Functional Characterization of the Human Tumor Necrosis Factor Receptor p75 in a Transfected Rat/Mouse T Cell Hybridoma

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Summary

We investigated the biological role of the human tumor necrosis factor p75 (hTNF-R75), making use of the species specificity of TNF responses in murine (m) T cell lines. Several TNF-mediated activities on mouse T cells, such as cytokine induction or proliferation, showed a 100–500-fold difference in specific biological activity between mTNF and hTNF. After transfection of hTNF-R75 cDNA in a rat/mouse T cell hybridoma (PC60), however, the 100-fold lower specific biological activity of hTNF was converted to the same specific biological activity as mTNF. The TNF-mediated induction of granulocyte/macrophage colony-stimulating factor was strongly synergized by the addition of interleukin 1. In the presence of the latter cytokine, ligand-competing monoclonal antibodies against hTNF-R75 (utr-1, utr-2, utr-3) were agonistic on transfected PC60 cells. This agonistic activity was further enhanced by crosslinking with sheep anti-murine immunoglobulin antibodies. These data provide direct evidence for a functional role of TNF-R75, without ligand-dependent TNF-R55 involvement, in the induction of cytokine secretion in T cells.

TNF is a pleiotropic cytokine, mainly produced by monocytes, macrophages, and T lymphocyte subsets. Its many different activities in inflammatory and immunological reactions, in septic shock, or in autoimmune diseases have been reviewed (1–5). Two distinct TNF receptors of 55–60 kD (TNF-R55) and 75–80 kD (TNF-R75) have been identified (6–8) and molecularly cloned in mice and humans (9–14). The amino acid sequence and a four-domain pattern characterized by a six-cysteine repeat motif of the extracellular parts of TNF-R55 and TNF-R75 are fairly homologous and have similarities also with those of other receptors, such as nerve growth factor receptor (NGF-R), CD40, CD27, and Fas antigen (15), and thus define a new receptor gene family (16). However, there is a remarkable absence of sequence similarity between the intracellular regions of the two TNF-Rs, suggesting different signal transduction pathways and functions.

TNF-R55 seems to be ubiquitous and occurs on, among others, epithelial cells and fibroblasts. Both polyclonal and monoclonal antibodies against human (h)[1]TNF-R55 have been shown to act agonistically and to exert a number of TNF activities, such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, activation of NF-κB, and synthesis of prostaglandin (17–19). Using polyclonal antibodies binding to murine (m)TNF-R55, it was demonstrated that TNF-R55 triggering is sufficient to mediate cytotoxicity and to induce MnSOD mRNA (20). The expression of TNF-R75 has been investigated in cells of hematopoietic origin, such as T cells (12, 21) and B cells (22).

There is little, if any, species specificity between mTNF and hTNF in TNF-R55-mediated activities. In contrast, mTNF-R75 is only triggered by mTNF (13) and not by hTNF, which explains the species specificity of, for example, several T cell responses to mTNF (23–25). To more specifically investigate the role of TNF-R75 and to exclude that a biochemically undetected, small number of TNF-R55 contributes to the cellular response thought to depend on TNF-R75, we used the rat/mouse T hybridoma PC60 (26), transfected with hTNF-R75 cDNA. In previous studies, the involvement of TNF-R75 has been demonstrated indirectly by the use of antagonistic anti-hTNF-R75 mAb, which in all cases resulted in at most a partial neutralization of the TNF-dependent biological response (19, 22, 27). Recently, an agonistic activity of a polyclonal anti-mTNF-R75 antiserum in the stimulation of the proliferation of murine thymocytes and of a cytotoxic T cell line (CT6) has been described (28). Interestingly,

[1] *Abbreviations used in this paper:* h, human; m, murine.

proliferative signals are mediated independently by both TNF-R75 and TNF-R55 in human mononuclear cells (29).

We report here that transfection of hTNF-R75 cDNA in a rat/mouse T cell hybridoma is sufficient to render these cells responsive to hTNF. This specifically hTNF-R75-mediated stimulation leads to synthesis of a set of cytokines, such as GM-CSF. This activity could also be mimicked by R75 crosslinking by means of anti-hTNF-R75 mAb.

Materials and Methods

Cytokines, Assays, and Antibodies. Purified *Escherichia coli*–derived hTNF and mTNF were prepared in our laboratory and had a specific biological activity of 0.94 and 2.24 × 10^8 IU/mg, respectively, in a standardized cytotoxic assay on WEHI-164 cl13 cells (30). Recombinant mGM-CSF was generously provided by Dr. J. DeLamarter (Glaxo IMB, Geneva, Switzerland) and had a specific biological activity of ~2.5 × 10^8 U/mg in the FDCp1 proliferation assay (31, 32). Recombinant hIL-1β (5 × 10^8 U/mg) was provided by Dr. A. Shaw (formerly of Biogen, Geneva, Switzerland) and was quantified by the RPMI 1788 proliferation assay (33). In all assays, 1 U was arbitrarily defined as the amount of cytokine required to induce half-maximal proliferation, except for the WEHI-164 cl13 test in which international standards for TNF quantification (IU/ml) were used (obtained from the National Institute for Biological Standards and Control, Potters Bar, UK).

Anti-hTNF-R55 (htr-9) and anti-hTNF-R75 mAb (utr-1, utr-2, utr-3, utr-4, utr-10) have been described elsewhere (7, 8); they all belong to the IgG1 isotype. Sheep anti-mIg (SAM) (Sera-Lab, Crawley Down, UK) was freed of NaN_3 by dialysis against PBS.

Cells. The hybridoma PC60.21.14.4 (PC60) is derived from a fusion between an IL-2-dependent murine CTL line B6.1SF.1 and a rat thymoma (C58.NT)D (26). LBRM-33-1A5, a murine T cell lymphoma (34); NOB-1, a murine thymoma (35); CT6, an IL-2-dependent murine cytotoxic T cell line (25); WE17/10, an IL-2-dependent human T cell line (36); and PC60 cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin G (50 U/ml), streptomycin sulfate (50 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and 2-ME (5 × 10^{-5} M). The factor-dependent FDCp1 cells were grown in the same medium, but supplemented with 10% WEHI-3 supernatant as a source of mIL-3.

