

INTERFERON  $\gamma$  AND LYMPHOTOXIN OR TUMOR  
NECROSIS FACTOR ACT SYNERGISTICALLY TO INDUCE  
MACROPHAGE KILLING OF TUMOR CELLS AND  
SCHISTOSOMULA OF *SCHISTOSOMA MANSONI*

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Macrophages play a crucial role in the defense against tumors and parasites. Activation of tumoricidal and microbicidal effector mechanisms requires stimulation of macrophages with macrophage-activating factors (MAF). One such MAF is interferon  $\gamma$  (IFN- $\gamma$ ). In some assays, substantial activity of IFN- $\gamma$  on murine macrophages, however, is only observed in synergy with lipopolysaccharide (LPS) or other cytokines (1). In addition, certain cytokines have been shown to induce monocyte or macrophage activation in the absence of IFN- $\gamma$  (2-5). We previously described lymphokines in the supernatant of a murine T cell clone that synergized with IFN- $\gamma$  in the induction of tumoricidal and schistosomulicidal murine macrophages (1). We called this lymphokine(s) macrophage cytotoxicity-inducing factor 2 (MCIF2) (1). A candidate for MCIF2 was lymphotoxin (LT), because the T cell clone supernatant contained high amounts of LT. LT is functionally homologous and structurally related to the macrophage product tumor necrosis factor (TNF). Therefore, we tested whether recombinant (r) LT or rTNF can function as MAF. We report here that rLT or rTNF synergize with rIFN- $\gamma$  in the induction of tumoricidal and schistosomulicidal murine macrophages.

### Materials and Methods

*Reagents and Cytokines.* Bacterial LPS (from *Shigella flexneri*) was purchased from Sigma Chemical Co. (Deisenhofen, Federal Republic of Germany). Human rTNF was a gift from BASF, Ludwigshafen (Federal Republic of Germany); human rLT was a gift provided by Genentech (2715/88, specific activity  $3 \times 10^7$  U/mg; South San Francisco, CA). The specific activity of rTNF tested on actinomycin D-treated mouse L929 cells was  $2 \times 10^7$  U/mg. rLT and rTNF showed the same specific activity as to lysis of L929 cells.

*Macrophage Tumor Cell and Schistosomula Killing.* As described previously (1), resident peritoneal macrophages from C3H/HeJ mice ( $2.5 \times 10^5$  peritoneal cells) were cultured in 200  $\mu$ l of medium. After 2 h, nonadherent cells were removed and the remaining adherent cells (>95% macrophages by nonspecific esterase staining) were incubated

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overnight in the presence of serial dilutions of rLT or rTNF in the presence or absence of rIFN- $\gamma$ . After two washes of the cells,  $4 \times 10^4$  6-[ $^3\text{H}$ ]TdR-labeled P815 target cells (Amersham Corp., Braunschweig, Federal Republic of Germany, sp act, 5.0 Ci/mmol; 4 h at 37°C in 5% CO $_2$ ; 10  $\mu\text{Ci}$  per  $10^6$  cells) in 200  $\mu\text{l}$  medium were added. After 20 h of incubation, [ $^3\text{H}$ ]TdR release was determined. The percent specific P815 killing was calculated as  $100 \times [(\text{experimental release}) - (\text{control release})] / [(\text{maximum release}) - (\text{control release})]$ . Schistosomula killing using skin schistosomula from a Puerto Rican strain of *Schistosoma mansoni* has previously been described (1) and was performed in the same way as the tumoricidal assay except that 200  $\mu\text{l}$  of a schistosomula suspension (100 larvae) were added to the macrophages preincubated with the corresponding cytokines. The viability of schistosomula was visually determined after 24 h of culture.

**Tumor Cell Killing with Bone Marrow-derived Macrophages.** Cells from femurs of C3H/HeJ mice were incubated for 10 d at a concentration of  $3 \times 10^5$  cells/ml in Petri dishes (non-tissue culture grade; Greiner and Söhne, Nürtingen, Federal Republic of Germany) in RPMI 1640 supplemented with L-glutamine (2 mM), streptomycin (100  $\mu\text{g}/\text{ml}$ ), penicillin (100 U/ml), essential amino acids (50  $\mu\text{g}/\text{ml}$ ), nonessential amino acids (50  $\mu\text{g}/\text{ml}$ ), 5% heat-inactivated horse serum, 10% heat-inactivated FCS, and 20% L cell-conditioned medium with two changes of culture medium on days 5 and 8. The cells (100% nonspecific esterase-positive) were detached by gentle pipetting with cold PBS (Ca $^{2+}$  and Mg $^{2+}$  free), washed, and resuspended in RPMI 1640 medium supplemented with L-glutamine (2 mM), streptomycin (100  $\mu\text{g}/\text{ml}$ ), penicillin (100 U/ml), and 10% FCS.  $10^5$  cells/culture were used for tumor cell killing.

