretion (4, 5). In our studies on activation of antileishmanial defense, we serendipitously encountered a process of CD4<sup>+</sup> T lymphocyte-mediated macrophage activation that occurs without MAF secretion, is not cytotoxic to host cells, and requires intimate contact between effector lymphocytes and target macrophages (6–9). Until recently, the membrane-associated effector molecule(s) involved in this defense remained obscure. The demonstration that TNF has macrophage-activating properties (10–12), and exists in both a soluble and a membrane-anchored form (13–15), led us to consider membrane TNF as a possible effector molecule in the defense we were investigating.

## Materials and Methods

**Parasites.** *Leishmania major*, NIH Seidman strain (WHO strain designation, MHOM/SN/74 Seidman), was originally isolated by

one of us (D.J. Wyler) from a Peace Corps volunteer in Senegal (16). Amastigotes were propagated in mice by serial infection and were obtained from infected footpad tissue, as described (6).

**Mice.** Female BALB/cAnNTacFBr (amastigote donor), C57BL/6TacFBr, and B6C3F<sub>1</sub> (macrophage and lymphocyte donors) mice were obtained from Taconic (Germantown, NY).

**Effector T Cells.** T lymphocytes were derived from draining popliteal lymph nodes excised from C57BL/6 mice infected for 4–5 wk with *L. major* (6). The 1B6 hybridoma cell line (a cloned *Leishmania*-specific CD4<sup>+</sup> CD3<sup>+</sup> Th1 hybridoma line, I-A<sup>b/k</sup> restricted, [9]) was originally produced from the lymph node T lymphocyte population without prior in vitro expansion and was cloned by customary methods (17).

**Antibodies and Flow Cytometry.** The anti-murine TNF- $\alpha$  antibody (rabbit hyperimmune serum) was obtained from Genzyme (Boston, MA). For isolation of CD4<sup>+</sup> cells, anti-L3T4 antibody (rat IgG2b-PE conjugate) from Becton Dickinson Immunocytometry Systems (Mountain View, CA) was used. For indirect immunofluorescence, FITC-conjugated goat anti-rabbit IgG (affinity purified) from Fisher Scientific (Pittsburgh, PA; Southern Biotechnology Associates, Inc., Birmingham, AL) was used. For blocking, normal goat (S-2007) and normal rat (S-2757) serum from Sigma Chemical Co. (St. Louis, MO) was used. All staining for FACS<sup>®</sup> analysis was carried out in the presence of 0.1% NaN<sub>3</sub>; dead cells were eliminated from analysis after DNA staining with 1  $\mu$ g propidium iodide. The cells were sorted on a FACS Star Plus<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA) or a Coulter Epics 541 Series flow cytometer (Coulter Scientific Instruments, Hialeah,

<sup>1</sup> Abbreviation used in this paper: MAF, macrophage-activating lymphokine.

FL). Variation in the percentage of cells positive for cell surface TNF, as determined by analysis of immunofluorescence histograms, was never >1–2% between either instrument.

**Assay for Antileishmanial Activity.** The ability of 1B6 cells to activate *Leishmania*-infected macrophages was assessed by our published methods (6). Resident macrophages from uninfected mice (B6C3F<sub>1</sub> strain; Taconic) were harvested by peritoneal lavage with divalent cation-deficient HBSS. Peritoneal cells were washed and resuspended in supplemented RPMI 1640 containing 10% FCS (Hyclone Laboratories, Logan, UT),  $5 \times 10^{-5}$  M 2-ME, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 4 mM L-glutamine.  $2 \times 10^5$  peritoneal cells were combined with  $2-4 \times 10^5$  *Leishmania* amastigotes in a total volume of 0.8 ml in 12  $\times$  75-mm propylene tubes (No. 2063; Falcon Labware, Becton Dickinson & Co., Lincoln, NJ). Effector T lymphocytes were added to infected macrophages 24 h later (one to two hybrid cells per macrophage; two to five lymphocytes per macrophage). The suspension cultures were then incubated for an additional 48 h at 37°C with or without the addition of anti-TNF- $\alpha$  antibody (1:100 dilution of hyperimmune serum). Cells were harvested onto glass slides by cytocentrifugation (Shandon-Southern, Sewickly, PA) and were stained with Wright's-Giemsa (Diff-Quick, Dade Diagnostics, Aquada, PR). The percentage of infected macrophages and the mean number of amastigotes per infected macrophage were determined microscopically. 100 macrophages in remote fields were scored; mean  $\pm$  SEM of amastigotes per 100 macrophages in triplicate culture with or without the addition of lymphocytes was compared by using a one-tailed Student's *t* test. The antileishmanial effect was calculated from the raw data to express the percentage reduction in parasites per 100 macrophages cultured with lymphocytes compared to ones cultured without.