DNA Transfection. Plasmids were constructed and prepared by standard techniques. HTNF-R75 cDNA (12) was cloned as a HindIII-Asp718 1,401-bp restriction fragment in pSV25S, a eukaryotic expression vector containing the SV40 early promoter, polyadenylation, and splicing signal (pSV25S-HTNFR75). As selection plasmid for the PC60 cell transformation, we used pBSΔppac (37), which contains the gene for *N*-acetyl puromycin transferase under control of the early SV40 promoter. PC60 cells were transfected by electroporation (Gene Pulser Apparatus; Bio-Rad Laboratories, Richmond, CA). Exponentially growing cells were washed once in cold transfection buffer (PBS without $MgCl_2$ and $CaCl_2$) and 5 × 10^6 cells were resuspended in 800 μl of the same buffer. EcoRI-linearized pSV25S-HTNFR75 (10 μg/800 μl) and pBSΔppac (1 μg/800 μl) plasmids were added to the cell suspension and kept for 5 min on ice. The mixture was aspirated into an ice-cooled 4-mm electroporation chamber (Bio-Rad Laboratories) and exposed to a single voltage pulse (1280 V and 25 μF). Cells were kept for another 10 min on ice, resuspended in 100 ml complete medium at room temperature, and put in culture. 3 d later, puromycin (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 3 μg/ml. 18 d later, cells were screened for expression of hTNF-R75 by flow fluorocytometric analysis (25–42% of the pool of cotransfected and antibiotic-selected PC60 cells were positive). Next, cells were subcloned by limiting dilution. Even after 2 mo of culturing in the absence of further selection for puromycin resistance, most of the transfected PC60 clones showed stable expression of hTNF-R75.

Flow Fluorocytometry. Transfectants were stained for 30 min at 4°C with mAb against hTNF-R75 (0.4 μg utr-1/5 × 10^5 cells in 200 μl), followed by fluorescein-conjugated SAM (Sera-Lab), and analyzed with an Epics 753 equipped with an argon-ion laser (Coulter Immunology, Hialeah, FL).

Induction of GM-CSF in PC60 Cells. Previously, we have demonstrated that rat GM-CSF is the most abundantly produced cytokine, of a series tested, after induction of PC60 cells (32). Induction experiments were performed in 96-well microtiter plates. 3 × 10^4 PC60 cells/well were exposed to a serial dilution of mTNF or hTNF, in the absence or presence of a constant amount of synergistically acting hIL-1β (1 ng/ml). When antagonistic or agonistic activities of anti-hTNF-R75 mAb (utr-1, utr-2, utr-3, utr-4, utr-10) were investigated, 3 × 10^4 hTNF-R75-expressing PC60 cells/well were preincubated for 1 h at 4°C with serial dilutions of the abovementioned mAb. Then, serial dilutions of hTNF or crosslinking SAM were added. After 24 h of incubation, supernatants were tested for GM-CSF activity.

Radiolabeling of TNF. ^{125}I-mTNF and ^{125}I-hTNF were prepared with Iodogen iodination agent (Pierce Chemical Co., Rockford, IL). A specific radioactivity of 10–30 μCi/μg was routinely achieved and its biological activity, normally between 50 and 100% of starting material, was assessed in the cytotoxic assay on WEHI-164 cl13 cells (18). The labeled TNF was separated from unincorporated label on a G25 column (PD10; Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with PBS-A (PBS without $CaCl_2$ or $MgCl_2$) containing 0.25% gelatin and 50 μg/ml gentamycin.

Scatchard Analysis. Serial dilutions of labeled TNF (10–200 pM for mTNF and 10–2,000 pM for hTNF) were added to 2 × 10^6 cells in a final vol of 1 ml (PBS-A, 0.5% BSA, 0.02% NaN_3) and left for 3 h at 4°C. Background binding was measured in the presence of a 150-fold molar excess of cold ligand. Cells were washed once and bound ligand was determined by pelleting the cells through a silicon oil/paraffin cushion (84:16) and cutting off the tip of the tube for counting the radioactivity.

Results

TNF-mediated Activities on Murine T Cell Lines Show a Strong Species Specificity. We tested a panel of murine T cell lines for their responsiveness to mTNF and hTNF. LBRM-33-1A5, a murine T cell lymphoma (34), and NOB-1, a murine thymoma (35), were tested for TNF-mediated induction of IL-2; PC60 cells, a rat/mouse T hybridoma (26), were tested for TNF-driven GM-CSF secretion, and CT6 cells (25) were tested for TNF-dependent proliferation. In all four T cell lines the specific biological activity of mTNF was between 100- and 500-fold higher than that of hTNF, whereas the cytotoxic activity on L929 cells of the same TNF preparations only indicated a three-fold difference. Although we did not detect specific binding with ^{125}I-hTNF in these murine T cells, hTNF exerted some minor bioactivity (Table 1). In contrast to Ranges et al. (25), we observed some minor biological activity of hTNF on CT6 proliferation. These observations most probably reflect a very low expression of endogenous

Table 1. *Specific Biological Activities and Binding of mTNF and hTNF on Several Murine T Cell Lines*

Cells	Assay system	Specific biological activity		Binding	
		mTNF	hTNF	^{125}I-mTNF	^{125}I-hTNF
		U/mg		*M*	
L929	Cytotoxicity	7.7×10^7	2.2×10^7	5.3×10^{-11} (217)	8.0×10^{-10} (383)
WE17/10	-	-	-	1.6×10^{-10} (636)	1.0×10^{-10} (757)
PC60	GM-CSF induction	3.3×10^4	2.5×10^2	5.0×10^{-11} (430)	No binding*
LBRM	IL-2 induction	1.0×10^5	1.0×10^3	1.3×10^{-11} (605)	No binding*
NOB-1	IL-2 induction	5.0×10^5	1.0×10^3	3.2×10^{-11} (1,092)	No binding*
CT6	Proliferation	5.0×10^5	1.0×10^3	2.0×10^{-11} (1,000)‡	No binding‡

TNF-mediated L929 cytotoxicity was performed in the presence of 1 μg/ml actinomycin D. 18 h later, viability was measured by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 53). GM-CSF secretion by PC60 cells was determined as described in Fig. 3. IL-2 induction in LBRM of NOB-1 cells was described previously (32). CT6 proliferation was performed as described (25). Specific biological activities were calculated on the basis of the half-maximal response induced by mTNF or hTNF. Corresponding amounts on mTNF and hTNF response curves are defined as 1 U/ml (see also Fig. 3). Characterization of the TNF-R by binding: K_d-values and number of TNF-binding sites (in parentheses) were calculated from Scatchard analyses of specific binding data.

* Concentrations up to 5 nM ^{125}I-hTNF were used.

‡ Data taken from reference 25.

mTNF-R55 molecules, which are not detected in the binding assays. This conclusion is supported by the fact that long exposure of Northern blots revealed very low TNF-R55 mRNA levels in CT6 cells (38). The strongly reduced bioactivity and the apparent absence of specific binding of hTNF on these murine T cell lines thus can be explained by the species specificity of mTNF-R75 (13, 38). The similar binding of iodinated mTNF and hTNF on WE17/10 cells, a TNF-R55$^-$, and TNF-R75$^+$ human T cell line (determined by flow fluorocytometric analysis with htr-9 and utr-1 mAbs, respectively; data not shown) (36), reflects the absence of species preference in the human system.

