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)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,
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 cross-linking 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 FDCp proliferation assay (31, 32). Recombinant hIL-1β (5 × 10^6 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-R75 (utr-1, utr-2, utr-3, utr-4, utr-10) and anti-hTNF-R55 (utr-9) mAb were used at appropriated concentrations. 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-mlg (SAM) (Sera-Lab, Crawley Down, UK) was freed of NaN3 by dialysis against PBS.

Cells. The hybridoma PC60.21.14.4 (PC60) is derived from a fusion between an IL2-dependent murine CTL line B6.1S.F1 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 IL2-dependent murine cytotoxic T cell line (25); WE17/10, an IL2-dependent FDCpl cells were grown in the same medium, but supplemented with 10% WEHI-3 supernatant as a source of mIg3. LBRM-33-1A5, a routine T cell lymphoma (34); NOB-1, a murine thymoma (35); CT6, an IL2-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 #g/ml), t-glutamine (2 mM), sodium pyruvate (1 mM), and 2-ME (5 × 10^-3 M). The factor-dependent FDCp cells were grown in the same medium, but supplemented with 10% WEHI-3 supernatant as a source of mIg3.

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 a selection plasmid for the PC60 cell transformation, we used pBSΔppac (37), which contains the gene for N-acetyl muramoyl 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 MgCl2 and CaCl2) 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 fluorometric 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 Fluorometry. Transfectants were stained for 30 min at 4°C with mAb against hTNF-R75 (0.4 μg utr-1/5 × 10^6 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.

Radio labeling of TNF. 125I-mTNF and 125I-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 CaCl2 or MgCl2 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 × 10^4 cells in a final vol of 1 ml (PBS-A, 0.5% BSA, 0.02% NaN3) 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 125I-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
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 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 to 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 pSV255-HTNF75, coding for the hTNF-R75 under the SV40 early promoter control, and pBSAppac, a puromycin-based selection system. In Fig. 1, we show the binding of 12SI-hTNF and 12SI-mTNF to a representative transfected PC60 subclone (PC60 c126). Scatchard plots on transfected PC60 c126 cells both with 125I-hTNF and 125I-mTNF reveal the presence of 5,180 and 5,640 receptors/cell, respectively, and a dissociation constant of 189 and 233 pM (Fig. 2 A). 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 125I-hTNF preparations, while Scatchard plots based on 125I-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 c126. 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 c126 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 c126 cells (note that the scales in Fig. 3 are logarithmic). The much higher levels of TNF-mediated GM-CSF induction in PC60 c126 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-1B (1 ng/ml) in parental and transfected PC60 cells proved to be strongly synergistic with TNF. The synergism of TNF/IL-1 in PC60 c126 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 hIL-1 and hIL-1B in transfected PC60 cells were studied in a checkerboard pattern. Half-maximal GM-CSF

### 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 | 125I-mTNF | 125I-hTNF |
| L929           | Cytotoxicity                       | 7.7 x 10⁶ | 2.2 x 10⁵ | 5.3 x 10⁻¹¹ (217) | 8.0 x 10⁻¹⁰ (383) |
| WE17/10        |                                   | 1.6 x 10⁻¹⁰ (636) | 1.0 x 10⁻¹⁰ (757) | No binding* |
| PC60           | GM-CSF induction                   | 3.3 x 10⁴ | 2.5 x 10³ | 5.0 x 10⁻¹¹ (430) | No binding* |
| LBRM           | IL-2 induction                     | 1.0 x 10⁴ | 1.0 x 10³ | 1.3 x 10⁻¹¹ (605) | No binding* |
| NOB-1          | IL-2 induction                     | 5.0 x 10⁴ | 1.0 x 10³ | 3.2 x 10⁻¹¹ (1,092) | No binding* |
| CT6            | Proliferation                      | 5.0 x 10⁴ | 1.0 x 10³ | 2.0 x 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-diphenyterazolium 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 of mTNF and hTNF response curves are defined as 1 U/ml (see also Fig. 3). Characterization of the TNF-R by binding: Kd-values and number of TNF-binding sites (in parentheses) were calculated from Scatchard analyses of specific binding data.

- Concentrations up to 5 nM 12SI-hTNF were used.
- Data taken from reference 25.
Figure 1. Binding of utr-1 to PC60 cl26 cells (A). Flow cytometric 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 2. Specific binding of 125I-labeled mTNF (●) and 125I-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 125I-labeled TNF alone or with excess unlabeled TNF. Data were represented as Scatchard analyses.

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 x 10^6 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
Table 2. Synergism between TNF and IL-1 and/or IL-2 to Induce GM-CSF

| Parental PC60 cells | PC60 cl26 cells |
|---------------------|----------------|
| TNF                 | GM-CSF activity |
|                     | Parental PC60 cells | PC60 cl26 cells |
|                     | Control | IL-2 | IL-1 | IL-1/IL-2 | Control | IL-2 | IL-1 | IL-1/IL-2 |
| ng/ml               | 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 x 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 x 10^4 cells/well in the presence of a serial dilution of hTNF and constant concentrations of hIL-1β (100 ng/ml [O]; 10 ng/ml [●]; 1 ng/ml [▼]; 100 pg/ml [▲]; 10 pg/ml [☐]; 1 pg/ml [■]; no IL-1 [A]) (A) and in the presence of a serial dilution of hIL-1β and constant concentrations of hTNF (500 ng/ml [O]; 100 ng/ml [●]; 20 ng/ml [▼]; 4 ng/ml [▲]; 0.8 ng/ml [☐]; 0.16 ng/ml [■]; no TNF [A]) (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 × 10⁴ cells/well were pretreated for 1 h at 4°C in the presence of utr-1 (V), 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 hlL-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 hlL-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 Mr 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 hlL-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, 25).

### 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/IL-1 |
| SAM           |          |          |
| Control       | 0.08 (0.02) | 0.11 (0.06) |
| mTNF          | 3.63 (0.44) | 3.97 (0.57) |
| hTNF          | 3.13 (0.61) | 3.24 (0.35) |
| utr-1         | 0.10 (0.02) | 0.39 (0.04) |
| utr-2         | 0.08 (0.02) | 0.39 (0.04) |
| utr-3         | 0.12 (0.02) | 0.14 (0.02) |
| utr-1/2/3     | 0.10 (0.05) | 0.34 (0.03) |
| utr-4         | 0.09 (0.02) | 0.10 (0.02) |
| utr-10        | 0.07 (0.02) | 0.09 (0.02) |
| htr-9         | 0.09 (0.01) | 0.10 (0.01) |

5 × 10⁴ cells/well were preincubated for 1 h at 4°C in the presence of anti-hTNF-R75 mAbs. Then, SAM and hlL-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

| SAM  | Utr-1 (μg/ml) | Utr-2 (μg/ml) | Utr-3 (μg/ml) |
|------|--------------|--------------|--------------|
|      | 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 |
| 24   | 14 15 22 28 26 | 14 15 22 28 26 | 14 15 22 28 26 |
| 2.4  | 14 15 22 28 26 | 14 15 22 28 26 | 14 15 22 28 26 |
| 0.312| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.625| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 1.25 | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 2.5  | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 3    | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.312| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.625| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 1.25 | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 2.5  | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 3    | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.312| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.625| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 1.25 | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 2.5  | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 3    | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.312| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 0.625| 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 1.25 | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 2.5  | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |
| 3    | 2.4 14 15 22 28 | 2.4 14 15 22 28 | 2.4 14 15 22 28 |

5 × 10⁴ 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-1B (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.

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(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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