

# TUMOR NECROSIS FACTOR $\alpha$ /CACHECTIN IS A GROWTH FACTOR FOR THYMOCYTES

## Synergistic Interactions with Other Cytokines

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TNF- $\alpha$ /cachectin, initially described for its ability to induce hemorrhagic necrosis in vivo (1), also exerts profound effects on several biological processes shared with IL-1, including endogenous pyrogen activity, stimulation of bone resorption, collagenase and prostaglandin E<sub>2</sub> synthesis by fibroblasts (2), IL-2-R expression and T cell growth (3). Among those activities which have not been reported to be shared between IL-1 and TNF- $\alpha$  are the ability to stimulate thymocyte proliferation. The purpose of our study was to determine the effects of TNF- $\alpha$  on the in vitro proliferative response of murine thymocytes.

### Materials and Methods

**Reagents.** Recombinant murine TNF- $\alpha$  (rmTNF- $\alpha$ ) and recombinant human TNF- $\alpha$  (rhTNF- $\alpha$ ) were cloned and expressed in *Escherichia coli* (4, 5) (specific activities  $5 \times 10^7$  and  $7 \times 10^7$  U/mg of protein, respectively). Recombinant murine interferon  $\gamma$  (rmIFN- $\gamma$ , sp act  $1-2 \times 10^7$  U/mg of protein) was cloned and expressed in *E. coli* (6). By limulus amoebocyte lysate assays, the recombinant proteins were found to contain  $<0.025$   $\mu$ g of endotoxin/ $\mu$ g of protein. Natural murine TNF- $\alpha$  had a specific activity of  $2 \times 10^7$  U/mg protein (7). Purified natural human IL-1 (specific activity  $10^7$  U/mg protein) was obtained from Genzyme (Boston, MA). Human rIL-2 (sp act  $10^7$  U/mg protein) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Murine rIL-4 (sp act  $10^7$  U/mg protein) was obtained from DNAX Research Institute (Palo Alto, CA).

**Thymocyte Comitogenic Assay.** C3H/HeJ thymocytes (The Jackson Laboratories, Bar Harbor, ME) were cultured in 96-well flat-bottomed culture plates ( $1.5 \times 10^6/0.1$  ml) (Costar, Cambridge, MA) in Eagle's MEM supplemented with 1% nonessential amino acids, 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 1% L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and  $5 \times 10^{-5}$  M 2-ME (CMEM) in the presence of a 1:2,000 final dilution of phytohemagglutinin P (PHA-P, Difco Laboratories, Detroit, MI). Cytokines were added to a final volume of 0.2 ml. After 60 h at 37°C, cultures were pulsed with 1  $\mu$ Ci [<sup>3</sup>H] thymidine (5 Ci/mmol; New England Nuclear, Bos-

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ton, MA) for 12 h, harvested onto glass fiber filters (PhD, Cambridge Technology, Inc., Watertown, MA), and mean [ $^3\text{H}$ ] thymidine incorporation (cpm) of triplicate cultures was determined.

**TNF- $\alpha$  Bioassay.** TNF- $\alpha$  activity was assayed on WEHI-164 clone 13 cells (8). This assay is sensitive to 10 fg/ml of mTNF- $\alpha$ . Rabbit anti-rmTNF- $\alpha$ , developed by methods similar to those described for rabbit antibodies to rhTNF- $\alpha$ , has a neutralization titer of  $\sim 10^6$  U/ml (9).

### Results and Discussion

As shown in Fig. 1 A, rmTNF- $\alpha$  but not rhTNF- $\alpha$  (even at doses as high as  $10^5$  U/ml) stimulates thymocyte proliferation in the presence of PHA-P, albeit (on a unit per unit basis) to a significantly lesser degree than IL-1. At the doses tested, natural mTNF- $\alpha$ /cachectin shows similar stimulatory effects compared with rmTNF- $\alpha$  on thymocytes (Fig. 1 B) (7). Previous studies using mTNF- $\alpha$ /cachectin were performed at concentrations below those required to stimulate thymocyte proliferation, which may explain the failure of earlier studies to detect proliferative activity by thymocytes (10). While species preferences for TNF- $\alpha$  activities have been observed (5, 11), the species specificity displayed on thymocytes was unexpected and explains the failure by previous studies (12) to detect thymocyte activation by this cytokine. mTNF- $\beta$  was not available for testing, but rhTNF- $\beta$  (lymphotoxin), like rhTNF- $\alpha$ , was not active in this assay (data not shown).