Transfection of hTNF-R75 cDNA in PC60 Cells. PC60 cells were cotransfected with pSV25S-HTNF75, coding for the hTNF-R75 under the SV40 early promoter control, and pBSΔppac, a puromycin-based selection system. In Fig. 1, we show the binding of utr-1, a mAb directed against an extracellular epitope of hTNF-p75, to a representative transfected PC60 subclone (PC60 cl26). Scatchard plots on transfected PC60 cl26 cells both with ^{125}I-hTNF and ^{125}I-mTNF reveal the presence of 5,180 and 5,640 receptors/cell, respectively, and a dissociation constant of 189 and 233 pM (Fig. 2 *B*). These results indicate that the affinity of the transfected gene product is equal to that of natural hTNF-R75, for example, on WE17/10 cells (see Table 1). Parental PC60 cells did not show specific binding with the same ^{125}I-hTNF preparations, while Scatchard plots based on ^{125}I-mTNF binding indicated the presence of 285 high affinity binding sites (45 pM) (Fig. 2 *A*).

PC60 Cells Transfected with hTNF-R75 Respond to mTNF and hTNF by Secretion of GM-CSF. The functionality of the transfected hTNF-R75 was studied in the PC60 subclone cl26. Other subclones had similar responses, although of various magnitudes. Rat GM-CSF secretion was assayed, because it was identified as a major cytokine produced by PC60 cells in response to TNF (or IL-1) stimulation (32). In Fig. 3, the capacities of mTNF and hTNF to induce GM-CSF secretion in parental PC60 or in transfected PC60 cl26 cells are compared. The specific biological activity of hTNF on parental PC60 cells is ~100-fold lower than that of mTNF, but the bioactivities of both TNF species are almost equal in PC60 cl26 cells (note that the scales in Fig. 3 are logarithmic). The much higher levels of TNF-mediated GM-CSF induction in PC60 cl26 cells are most probably correlated with the enhanced TNF-R expression (see Fig. 2), since the TNF responses in other transfected PC60 clones were also increased (data not shown).

TNF and hIL-1 Synergize in the Induction of GM-CSF Secretion. IL-1 and IL-2 promote the optimal induction of rat GM-CSF (32) and differentiation of PC60 cells to CTL (39). We therefore investigated whether addition of these cytokines might also enhance the TNF-mediated responses illustrated in Fig. 3. The induction of GM-CSF secretion by saturating concentrations of hIL-1β (1 ng/ml) in parental and transfected PC60 cells proved to be strongly synergistic with TNF. The synergism of TNF/IL-1 in PC60 cl26 cells in most experiments was not affected by the addition of hIL-2 (100 IU/ml) (Table 2); parental PC60 cells, however, generally showed a twofold enhancement of the TNF/IL-1-induced GM-CSF levels in the presence of IL-2 (Table 2). None of the stimulation conditions of the PC60 cells influenced the subsequent quantification of GM-CSF in FDCp1 cell assays (data not shown).

To examine mutual influence of IL-1 and TNF, serial dilutions of hTNF and hIL-1β in transfected PC60 cells were studied in a checkerboard pattern. Half-maximal GM-CSF

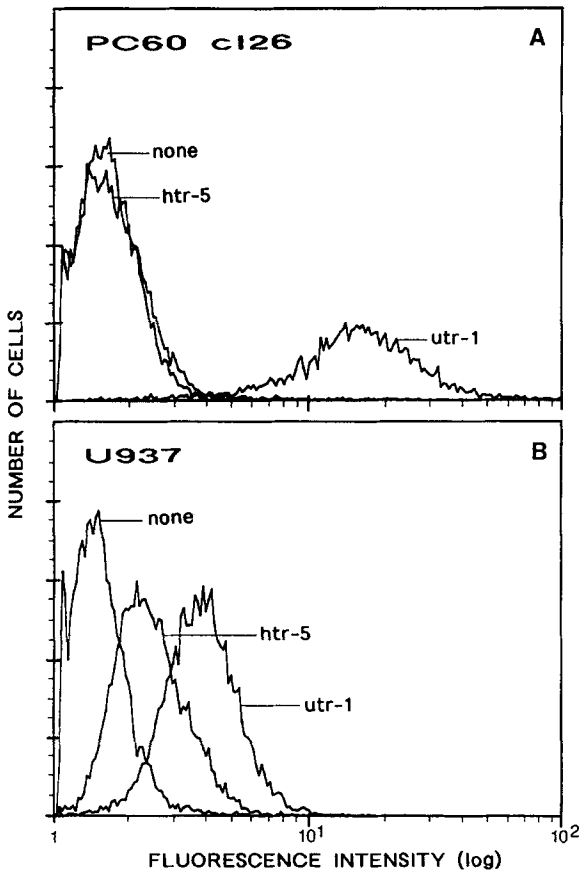

Figure 1. Binding of utr-1 to PC60 cl26 cells (*A*). Flow fluorocytometric analysis of cells stained with utr-1 (2 μg/ml) and fluorescein-conjugated SAM (1:100 diluted). Utr-1 staining is compared with htr-5 staining and with second antibody alone as negative controls. For comparison, utr-1 and htr-5 binding to U937 cells is also displayed (*B*).

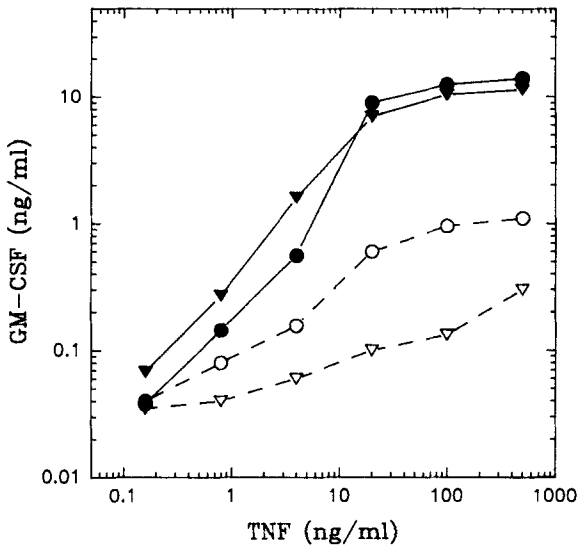

Figure 3. TNF activity on parental and transfected PC60 cells. Parental PC60 cells (*open symbols*) and transfected PC60 cl26 cells (*filled symbols*) were incubated for 24 h at 5 × 10^4 cells/well in the presence of a serial dilution of hTNF (▽, ▼) or mTNF (○, ●). GM-CSF activity in the supernatant is expressed as ng/ml per 10^6 cells. Note that both scales are logarithmic.

induction in the absence of hIL-1β is reached at ~30 ng/ml hTNF (Fig. 4 *A*). The dose dependence of GM-CSF induction at constant hTNF or IL-1β concentration and increasing amounts of hIL-1β or TNF, respectively, is shown in Fig. 4.