**Preparation of Fixed Cells.** Cells that were fixed were treated with a 1% paraformaldehyde solution in HBSS for 15 min at room temperature. The paraformaldehyde was removed, and the cells were washed three times with HBSS. After the third wash, the cells were incubated in supplemented culture medium for 30 min at 37°C. This incubation period was necessary to remove residual amounts of paraformaldehyde. The cells were washed three more times and were used without further treatment.

**Preparation of Cell Membrane Lysates.** 1B6 cells were stimulated with Con A (5 mg/ml; Miles Laboratories, Kankakee, IL) and anti-murine CD3 mAb (hamster IgG, 10 mg/ml; PharMingen, San Diego, CA) for 12–18 h in supplemented media at 37°C. Stimulation under these conditions was used to avoid contamination with macrophages if used as stimulator cells. After stimulation, cells were washed three times with PBS.  $10^6$  cells were lysed for 1–2 h at 4°C in lysis buffer containing 1% Triton X-100, 10 mM Hepes at pH 7.4, 200 mM NaCl, 2 mM CaCl<sub>2</sub>, 2.5 mM MgCl<sub>2</sub>, and protease inhibitors, which included 2 mM chymostatin, 5 mM leupeptin, 10 mg/ml soybean trypsin inhibitor, 10 mM iodoacetamide, 10 mM EDTA, 0.02% NaN<sub>3</sub>, and 1–2 mM PMSF. Cell lysates were centrifuged at 32,000 *g* in an ultracentrifuge with a 70 Ti rotor (Beckman Instruments, Palo Alto, CA) for 1 h at 10°C to sediment nuclear and other cellular debris. The membrane protein-enriched fraction was suspended in 1 ml of buffer and assayed as described.

**Immunological Assay for TNF- $\alpha$ .** An ELISA (Genzyme) using mAb specific for murine TNF- $\alpha$ , which has been previously described (18), was used to quantify the amount of TNF present in culture supernatants of 1B6 T hybridoma cells. A standard curve for each assay was generated with known concentrations of murine TNF- $\alpha$  containing 0, 50, 100, 200, 400, 800, 1,600, and 3,200 pg/ml (concentrations defined according to the manufacturer).

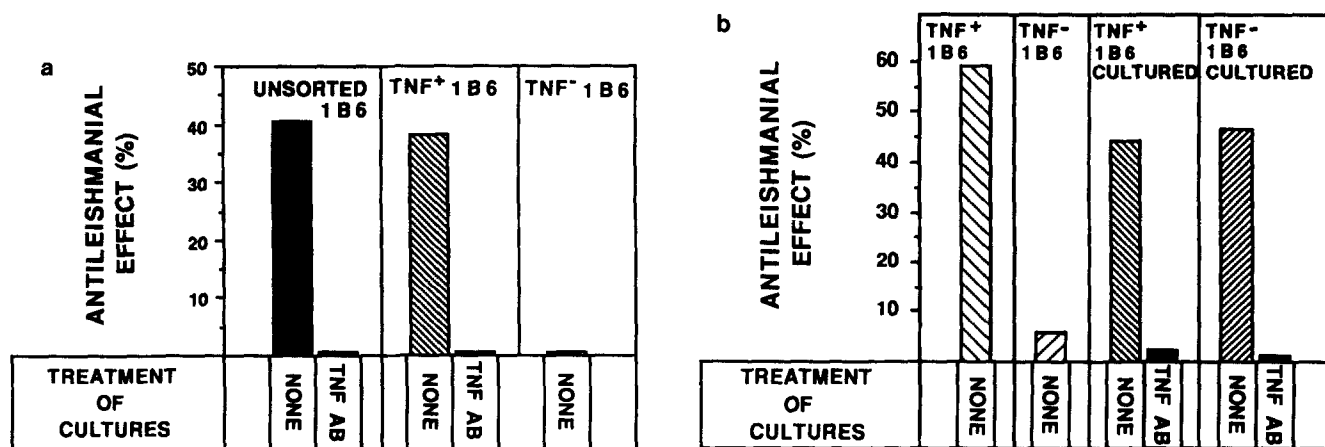
**Western Blot Analysis.** A total of  $10^7$  1B6 cells were lysed for 1–2 h at 4°C in lysis buffer containing protease inhibitors. Cell lysates were centrifuged at 32,000 *g* in an ultracentrifuge with a 70 Ti rotor (Beckman Instruments) for 1 h at 10°C to sediment nuclear and other cellular debris. The membrane protein-enriched pellet after high speed centrifugation (55,000 *g*, 2 h) was then extracted and reduced in sample buffer containing 150 mM Tris at pH 6.8, 10% SDS, 10% glycerol, 10% 2-ME, and 0.015% pyronin Y. The sample was boiled for 5 min before loading onto a SDS/15% polyacrylamide gel. Gel electrophoresis and Western blot analysis were done essentially as described (19, 20). The immunoblots were developed with the rabbit anti-murine TNF- $\alpha$  antibody and alkaline phosphatase-labeled goat anti-rabbit IgG (Proto Blot System; Promega Corp., Madison, WI), as described (21).