**Chemiluminescence (CL) by Bone Marrow-derived Macrophages.**  $3 \times 10^5$  cells in 0.5 ml medium were added to cylindrical glass tubes and incubated for 18 h in the presence of medium alone or medium containing cytokines. The cells were then washed and CL measured at 37°C in Dulbecco's PBS supplemented with BSA (1 mg/ml), glucose (1 mg/ml), luminol (20  $\mu\text{M}$ ), and PMA (100 nM).

## Results and Discussion

**Induction of Tumoricidal and Schistosomulicidal Murine Resident Peritoneal Macrophages with rIFN- $\gamma$  and rLT or rTNF.** We tested several recombinant cytokines for in vitro activation of tumoricidal and schistosomulicidal macrophages. Purified murine rIL-1, rIL-3, and purified natural murine IL-2 (3–100 U/ml) in the presence or absence of murine rIFN- $\gamma$  (20 U/ml) failed to induce macrophage activation. Likewise, rIFN- $\gamma$  at a concentration up to 100 U/ml was inactive. rIFN- $\gamma$  (20 U/ml), however, served as a priming signal in the presence of LPS as the triggering signal (data not shown). In contrast, human rLT and rTNF, active on mouse cells, synergized with rIFN- $\gamma$  in the induction of both macrophage activities (Fig. 1). In the absence of rIFN- $\gamma$ , no effect was observed with rLT or rTNF up to a concentration of 10  $\mu\text{g}/\text{ml}$  (Fig. 1). The effective amounts of rLT and rTNF to induce a 50% cytotoxic response varied in a wide range of concentrations depending on each particular experiment (2–250 ng/ml; 12 experiments). Induction of macrophage activation by rIFN- $\gamma$  and rLT or rTNF was not due to contamination with LPS, because the concentrations of LPS present in the rIFN- $\gamma$ , rLT, and rTNF preparations used (<1.0 ng/ml) were inactive. In addition, macrophage activation was not impaired in the presence of polymyxin B (50  $\mu\text{g}/\text{ml}$ ), neutralizing at least 200 ng/ml of LPS in the macrophage tumoricidal assay (data not shown). Furthermore, in most experiments and in the experiments shown here we used macrophages from C3H/HeJ mice (LPS low responder). Finally, treatment of rLT or rTNF at 100°C for 10 min destroyed the capacity of these cytokines to induce tumoricidal and schistosomulicidal activities.