Anti-TNF-R75 mAbs Inhibit hTNF-mediated GM-CSF Secretion. To confirm that the transfected hTNF-R75 in PC60 cl26 was functionally active, we tested whether hTNF-mediated

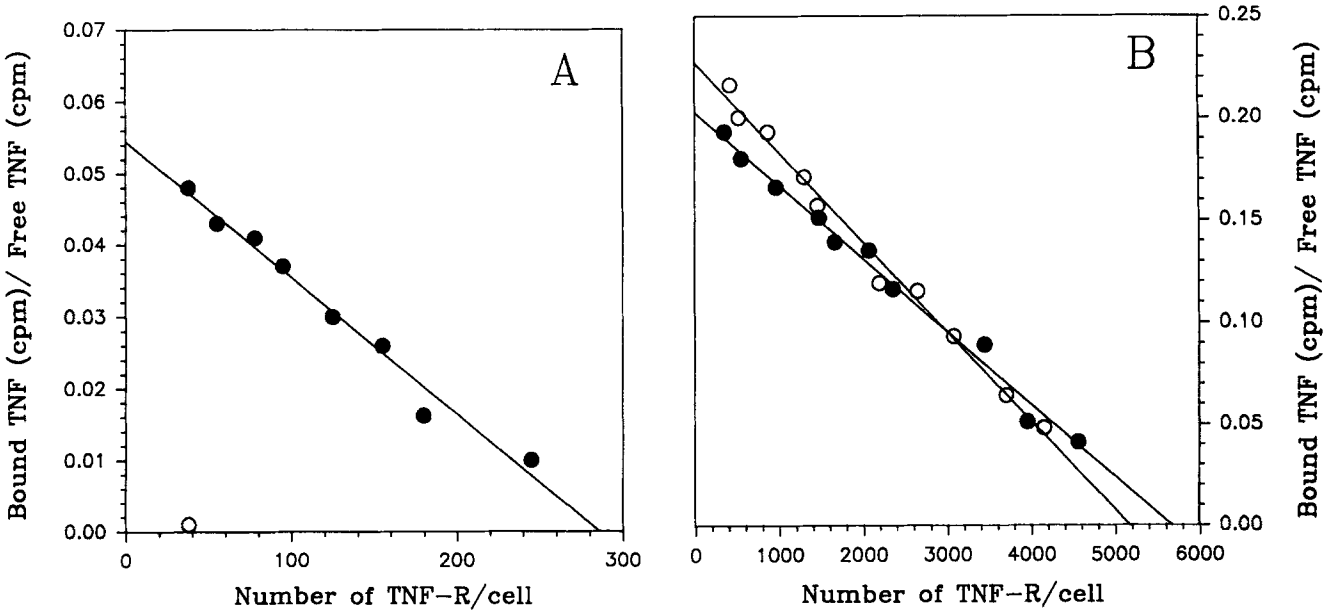

Figure 2. Specific binding of ^{125}I-labeled mTNF (●) and ^{125}I-labeled hTNF (○) on parental PC60 cells (*A*) and on transfected PC60 cl26 cells (*B*). Stably transfected PC60 cl26 cells were incubated with increased concentrations of ^{125}I-labeled TNF alone or with excess unlabeled TNF. Data were represented as Scatchard analyses.

Table 2. *Synergism between TNF and IL-1 and/or IL-2 to Induce GM-CSF*

	GM-CSF activity							
	Parental PC60 cells				PC60 cl26 cells			
TNF	Control	IL-2	IL-1	IL-1/IL-2	Control	IL-2	IL-1	IL-1/IL-2
ng/ml	*ng/ml per 10^6 cells*							
	0.025	0.04	2.9	4.4	0.04	0.04	19	21
mTNF								
0.16	0.03	0.06	2.6	4.2	0.04	0.06	18	22
0.8	0.08	0.12	2.8	4.6	0.14	0.44	20	25
4	0.16	0.36	3.9	5.1	0.6	2.2	25	50
20	0.6	2.2	7.5	8.3	9	18	125	110
100	1.0	3	10	14.0	13	25	140	140
500	1.1	3	9	16.5	14	23	180	155
hTNF								
0.16	0.025	0.05	3.0	4.7	0.07	0.16	22	18
0.8	0.04	0.04	2.8	4.5	0.27	0.88	28	28
4	0.06	0.08	2.9	4.5	2	3.1	65	90
20	0.10	0.22	3.8	4.3	5	13	138	156
100	0.13	0.3	3.3	7.5	9	16	138	156
500	0.3	0.64	6.5	9.0	8	19	138	156

3 × 10^4 cells/well were incubated in the presence of a serial dilution of TNF (500–0.16 ng/ml) with or without IL-1 (1 ng/ml) and/or IL-2 (100 IU/ml. After 24 h, GM-CSF activity was determined in the supernatant. SD on these induction experiments was <10%.