Previous studies have indicated that human TNF- $\alpha$  competes with mTNF- $\alpha$  for receptor binding on murine cells (11). As it was of interest to determine whether the failure of rhTNF- $\alpha$  to induce murine thymocyte proliferation was due to the inability to specifically bind to thymocytes, competition assays were performed to determine whether rhTNF- $\alpha$  could inhibit the modulation of thymocyte proliferation by rmTNF- $\alpha$ . The results demonstrated that rmTNF- $\alpha$ -induced thymocyte proliferation was inhibited, in a dose-dependent manner, by rhTNF- $\alpha$  (Fig. 2). The amounts of rhTNF- $\alpha$  required to achieve this suppression were 5–10-fold higher than the amount of rmTNF- $\alpha$  signaling proliferation, suggesting that rhTNF- $\alpha$  may bind to the TNF receptor with less affinity than rmTNF- $\alpha$  (11).

Several recent studies indicate that TNF- $\alpha$  can synergize with one or more lymphokines during the induction of certain biological activities (3, 13). The following studies were designed to determine whether rMuTNF- $\alpha$  could synergize

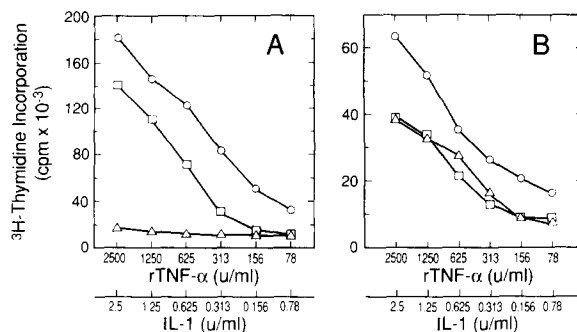


FIGURE 1. (A) rmTNF- $\alpha$  but not rhTNF- $\alpha$  enhances murine thymocyte proliferation. rmTNF- $\alpha$  ( $\square$ ), rhTNF- $\alpha$  ( $\Delta$ ), or hIL-1 ( $\circ$ ) were added at the indicated concentrations. mAbs to rmTNF- $\alpha$  abolished the proliferative effects of rmTNF- $\alpha$  on murine thymocytes (data not shown). (B) Both natural and rmTNF- $\alpha$  enhance PHA-P-induced murine thymocyte proliferation. rmTNF- $\alpha$  ( $\square$ ), natural mTNF- $\alpha$  ( $\Delta$ ), or hIL-1 ( $\circ$ ) were added to thymocyte cultures at indicated concentrations.

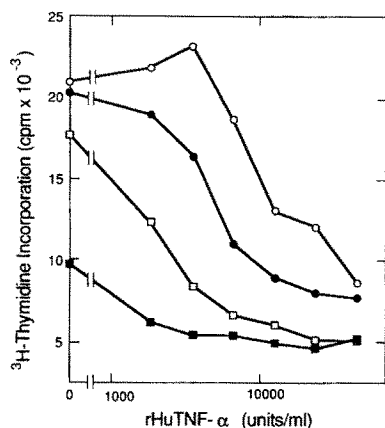


FIGURE 2. Competitive inhibition of rmTNF- $\alpha$ -induced thymocyte proliferation by rhTNF- $\alpha$ . rmTNF- $\alpha$ : (○), 2,500 U/ml; (●), 1,250 U/ml; (□), 625 U/ml; (■), 313 U/ml were assayed in the presence of PHA-P and in the absence or presence of rhTNF- $\alpha$  concentrations ranging from  $1.3 \times 10^3$ – $5.0 \times 10^5$  U/ml.