## Results and Discussion

**Activation of Antileishmanial Effects by T Cell Hybridomas Is Inhibited by Anti-TNF- $\alpha$  Antibody.** Using published methods (17), we prepared and cloned T-T cell hybridomas from popliteal lymph node lymphocytes of mice with hind footpads infected with *Leishmania major*. We selected clone 1B6 (CD4<sup>+</sup>) for its failure to secrete MAFs and its ability in vitro to induce antileishmanial effects in *L. major*-infected macrophages (9). When we added polyclonal rabbit anti-murine TNF- $\alpha$  antibody (IgG) to cocultures of 1B6 cells and infected macrophages, the antileishmanial effects of the T cell were abrogated (Fig. 1 *a*); nonimmune rabbit IgG had no effect in this assay (data not shown).

**1B6 Hybridoma Cells Express TNF on Their Cell Surface.** We determined by fluorescence flow cytometric analysis with anti-TNF that 40–60% of the 1B6 cells expressed TNF on their surface (six experiments). Interestingly, when these hybridoma cells were isolated by FACS<sup>®</sup>, only the TNF<sup>+</sup> (and not the TNF<sup>-</sup>) population induced antileishmanial effects in cultures of infected macrophages (Fig. 1 *a*); these effects were markedly reduced in the presence of anti-TNF antibody. Particularly noteworthy was our observation that when the TNF<sup>-</sup> 1B6 cells were maintained in culture for 3–7 d after the initial sorting and were then reanalyzed, 40–60% of the cells had become TNF<sup>+</sup> and the population concomitantly had acquired the ability to induce antileishmanial effects (Fig. 1 *b*). After incubating for 3–7 d, the sorted cells that were initially 96.5% TNF<sup>+</sup>, were also noted to be 40–60% TNF<sup>+</sup>.

**1B6 Hybridoma Cells Stimulated by *Leishmania*-infected Macrophages Do Not Secrete TNF.** The correlation of membrane expression of TNF on 1B6 cells and the ability of the cells to exert an antileishmanial effect as revealed by cell sorting studies strongly suggested that surface TNF was involved in the macrophage activation process. We found that 1B6 cells do not secrete TNF when cocultured with infected macrophages. In the coculture supernatants of viable 1B6 cells that were coincubated with live infected macrophages, we could detect TNF activity (250–500 pg/ml; limit of sensitivity, 50 pg/ml; measured by a TNF-specific ELISA [Table 1] and by L929 cytotoxicity assay; reference 22). When, however, live 1B6 cells were coincubated with paraformaldehyde-fixed macrophages (1% paraformaldehyde, washed extensively