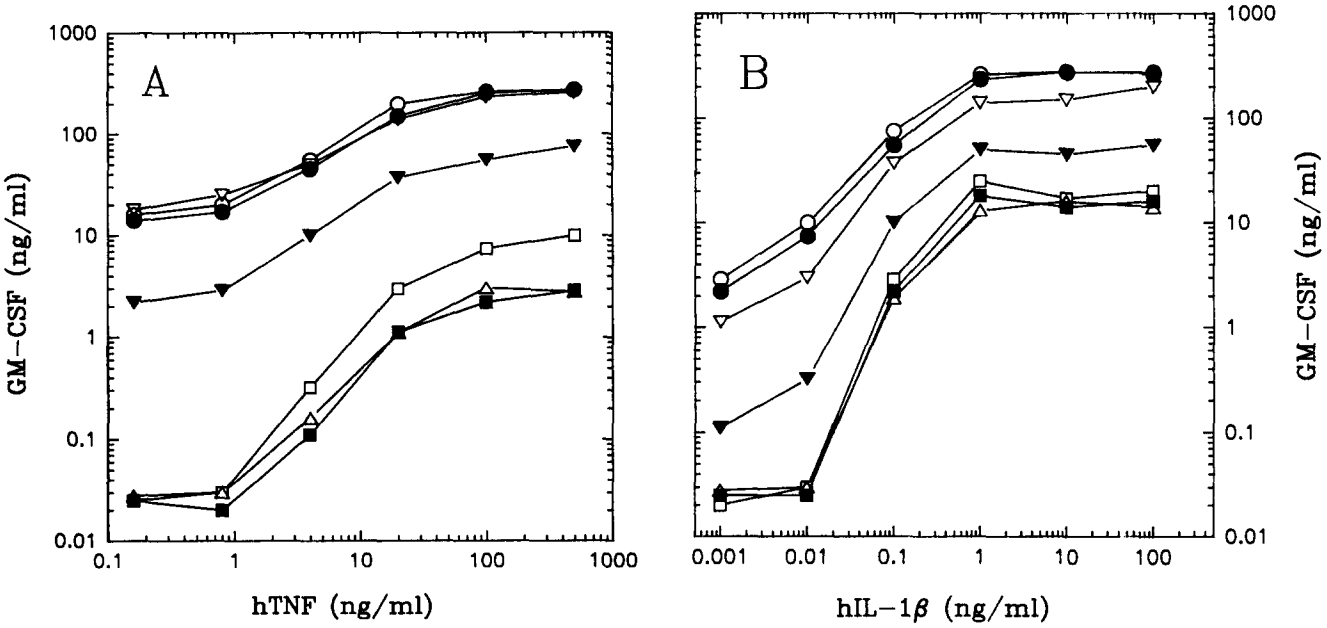

Figure 4. Synergism between IL-1 and TNF on transfected PC60 cells. PC60 cl26 cells were incubated for 24 h at 5 × 10^4 cells/well in the presence of a serial dilution of hTNF and constant concentrations of hIL-1β (100 ng/ml [○]; 10 ng/ml [●]; 1 ng/ml [▽]; 100 pg/ml [▼]; 10 pg/ml [□]; 1 pg/ml [■]; no IL-1 [△]) (*A*) and in the presence of a serial dilution of hIL-1β and constant concentrations of hTNF (500 ng/ml [○]; 100 ng/ml [●]; 20 ng/ml [▽]; 4 ng/ml [▼]; 0.8 ng/ml [□]; 0.16 ng/ml [■]; no TNF [△]) (*B*). The amount of GM-CSF secreted in the absence of TNF and IL-1 was 0.030 ng/ml. Note that both scales are logarithmic.

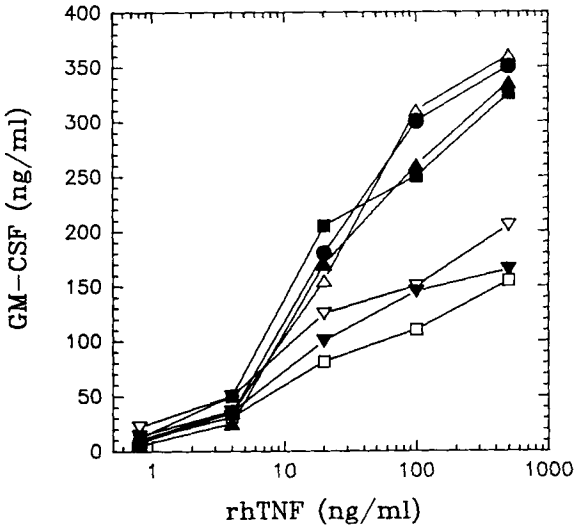

Figure 5. Inhibition of hTNF-dependent GM-CSF secretion by mAb against hTNF-R75. 3 × 10^4 cells/well were pretreated for 1 h at 4°C in the presence of utr-1 (▽), utr-2 (▼), utr-3 (□), utr-4 (■), utr-10 (△), htr-9 (▲), and on antibodies (●). Antibodies were used at 5 μg/ml. Serial dilutions of hTNF were added in the presence of a constant amount of hIL-1β (1 ng/ml). 24 h later, the supernatant was tested for GM-CSF activity. The addition of utr-1, utr-2, or utr-3 in the presence of hIL-1β was slightly agonistic, as clearly shown in Table 3 (even in the absence of a crosslinking second antibody).

GM-CSF induction could be inhibited by mAbs against hTNF-R75 (utr-1, utr-2, utr-3, utr-4, utr-10). Since the half-maximal induction in the assay system required 20–30 ng/ml TNF (~400–600 nM, based on the M_r of trimeric TNF), the inhibition by the neutralizing mAbs utr-1, utr-2, and utr-3 (8) at 5 μg/ml (~50-fold molar excess) was only partial and not always reproduced (Fig. 5). However, the nonneutralizing antibodies utr-4 and utr-10 never affected the hTNF-dependent GM-CSF induction in hTNF-R75-transfected PC60 cells.

Neutralizing Anti-TNF-R75 mAbs Gain Agonistic Activity After Crosslinking. To demonstrate signal transduction via hTNF-R75 in PC60 cl26 cells, we explored conditions under which anti-hTNF-R75 mAbs gained agonistic activities. None of the mAbs were agonistic on their own. However, in the presence of hIL-1β, utr-1, utr-2, and utr-3 showed minor agonistic activity (see Table 3). But crosslinking of these neutralizing anti-TNF-R75 mAbs by SAM significantly enhanced the response, whereas crosslinking of the nonneutralizing mAbs utr-4 and utr-10 hardly had any effect (Tables 3 and 4). However, even under optimal conditions, antibody-mediated GM-CSF secretion was always lower than that elicited by TNF, even when the three agonistic mAbs were combined. These results clearly demonstrate that TNF activity can be mimicked by crosslinking anti-TNF-R75 mAbs, indicating that, indeed, transfected TNF-R75 is fully functional and that clustering plays a key role also in TNF-R75-mediated signal transduction.

Discussion

The molecular cloning and expression of both mTNF-Rs revealed that mTNF, but not hTNF, binds to mTNF-R75 (13). This undoubtedly is the reason for the various species-specific bioactivities of hTNF observed on murine T cells (23, 24,

Table 3. *Effect of mAbs against TNF-R75 on the Induction of GM-CSF in PC60 cl26 Cells*

	GM-CSF activity			
	No IL-1		1 ng/ml IL-1	
SAM	−	+	−	+
	ng/ml per 10^6 cells			
Control	0.08 (0.02)	0.11 (0.06)	2.0 (0.4)	2.2 (0.4)
mTNF	3.63 (0.44)	3.97 (0.57)	1,088 (106)	972 (155)
hTNF	3.13 (0.61)	3.24 (0.35)	1,004 (86)	966 (79)
utr-1	0.10 (0.02)	0.39 (0.04)	21 (3.5)	164 (14)
utr-2	0.08 (0.02)	0.39 (0.04)	12 (5.8)	134 (45)
utr-3	0.12 (0.02)	0.14 (0.02)	16 (1.8)	96 (14)
utr-1/2/3	0.10 (0.05)	0.34 (0.03)	28 (59)	154 (13)
utr-4	0.09 (0.02)	0.10 (0.02)	2.0 (0.4)	13 (2)
utr-10	0.07 (0.02)	0.09 (0.02)	2.5 (0.2)	4.9 (0.4)
htr-9	0.09 (0.01)	0.10 (0.01)	1.7 (0.5)	2.2 (0.3)

5 × 10^4 cells/well were preincubated for 1 h at 4°C in the presence of anti-hTNF-R75 mAbs. Then, SAM and hIL-1β (1 ng/ml) were added. Optimized ratios between utr and SAM were deduced from data represented in Table 4. Utr-1, 1.25 μg/ml: SAM, 2.5 μg/ml; utr-2, 1.25 μg/ml: SAM, 2.5 μg/ml; utr-3, 2.5 μg/ml: SAM, 2.5 μg/ml; utr-4, 5 μg/ml: SAM, 2.5 μg/ml; utr-10, 5 μg/ml: SAM 2.5 μg/ml; and htr-9, 5 μg/ml: SAM, 2.5 μg/ml. Combined addition of utr-1, -2, and -3 was performed at 1.25, 1.25, and 2.5 μg/ml, respectively, in the presence of 5 μg/ml SAM. After 24 h, GM-CSF activity was measured. SD is in parentheses.