with several lymphokines known to have immunologic activity in the thymocyte comitogenic assay (Fig. 3). Both hIL-1 and rhIL-2 induced thymocytes to proliferate in the absence or presence of PHA-P although rhIL-2 was far more potent under either condition. rmIFN- $\gamma$  was ineffective at inducing activity whether or not PHA-P was present. When rmTNF- $\alpha$  was added to cultures containing either hIL-1 or rhIL-2, the response was enhanced substantially beyond additive responses of the two lymphokines alone, both in the absence and presence of PHA-P.

As rmIL-4 is also comitogenic for murine thymocytes (14), we examined the relationships of rmTNF- $\alpha$  and rmIL-4 in augmenting thymocyte proliferation (Fig. 4). The data indicate that rmIL-4 is mitogenic for murine thymocytes, albeit to a much greater degree in the presence of PHA-P. In contrast to the results obtained with hIL-1 or rhIL-2, the addition of rmTNF- $\alpha$  did not enhance the mitogenic signal of rMuIL-4 alone, and only marginally increased the proliferative response of thymocytes to rmIL-4 in the presence of PHA-P beyond the additive response of the two lymphokines alone.

Among the lymphokines that are comitogenic for thymocytes in the presence of PHA-P are IL-1, IL-2, and IL-4 (14, 15). To test whether rmTNF- $\alpha$  was either directly comitogenic or indirectly by stimulating the synthesis of one or more of these lymphokines which in turn acted directly on the thymocytes, supernatants were assayed from rmTNF- $\alpha$ /PHA-P-activated thymocyte cultures for the presence of these factors. The IL-1 assay, performed with subclone D10.5 of the murine helper T cell line, D10.G4.1, is sensitive to 1–10 fg/ml of IL-1 $\alpha$  or IL-1 $\beta$  (16). The IL-2 and IL-4 assays, performed on HT-2 cells, are sensitive to 0.15 U rhIL-2/ml and 1 U rIL-4/ml (17). The levels of IL-1, IL-2, and IL-4 in supernatants from rmTNF- $\alpha$ /PHA-P-activated thymocyte cultures were below the detection limit for these assays. Because the levels of lymphokines detectable by these assays fall below those levels that synergize with rmTNF- $\alpha$ , our results suggest that the proliferative response by thymocytes to TNF- $\alpha$  does not require the intermediate production of any of these lymphokines.

Having established that rmTNF- $\alpha$  provides a comitogenic signal for thymocyte proliferation, we examined whether rmTNF- $\alpha$  was produced by thymocytes stim-