**Figure 1.** (a) Effects of anti-TNF antibody on the ability of the 1B6 hybridoma clone to induce antileishmanial defense in vitro. 1B6 cells (T cell hybridoma specific for *Leishmania*; I-A<sup>b/k</sup> restricted; reference 9) were added to syngeneic (B6C3F<sub>1</sub>) peritoneal macrophages infected for 24 h with *L. major* at a parasite/macrophage ratio of 2:1. Cocultures were maintained for 48 h with or without the addition of anti-TNF- $\alpha$  antibody (TNF AB; rabbit hyperimmune serum, 1:100 dilution). TNF-expressing 1B6 cells were enriched (96.5  $\pm$  1.8% positive, mean  $\pm$  SE; three experiments) or depleted (97.9  $\pm$  1.3% negative) by FACS<sup>®</sup> using anti-TNF antibody. Control cultures of infected macrophages were maintained without lymphocytes in medium alone or with anti-TNF antibody. Data were derived from triplicate determinations in a representative experiment of six performed. (b) Effects of anti-TNF antibody on the ability of sorted and recultured 1B6 hybridoma clone to induce antileishmanial defense in vitro. 1B6 cells were enriched or depleted for surface TNF by FACS<sup>®</sup>. Aliquots of the cells were either tested immediately in the antileishmanial defense assay or were incubated alone for an additional 4–7 d before testing (cultured). Flow cytometry revealed that after reculture of the originally TNF<sup>-</sup> population, 40–60% of the 1B6 cells expressed TNF on their surface. Data are shown for triplicate determinations in a representative experiment of three performed.

in HBSS, and subsequently in supplemented media), we could detect no TNF activity in the coculture supernatants. On the other hand, using these assays, we could detect TNF activity (100–300 pg/ml [equivalent to the membrane protein-

**Table 1.** TNF Level Present in Cell-free Supernatants from Cultures of 1B6 T Hybridoma Cells under Various Culture Conditions

Supernatant*	TNF <sup>†</sup>
	pg/ml
M $\Phi$ I alone	10 $\pm$ 7
1B6 alone	0 $\pm$ 0
M $\Phi$ I + 1B6 <sup>§</sup>	340 $\pm$ 55
Fixed M $\Phi$ I + 1B6 <sup>  </sup>	0 $\pm$ 0
1B6 cell membrane lysate <sup>¶</sup>	225 $\pm$ 65
M $\Phi$ I + fixed 1B6	690 $\pm$ 160

An ELISA using a mAb specific for murine TNF- $\alpha$  that has been previously described (25) was used to quantify the amount of TNF present in culture supernatants of 1B6 T hybridoma cells.

\* Tested as a final dilution of 1:2. M $\Phi$ I, *Leishmania*-infected macrophages.

<sup>†</sup> Mean  $\pm$  SEM; three separate experiments.

<sup>§</sup> Viable hybridomas were added to live 24-h infected macrophages at a 2:1 E/T cell ratio.

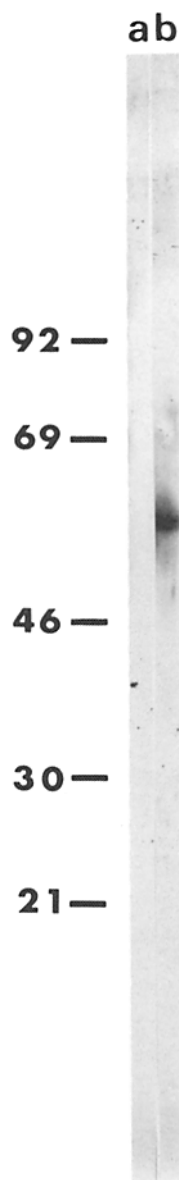
<sup>||</sup> Cells that were fixed were treated with a 1% paraformaldehyde solution in HBSS and extensively washed before use.

<sup>¶</sup> 1B6 cells were stimulated with Con A (5  $\mu$ g/ml) and anti-murine CD3 mAb (hamster IgG, 10  $\mu$ g/ml) for 12–18 h in supplemented media at 37°C. After stimulation, cells were washed and were lysed in lysis buffer containing protease inhibitors. After isolation, the membrane protein-enriched fraction of the total cell lysate was suspended in 1 ml of buffer and assayed as described.

enriched fraction of the total cell lysate suspended in 1 ml derived from a total of 10<sup>6</sup> cells) in the cell membrane protein-enriched fraction prepared from 1B6 cells after a 12–18 h stimulation with Con A (5  $\mu$ g/ml) and anti-murine CD3 mAb (hamster IgG, 10  $\mu$ g/ml). Furthermore, paraformaldehyde-fixed 1B6 cells could trigger infected macrophages to secrete 0.5–1.0 ng/ml TNF into the culture supernatants (Table 1). These observations indicate that 1B6 cells express TNF on their surface but do not secrete TNF.