Table 4. *Effect of Crosslinking SAM on Anti-TNF-R75-mediated GM-CSF Production in PC60 c126 Cells*

		Utr-1 (μg/ml)					Utr-2 (μg/ml)					Utr-3 (μg/ml)				
SAM	–	0.312	0.625	1.25	2.5	5	0.312	0.625	1.25	2.5	5	0.312	0.625	1.25	2.5	5
0	2.4	14	15	22	28	26	5	8	14	15	15	3.8	4.8	4.8	19	22
0.312	2.8	24	28	43	31	31	22	20	25	22	28	7.5	15	15	24	24
0.625	2.8	50	59	69	38	38	38	43	38	28	38	6.3	18	24	43	43
1.25	2.2	43	94	88	75	75	50	88	88	50	81	2.0	12	31	75	69
2.5	2.4	16	81	156	109	88	31	94	125	88	94	3.0	3.3	24	109	109
5	3.3	5.6	17.5	188	188	156	5	88	200	141	156	2.0	3.0	5.0	88	141
		Utr-4 (μg/ml)					Utr-10 (μg/ml)					Htr-9 (μg/ml)				
SAM	–	0.312	0.625	1.25	2.5	5	0.312	0.625	1.25	2.5	5	0.312	0.625	1.25	2.5	5
0	2.4	1.5	2.0	2.2	2.4	1.2	3.0	2.8	3.2	3.0	3.3	2.4	3.0	2.7	3.3	2.4
0.312	2.4	1.5	3.0	4.4	7.5	10.0	2.3	3.0	3.5	3.8	3.8	2.4	2.8	2.4	1.7	2.4
0.625	2.8	2.4	2.0	5.0	11.9	13.8	1.6	2.2	3.8	4.4	5.6	1.6	2.8	1.6	2.0	2.0
1.25	2.0	1.5	1.9	2.2	10.0	18.8	3.3	2.2	3.8	5.0	6.6	3.0	2.8	2.0	1.5	1.5
2.5	2.4	1.7	1.8	2.0	3.5	16.3	3.5	2.0	2.0	3.5	6.3	3.3	2.4	2.4	1.7	1.5
5	3.3	1.6	1.5	2.0	2.0	4.8	3.3	3.0	3.0	3.5	4.8	2.0	2.8	2.4	2.4	2.0

5×10^4 cells/well were preincubated for 1 h at 4°C in the presence of serial dilutions of anti-hTNF-R75 mAbs (utr-1, 2, 3, 4, 10) and, as a control, of an anti-hTNF-R55 mAb (htr-9). A serial dilution of SAM and a constant amount of hIL-1β (1 ng/ml) were added. After 24 h, GM-CSF activity was measured. Each value represents the mean of three replicates; SD was <10%. mTNF and hTNF, in the presence of IL-1, induced 916 and 880 ng/ml GM-CSF, respectively.

40, 41). It also explains the 100–500-fold lower specific biological activity of hTNF, and the lack of binding of ^{125}I-hTNF, on the LBRM-33-1A5, NOB-1, PC60, and CT6 cell lines, since TNF-R75 is the predominant or only receptor type expressed on these cells (13, 38).

PC60 cells are derived from a cross between an IL-2-dependent murine CTL line and a rat thymoma (26). Previous studies have shown that IL-1 combined with IL-2 induce these cells to become cytolytic and strongly increase the expression of a number of T cell–specific genes, including IL-2Rα (39), IL-6, and rat GM-CSF (32). These genes are also inducible by mTNF, but not by hTNF (23, 32). The finding that upon transfection of hTNF-R75 cDNA, hTNF also induces GM-CSF secretion in PC60 cells indicates that the human receptor is capable of functionally interacting with the PC60 signal transduction pathways. Remarkably, although hTNF-R75$^+$-transfected cells express a 20-fold higher number of TNF-R (~5,600/cell) than parental PC60 cells (285/cell), both show similar specific biological activities for mTNF. This demonstrates that the sensitivity of the TNF response is not altered by the number of TNF-R on the cell surface. However, the level of TNF-dependent GM-CSF induction in the transfected PC60 cells is ~10-fold higher. This is not a property of a particular cell clone, as it has also been observed in most other hTNF-R75 transfectants. Hence, the magnitude of the response seems to correlate with the number of receptors per cell.

The induction of GM-CSF in PC60 cells is an example of the many biological activities shared between IL-1 and TNF (42). However, compared on a molar basis, ~100-fold more TNF than IL-1 is required to induce similar levels of GM-CSF secretion. Analogous differences in specific biological activity between IL-1 and TNF have also been observed in other IL-1-mediated systems, such as the lymphocyte-activating factor (LAF) assay (24, 40), as well as the D10.G4.1 (43), EL-4 NOB-1, and LBRM-33-1A5 assays (33). Possibly, TNF-dependent T cell responses are only physiologically relevant under conditions of fairly high local TNF concentrations, such as those obtained in septic shock (44) and in meningococcal meningitis (45). Although the combination of IL-1 and TNF was strongly synergistic for the induction of GM-CSF secretion, a constant amount of IL-1 did not increase the sensitivity for TNF bioactivity; its specific biological activity remained at $3.3–5.0 \times 10^4$ U/mg. The augmentation of TNF-dependent GM-CSF secretion in the presence of IL-1 is, most probably, due to synergism between both intracellular signal transduction pathways. It is worthwhile to note that the combined addition of IL-1 and TNF is able to elicit GM-CSF levels of > 1μg/ml per 10^6 cells over 24 h. A similar response-amplifying mechanism might form the basis for the severe in vivo toxicity when low nonlethal doses of TNF are administered together with IL-1 (46, 47).