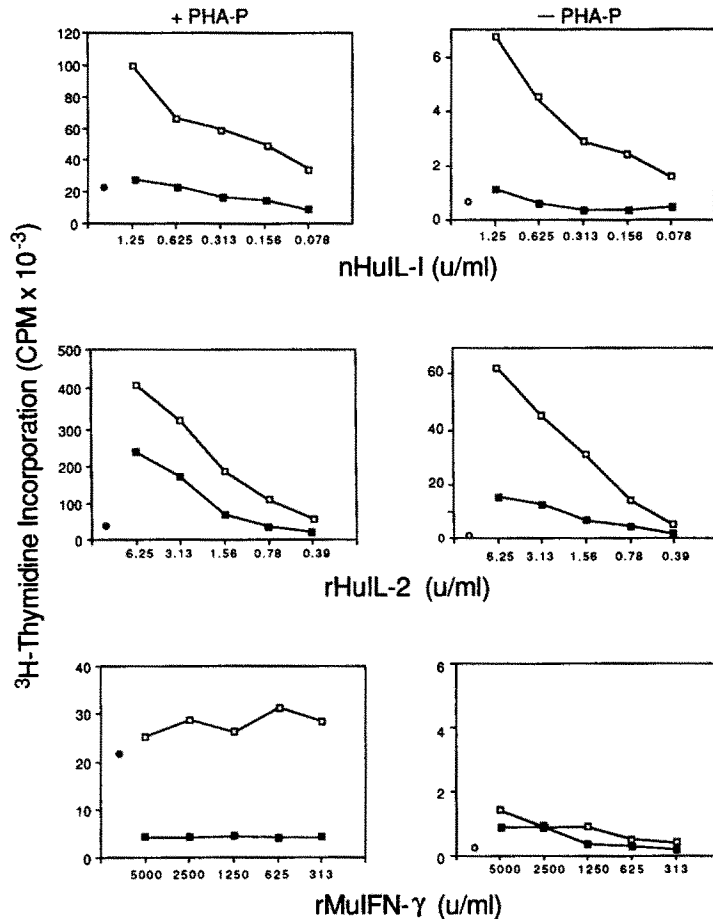


FIGURE 3. Interactions between hIL-1, rhIL-2, or rmIFN- $\gamma$  with rmTNF- $\alpha$ . hIL-1, rhIL-2, and rmIFN- $\gamma$  were used at the indicated doses in the absence (■) or presence (□) of 1,000 U/ml of rmTNF- $\alpha$  and in the absence or presence of a 1:2,000 final dilution of PHA-P. [ $^3$ H]thymidine incorporation of thymocytes stimulated with 1,000 U/ml of rmTNF- $\alpha$  alone (○) or with rmTNF- $\alpha$  in the presence (●) of a 1:2,000 final dilution of PHA-P was 874 and 22,008 cpm, respectively.

ulated with either PHA-P alone or in conjunction with hIL-1, rhIL-2, or rmIL-4. Previous reports have indicated the T cells from human peripheral blood can be induced to produce TNF- $\alpha$  (18). The results indicate that rmTNF- $\alpha$  was produced by PHA-P stimulation alone and that the addition of hIL-1, rhIL-2, or rmIL-4 at concentrations that induce significant thymocyte proliferation resulted in an augmentation of mTNF- $\alpha$  production (Table I).

The data presented here suggest that TNF- $\alpha$ , as well as other lymphokines, may play an important role in thymocyte proliferation and differentiation (17, 18). Our observations indicate that mTNF- $\alpha$  is not directly mitogenic for thymocytes but acts as a comitogen with PHA-P. The mechanism of activation of thymocytes by mTNF- $\alpha$  is not yet clear, but the failure to detect significant stimulatory levels of mIL-1, mIL-2, or mIL-4 in thymocyte cultures stimulated with rmTNF- $\alpha$  and PHA-P suggests that the production of these lymphokines is not