*T Hybridoma Cell Surface TNF Is ~50–60 kD.* Since the secreted form of TNF has a  $M_r$   $\sim$ 17 kD (13), whereas T lymphocyte membrane-anchored TNF has a  $M_r$   $\sim$ 50–60 kD (15, 23), we carried out studies to determine the  $M_r$  of 1B6-associated TNF. 1B6 cells were incubated for 12–18 h in the presence of 5  $\mu$ g/ml Con A and 10  $\mu$ g/ml anti-murine CD3 antibody, after which the membrane protein-enriched fraction of the total cell lysate was prepared, detergent solubilized, subjected to electrophoresis under reducing conditions, and finally analyzed by Western blot methods using anti-TNF antiserum. A single protein band, migrating with a  $M_r$   $\sim$ 46–69 kD, was identified (Fig. 2, lane b). In related experiments, 1B6 cells were metabolically labeled with <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine. A single protein band with a  $M_r$   $\sim$ 46–69 kD was immunoprecipitated from membrane protein-enriched fractions of <sup>35</sup>S-labeled cells with TNF-specific antibodies (data not shown). In neither experiment did normal rabbit serum identify any 1B6-associated proteins. These findings indicate that 1B6 synthesize the large  $M_r$  TNF that has been identified as the membrane anchored form (15, 23).

*Effector CD4<sup>+</sup> T Lymphocytes from Leishmania-infected Mice Induce Antileishmanial Effects in Macrophages by Cell Surface TNF.* We extended our observations with the 1B6 T-T hy-



**Figure 2.** Western blot analysis of 1B6 cell membrane proteins probed with anti-murine TNF- $\alpha$  antibody. Total cell lysates enriched for cell membrane proteins derived from a total of  $10^7$  1B6 cells were subjected to electrophoresis in each lane and immunoblotted with either normal rabbit serum (lane *a*) or anti-murine TNF- $\alpha$  antiserum (lane *b*). The migration positions of phosphorylase b (92 kD), BSA (69 kD), OVA (46 kD), carbonic anhydrase (30 kD), and trypsin inhibitor (21 kD) are indicated in lane *a*.

bridoma clone to T lymphocytes from lymph nodes draining leishmanial footpad infections in vivo (week 5 of *L. major* infection in C57BL/6 mice). Using two-color fluorescence flow cytometry, we determined that 5–25% of the CD4<sup>+</sup> lymph node lymphocytes express surface TNF (four experiments). When isolated by FACS<sup>®</sup>, these TNF<sup>+</sup> cells induced in vitro antileishmanial effects in infected macrophages ( $46.3 \pm 5.8\%$ , reduction in amastigotes per 100 macrophages compared to controls with no lymphocytes added; mean  $\pm$  SE, three determinations; three separate experiments), effects that were diminished (by 52.3%) in the presence of anti-TNF antibody.

The results of these studies indicate that CD4<sup>+</sup> T lymphocytes, like macrophages (13, 14), CD8<sup>+</sup> (15) and unclassified peripheral blood T lymphocytes (23), can express a membrane-associated form of TNF. Furthermore, our studies suggest that the surface TNF can induce antileishmanial defense in macrophages through a cell contact-dependent mechanism. The antigenic specificity (24, 25) and genetic restriction (7, 26) characteristic of the contact-mediated defense, which we have previously reported, indicates that CD4<sup>+</sup> T lymphocyte membrane-anchored TNF can only exert a macrophage-activating influence in a highly restricted manner. This contrasts with the more promiscuous influence of soluble MAFs in host defense (3). Since soluble TNF has a broad range of biological activities, some of which are deleterious to the host (12), the more precisely targeted delivery of this effector molecule to infected macrophages may be beneficial. Moreover, we have observed that inflammatory macrophages that are relatively refractory to soluble MAF-mediated activation of antileishmanial defense are fully susceptible to cell contact-mediated activation (6). Additionally, there exist certain strains of *L. mexicana* whose growth is not inhibited in resident macrophages cultured in the presence of soluble MAFs but is inhibited when the macrophages are cocultured with the effector lymphocytes (27). Thus, the physiological consequences in infected macrophages of interactions with CD4<sup>+</sup> T lymphocyte membrane-anchored TNF may differ significantly from those induced by soluble lymphokines. Our observations emphasize the need to evaluate membrane-membrane interactions between CD4<sup>+</sup> T lymphocytes and macrophages not only in antigen presentation, but also in effector functions of these lymphocytes.

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Address correspondence to Joseph P. Sypek, Division of Geographic Medicine and Infectious Diseases,

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