The trimeric structure of TNF (48–50), and the identification of three receptor-binding sites per native molecule

(51), already suggested that TNF-R-mediated signal transduction might result from ligand-dependent crosslinking. In the case of TNF-R55-mediated biological activities, this conclusion was supported by the finding of agonistically acting anti-hTNF-R55 polyclonal antisera and mAbs (17–19, 28, 29, 52), demonstrating that aggregation of TNF-R55 by itself is already sufficient to initiate intracellular signal transduction. PC60 cells transfected with hTNF-R75 allowed us to investigate the specific role of TNF-R75. The absence of a contribution from TNF-R55-mediated signals was indicated by the agonistic properties of several anti-hTNF-R75 mAbs (utr-1, utr-2, utr-3). However, it is intriguing that antibody-mediated clustering of TNF-R75 by itself was not sufficient to elicit biological response, but required synergistically acting IL-1. This suggests that TNF-R75-mediated intracellular signal pathways need the cooperation of other cytokine receptor–triggered pathways, such as IL-1R and/or TNF-R55 (see below). This might explain the fact that other investigators found an involvement of TNF-R75 by neutralization experiments, but did not observe agonistic activity with the utr-1 mAb (17, 19, 52). Furthermore, even enhanced crosslinking by polyclonal anti–mouse Ig antibodies resulted in only 10–20% of the response generated in the presence of TNF and IL-1. The latter observation might suggest that adequate triggering requires a trimeric configuration of TNF-R75 molecules, which is less efficiently reached with bivalent antibodies. Alternatively, TNF, besides its interaction with transfected hTNF-R75, may also trigger some rare, endogenous, and cooperatively acting TNF-R55 molecules. In this respect, one may refer to the hypothesis that TNF-R75 somehow facilitates TNF interaction with TNF-R55 (20). It is also quite remarkable that only neutralizing mAbs (utr-1, utr-2, utr-3) were able to mimic TNF effects. This suggests that neutralizing epitopes and agonistic epitopes are superimposable or topologically correlated. Nonneutralizing mAbs (utr-4, utr-10) were not or hardly able to evoke GM-CSF induction.

The present report provides direct evidence for a functional role of the hTNF-R75 in TNF-mediated cytokine production in a rat/mouse hybridoma. Anti-hTNF-R75 mAbs were strongly agonistic when crosslinked in the presence of IL-1. Our results further demonstrate that transfection of hTNF-R75 in PC60 cells is sufficient to overcome the species specificity of hTNF. Cotransfection of hTNF-R55 and hTNF-R75 in PC60 cells, and the use of TNF-mimicking mAbs against TNF-R55 (htr-1, htr-9; 18) and/or TNF-R75, will allow the dissection of intracellular signaling pathways initiated by either of the two receptors, and their possible interactions. This may contribute to a better understanding of the functional significance of the two intracellular domains, which are totally unrelated in sequence (12).

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References

1. Beutler, B., and A. Cerami. 1989. The biology of cachectin/TNF: a primary mediator of the host response. *Annu. Rev. Immunol.* 7:625.
2. Old, L.J. 1990. Tumor necrosis factor. *In* Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy. B. Bonavida and G. Granger, editors. Karger, Basel. 1–30.
3. Vilček, J., and T.H. Lee. 1991. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* 266:7313.
4. Aggarwal, B.B. (Guest Editor). 1991. Tumor necrosis factor. *Biotherapy.* 3:103.
5. Fiers, W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:199.
6. Fiers, W., P. Brouckaert, R. Devos, L. Fransen, G. Leroux-Roels, E. Remaut, P. Suffys, J. Tavernier, J. Van der Heyden, and F. Van Roy. 1986. Lymphokines and monokines in anticancer therapy. *In* Molecular Biology of Homo sapiens. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 587–595.
7. Hohmann, H.P., R. Remy, M. Brockhaus, and A.P.G.M. Van Loon. 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNFα). *J. Biol. Chem.* 264:14927.
8. Brockhaus, M., H.-J. Schoenfeld, E.-J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher. 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 87:3127.
9. Loetscher, H., Y.E. Pan, H.W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell.* 61:351.
10. Schall, T.J., M. Lewis, K.J. Koller, A. Lee, G.C. Rice, G.H.W. Wong, T. Gatanaga, G.A. Granger, R. Lentz, H. Raab, W.J. Kohr, and D.V. Goeddel. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell.* 61:361.