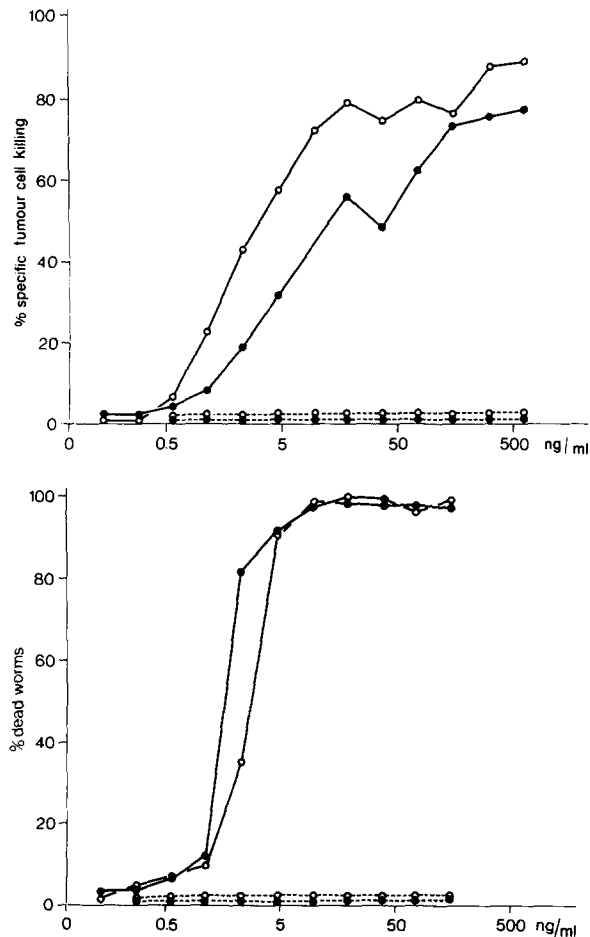


FIGURE 1. Induction of tumoricidal (a) and schistosomicidal (b) murine resident peritoneal macrophages upon incubation with different doses of rLT (○) or rTNF (●) in the presence (solid lines) or absence (dashed lines) of rIFN- $\gamma$  (20 U/ml). The percentage of specific killing for each point represents the mean of triplicate samples. The standard errors were <5% of the mean value. The results are from a representative experiment out of 12.

micidal macrophages. Such heat treatment, however, does not destroy LPS activity. Taken together, these data show that macrophage activation in our experiments was induced by rIFN- $\gamma$  and rLT or rTNF and not by rIFN- $\gamma$  and contaminating LPS.

*Induction of Tumoricidal Murine Bone Marrow-derived Macrophages with rIFN- $\gamma$  and rLT or rTNF.* Similar data as with adherent peritoneal cells were obtained with pure macrophages from 10-d bone marrow cell cultures. Fig. 2 shows that such cells killed P815 tumor cells upon activation by rLT or rTNF only in synergy with rIFN- $\gamma$ . Thus, macrophage activation by rIFN- $\gamma$  and rLT or rIFN- $\gamma$  and rTNF is mediated through the direct action of these cytokines on macrophages.

*Lack of P815 Tumor Cell Lysis and Schistosomula Killing by rIFN- $\gamma$  and rLT or rTNF in the Absence of Macrophages.* The above experiments did not formally distinguish between the possible dual role of LT and TNF as inducers of macrophage functions and as cytolytic molecules (6, 7). Furthermore, a synergy between IFN- $\gamma$  and TNF or LT for direct tumor cell cytotoxicity has previously been documented (8). Therefore, we devised experiments to show that the

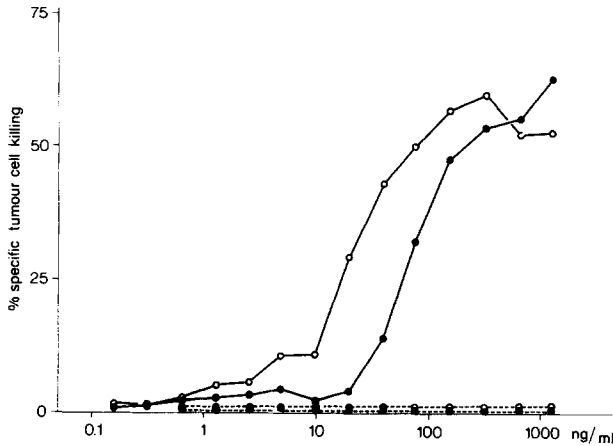


FIGURE 2. Induction of tumoricidal murine bone marrow-derived macrophages upon incubation with different doses of rLT (○) or rTNF (●) in the presence (solid lines) or absence (dashed lines) of rIFN- $\gamma$  (20 U/ml). The results are from one representative experiment out of five.

stimuli provided by rLT or rTNF in synergy with rIFN- $\gamma$  were truly inductive. P815 tumor cells were resistant and schistosomula not killed within 20 h of incubation with rIFN- $\gamma$  (20 U/ml) and rLT (0.075–5  $\mu$ g/ml) or rTNF (0.075–5  $\mu$ g/ml) in the absence of macrophages. rTNF alone had no effect. Longer incubation (72 h) of P815 tumor cells with rIFN- $\gamma$  (20 U/ml) and rTNF (0.025–2.5  $\mu$ g/ml) was also not effective. Schistosomula were even resistant to direct cytotoxicity of rTNF, when their efficient damage repair system was blocked with actinomycin D (50  $\mu$ g/ml), cycloheximide (10  $\mu$ g/ml), colchicine (50  $\mu$ g/ml), or mithramycin A (10  $\mu$ g/ml). However, under the same conditions but with resident peritoneal macrophages, pronounced tumoricidal (60–62% killing) and schistosomicidal (82–95% killing) effects were observed. Similar results were obtained with comparable doses of rLT. In addition, macrophages were incubated with rIFN- $\gamma$  and rLT or rTNF at doses that induce macrophage cytotoxicity and subsequently fixed with formaldehyde. These cells failed to kill P815 tumor cells or schistosomula. These results show that killing is exerted only by viable, activated macrophages and is unlikely to be explained by the mere absorption of rLT or rTNF to the macrophage surface.