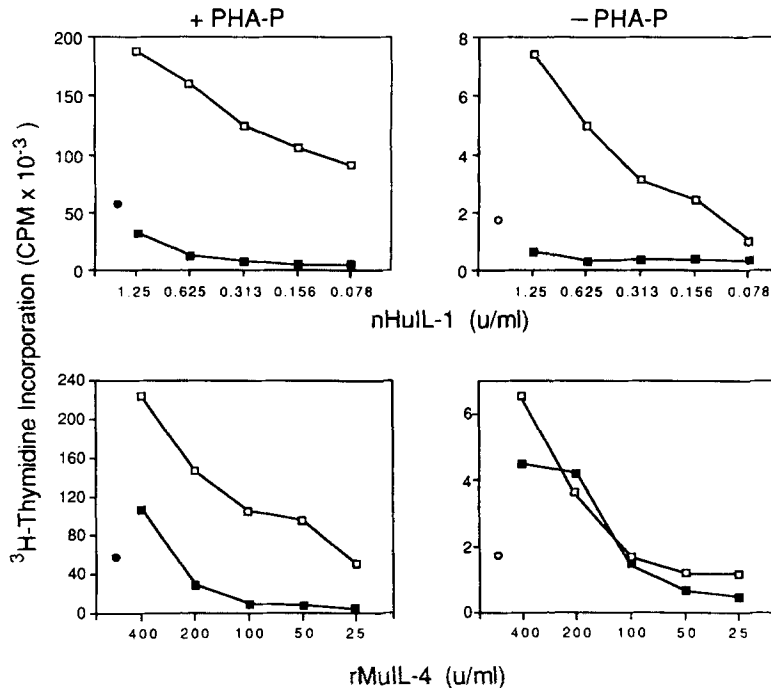


FIGURE 4. Interactions between hIL-1 or rMuIL-4 with rmTNF- $\alpha$ . hIL-1 and rMuIL-4 were used at the indicated doses in the absence (■) or presence (□) of 1,000 U/ml of rmTNF- $\alpha$  and in the absence or presence of a 1:2,000 final dilution of PHA-P. [ $^3$ H]thymidine incorporation of thymocytes stimulated with 1,000 U/ml rmTNF- $\alpha$  alone (○) or rmTNF- $\alpha$  in the presence (●) of a 1:2,000 final dilution of PHA-P was 1,940 and 57,590 cpm, respectively.

intermediate to this process. Alternatively, stimulation of thymocytes by these lymphokines in the presence of PHA-P is accompanied by the production of significant amounts of mTNF- $\alpha$ , possibly implicating mTNF- $\alpha$  as a common intermediate in thymocyte activation initiated by these materials.

TABLE I  
*Cytokine-induced TNF- $\alpha$  Production by Murine Thymocytes*

Thymocyte treatment*	mTNF- $\alpha$ (U $\times$ 10 $^{-3}$ /ml) $\pm$ SD	
	Alone	Anti-rmTNF- $\alpha$ †
None	0.27 $\pm$ 0.04	1.0 $\pm$ 0.01
PHA-P	4.2 $\pm$ 0.4	0.67 $\pm$ 0.01
PHA-P + 5 U/ml hIL-1	9.3 $\pm$ 1.0	0.92 $\pm$ 0.08
PHA-P + 5 U/ml rhIL-2	24.6 $\pm$ 1.2	1.07 $\pm$ 0.42
PHA-P + 250 U/ml rMuIL-4	11.0 $\pm$ 1.5	0.83 $\pm$ 0.01

\* C3H/HeJ thymocytes (1.5  $\times$  10 $^7$ /ml) were stimulated with PHA-P (1:2,000 final dilution) alone or with the indicated concentrations of cytokines. After 48 h of culture at 37°C, supernatants were assayed for mTNF- $\alpha$  activity on WEHI-164 clone 13 cells (11).

† Rabbit anti-rmTNF- $\alpha$  serum (at a 1:30 dilution) was added to the supernatants 30 min before assay. Normal rabbit serum at 1:30 dilution showed no anti-TNF- $\alpha$  activity or interference in this assay.

We have recently observed that rmTNF- $\alpha$  will induce production of substantial amounts of IL-6 by murine thymocytes and that IL-6 can act as a comitogen for thymocyte proliferation in the presence of PHA-P (unpublished observations). Investigations are in progress to determine the role of these factors in thymocyte proliferation and development.

### Summary

Recombinant murine (rm) TNF- $\alpha$  but not recombinant human (rh) TNF- $\alpha$  induces the proliferation of murine thymocytes in the presence of a comitogenic stimulus. This effect does not appear to be due to the production of significant levels of IL-1, IL-2, or IL-4. Although not directly mitogenic (i.e., in the absence of PHA-P) for thymocytes, rmTNF- $\alpha$  amplifies the direct mitogenic signals from hIL-1 and rhIL-2 but not rmIL-4. In the presence of PHA-P, thymocytes stimulated with hIL-1, rhIL-2, and rmIL-4 produced significant amounts of TNF- $\alpha$ . Although rhTNF- $\alpha$  does not induce a proliferative response, it will competitively inhibit the proliferative response of thymocytes to rmTNF- $\alpha$ . These data suggest a critical role for TNF- $\alpha$  in the intrathymic proliferation of developing T cells.

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