11. Smith, C.A., T. Davis, D. Anderson, L. Solam, M.P. Beckmann, R. Jerzy, S.K. Dower, D. Cosman, and R.G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science (Wash. DC).* 248: 1019.
12. Dembic, Z., H. Loetscher, U. Gubler, Y.E. Pan, H.-W. Lahm, R. Gentz, M. Brockhaus, and W. Lesslauer. 1990. Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. *Cytokine.* 2:231.
13. Lewis, M., L.A. Tartaglia, A. Lee, G.L. Bennett, G.C. Rice, G.H.W. Wong, E.Y. Chen, and D.V. Goeddel. 1991. Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc. Natl. Acad. Sci. USA.* 88:2830.
14. Goodwin, R.G., D. Anderson, R. Jerzy, T. Davis, C.I. Brannan, N.G. Copeland, N.A. Jenkins, and C.A. Smith. 1991. Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. *Mol. Cell. Biol.* 11:3020.
15. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233.
16. Loetscher, H., M. Steinmetz, and W. Lesslauer. 1991. Tumor necrosis factor: receptors and inhibitors. *Cancer Cells (Cold Spring Harbor).* 3:221.
17. Engelmann, H., H. Holtmann, C. Brakebusch, Y. Shemer Avni, I. Sarov, Y. Nophar, E. Hadas, O. Leitner, and D. Wallach. 1990. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem.* 265:14497.
18. Espevik, T., M. Brockhaus, H. Loetscher, U. Nonstad, and R. Shalaby. 1990. Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. *J. Exp. Med.* 171:415.
19. Shalaby, M.R., A. Sundan, H. Loetscher, M. Brockhaus, W. Lesslauer, and T. Espevik. 1990. Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med.* 172:1517.
20. Tartaglia, L.A., and D.V. Goeddel. 1992. Two TNF receptors receptors. *Immunol. Today.* 13:151.
21. Thoma, B., M. Grell, K. Pfizenmaier, and P. Scheurich. 1990. Identification of a 60-kD tumor necrosis factor (TNF) receptor as the major signal transducing component in TNF responses. *J. Exp. Med.* 172:1019.
22. Erikstein, B.K., E.B. Smeland, H.K. Blomhoff, S. Funderud, K. Prydz, W. Lesslauer, and T. Espevik. 1991. Independent regulation of 55-kDa and 75-kDa tumor necrosis factor receptors during activation of human peripheral blood B lymphocytes. *Eur. J. Immunol.* 21:1033.
23. Plaetinck, G., W. Declercq, J. Tavernier, M. Nabholz, and W. Fiers. 1987. Recombinant tumor necrosis factor can induce interleukin 2 receptor expression and cytolytic activity in a rat × mouse T cell hybrid. *Eur. J. Immunol.* 17:1835.
24. Ehrke, M.J., R.L.X. Ho, and K. Hori. 1988. Species-specific TNF induction of thymocyte proliferation. *Cancer Immunol. Immunother.* 27:103.
25. Ranges, G.E., M.P. Bombara, R.A. Aiyer, G.G. Rice, and M.A. Palladino, Jr. 1989. Tumor necrosis factor-α as a proliferative signal for an IL-2-dependent T cell line: Strict species specificity of action. *J. Immunol.* 142:1203.
26. Conzelmann, A., P. Corthésy, M. Cianfriglia, A. Silva, and M. Nabholz. 1982. Hybrids between rat lymphoma and mouse T cells with inducible cytolytic activity. *Nature (Lond.).* 298:170.
27. Naume, B., R. Shalaby, W. Lesslauer, and T. Espevik. 1991. Involvement of the 55- and 75-kDa tumor necrosis factor receptors in the generation of lymphokine-activated killer cell activity and proliferation of natural killer cells. *J. Immunol.* 146:3045.
28. Tartaglia, L.A., R.F. Weber, I.S. Figari, C. Reynolds, M.A. Palladino, Jr., and D.V. Goeddel. 1991. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc. Natl. Acad. Sci. USA.* 88:9292.
29. Gehr, G., R. Gentz, M. Brockhaus, H. Loetscher, and W. Lesslauer. 1992. Both tumor necrosis factor receptor types mediate proliferative signals in human mononuclear cell activation. *J. Immunol.* 149:In press.
30. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods.* 95:99.
31. DeLamarter, J.F., J.-J. Mermod, C.-M. Liang, J.F. Eliason, and D.R. Thatcher. 1985. Recombinant murine GM-CSF from E. coli has biological activity and is neutralized by a specific antiserum. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2575.
32. Vandenabeele, P., Y. Guisez, W. Declercq, G. Bauw, J. Vandekerckhove, and W. Fiers. 1990. Response of murine cell lines to an IL-1/IL-2-induced factor in a rat/mouse T hybridoma (PC60): differential induction of cytokines by human IL-1α and IL-1β and partial amino acid sequence of rat GM-CSF. *Lymphokine Res.* 9:381.
33. Vandenabeele, P., W. Declercq, C. Libert, and W. Fiers. 1990. Development of a simple, sensitive and specific bioassay for interleukin-1 based on the proliferation of RPMI 1788 cells. Comparison with other bioassays for IL-1. *J. Immunol. Methods.* 135:25.
34. Conlon, P.J. 1983. A rapid biological assay for the detection of interleukin 1. *J. Immunol.* 131:1280.
35. Gearing, A.J.H., C.R. Bird, A. Bristow, S. Poole, and R. Thorpe. 1987. A simple sensitive bioassay for interleukin-1 which is unresponsive to 103 U/ml of interleukin-2. *J. Immunol. Methods.* 99:7.
36. Willard-Gallo, K.E., F. Van de Keere, and R. Kettmann. 1990. A specific defect in CD3 γ-chain gene transcription results in loss of T-cell receptor/CD3 expression late after human immunodeficiency virus infection of a CD4$^+$ T-cell line. *Proc. Natl. Acad. Sci. USA.* 87:6713.
37. de la Luna, S., I. Soria, D. Pulido, J. Ortín, and A. Jiménez. 1988. Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. *Gene (Amst.).* 62:121.
38. Barrett, K., D.A. Taylor-Fishwick, A.P. Cope, A.M. Kissonerghis, P.W. Gray, M. Feldmann, and B.M.J. Foxwell. 1991. Cloning, expression, and cross-linking analysis of the murine p55 tumor necrosis factor receptor. *Eur. J. Immunol.* 21:1649.
39. Erard, P., P. Corthésy, K.A. Smith, W. Fiers, A. Conzelmann, and M. Nabholz. 1984. Characterization of soluble factors that induce the cytolytic activity and the expression of T cell growth factor receptors of a T cell hybrid. *J. Exp. Med.* 160:584.
40. Ranges, G.E., A. Zlotnik, T. Espevik, C.A. Dinarello, A. Cerami, and M.A. Palladino, Jr. 1988. Tumor necrosis factor α/cachectin is a growth factor for thymocytes. Synergistic interactions with other cytokines. *J. Exp. Med.* 167:1472.
41. Kramer, S.M., B.B. Aggarwal, T.E. Eessalu, S.M. McCabe, B.L. Ferraiolo, I.S. Figari, and M.A. Palladino, Jr. 1988. Characterization of the *in vitro* and *in vivo* species preference of human and murine tumor necrosis factor-α. *Cancer Res.* 48:920.

42. Oppenheim, J.J., K. Matsushima, T. Yoshimura, E.J. Leonard, and R. Neta. 1989. Relationship between interleukin 1 (IL1), tumor necrosis factor (TNF) and a neutrophil attracting peptide (NAP-1). *Agents Actions.* 26:134.
43. Hopkins, S.J., and M. Humphreys. 1989. Simple, sensitive and specific bioassay of interleukin-1. *J. Immunol. Methods.* 120:271.
44. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin-TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.).* 330:662.
45. Waage, A., A. Halstensen, R. Shalaby, P. Brandtzaeg, P. Kierulf, and T. Espevik. 1989. Local production of tumor necrosis factor α, interleukin 1, and interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. *J. Exp. Med.* 170:1859.
46. Waage, A., and T. Espevik. 1988. Interleukin 1 potentiates the lethal effect of tumor necrosis factor α/cachectin in mice. *J. Exp. Med.* 167:1987.
47. Everaerdt, B., P. Brouckaert, A. Shaw, and W. Fiers. 1989. Four different interleukin-1 species sensitize to the lethal action of tumour necrosis factor. *Biochem. Biophys. Res. Commun.* 163:378.
48. Arakawa, T., and D.A. Yphantis. 1987. Molecular weight of recombinant human tumor necrosis factor-α. *J. Biol. Chem.* 262:7484.
49. Wingfield, P., R.H. Pain, and S. Craig. 1987. Tumour necrosis factor is a compact trimer. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 211:179.
50. Lewit-Bentley, A., R. Fourme, R. Kahn, T. Prangé, P. Vachette, J. Tavernier, G. Hauquier, and W. Fiers. 1988. Structure of tumour necrosis factor by X-ray solution scattering and preliminary studies by single crystal X-ray diffraction. *J. Mol. Biol.* 199:389.
51. Van Ostade, X., J. Tavernier, T. Prangé, and W. Fiers. 1991. Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:827.
52. Hohmann, H.-P., M. Brockhaus, P.A. Baeuerle, R. Remy, R. Kolbeck, and A.P.G.M. Van Loon. 1990. Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-kB. TNFα is not needed for induction of a biological effect via TNF receptors. *J. Biol. Chem.* 265:22409.
53. Tada, H., O. Shiho, K. Kuroshima, M. Koyama, and K. Tsukamoto. 1986. An improved colorimetric assay for interleukin 2. *J. Immunol. Methods.* 93:157.