*CL by Bone Marrow-derived Macrophages with rIFN- $\gamma$  and rLT or rTNF.* To further support our contention that rIFN- $\gamma$  and rLT or rTNF were not merely cytotoxic but inducers of macrophage activation, we examined a macrophage function different from tumor or parasite killing, namely the release of oxygen intermediates by activated macrophages (9). The activity of rTNF and rLT (both in the presence or absence of rIFN- $\gamma$ ) on the oxidative respiratory burst of macrophages in a PMA-triggered, luminol-dependent CL assay was tested. Fig. 3 shows that bone marrow-derived macrophages preincubated during 18 h with rIFN- $\gamma$  (20 U/ml) and rTNF (100 ng/ml) demonstrated enhanced CL as compared to cells preincubated with either lymphokine alone. Experiments with rIFN- $\gamma$  and rLT gave superimposable CL curves (data not shown). This effect was specific for rTNF or rLT, since preincubation in the presence of rIFN- $\gamma$  and IL-2 did not show this effect. Taken together, these data demonstrate that LT and TNF synergize with IFN- $\gamma$  to directly induce tumoricidal and schistosomicidal macrophages. Thus, MCIF2 present in T cell supernatant and

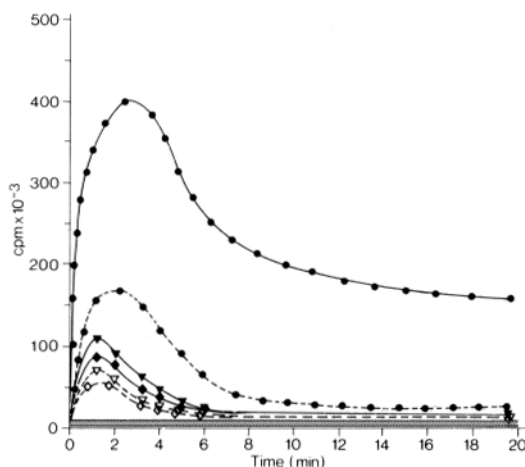


FIGURE 3. Chemiluminescence by bone marrow-derived macrophages upon incubation with different cytokines. The cells were incubated in the presence of medium alone ( $\nabla$ ) or medium containing: purified natural murine IL-2 (100 U/ml) ( $\diamond$ ), rIFN- $\gamma$  (20 U/ml) ( $\blacktriangledown$ ), rTNF (100 ng/ml) ( $\bullet$ ), rIFN- $\gamma$  (20 U/ml) + natural IL-2 (100 U/ml) ( $\blacklozenge$ ), rIFN- $\gamma$  (20 U/ml) + rTNF (100 ng/ml) ( $\bullet$ ). The shaded area represents background CL in the absence of the inducer PMA.

synergizing with IFN- $\gamma$  could be LT. Whether yet other T cell-derived MAF apart from IFN- $\gamma$  and LT exist and whether IFN- $\gamma$  and LT act as priming and triggering signal, respectively, remains to be shown. Previous papers have shown a synergy between rIFN- $\gamma$  and rLT or rTNF for (a) an antiproliferative effect on different normal and transformed cell lines (10), (b) the induction of NK-mediated target cell killing (11), (c) an inhibition of granulocyte/monocyte colony formation (14), and (d) a differentiation of human myeloid cell lines (13). Our data show that properties of LT and TNF as immunomodulators include induction of tumoricidal and schistosomicidal macrophages. IFN- $\gamma$  and LT are lymphokines secreted by activated T lymphocytes. Therefore, macrophage tumoricidal and parasiticidal activity could arise in vivo in the course of a T cell-mediated immune response. In addition, TNF is released by macrophages themselves upon stimulation with bacterial endotoxins (LPS) (14–16), or as a result of a virus infection (17). Thus, TNF could act either on other macrophages or in an autocrine manner on the same cell that produced it (6). This might result in resistance to a further viral or microbial infection.

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