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The Transmembrane Form of Tumor Necrosis Factor Is the Prime Activating Ligand of the 80 kDa Tumor Necrosis Factor Receptor

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Summary

The 60 kDa tumor necrosis factor receptor ($TNFR_{60}$) is regarded as the major signal transducer of TNF-induced cellular responses, whereas the signal capacity and role of the 80 kDa TNFR ($TNFR_{80}$) remain largely undefined. We show here that the transmembrane form of TNF is superior to soluble TNF in activating $TNFR_{80}$ in various systems such as T cell activation, thymocyte proliferation, and granulocyte/macrophage colony-stimulating factor production. Intriguingly, activation of $TNFR_{80}$ by membrane TNF can lead to qualitatively different TNF responses such as rendering resistant tumor cells sensitive to TNF-mediated cytotoxicity. This study demonstrates that the diversity of TNF effects can be controlled through the differential sensitivity of $TNFR_{80}$ for the two forms of TNF and suggests an important physiological role for $TNFR_{80}$ in local inflammatory responses.

Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine that is primarily produced by activated macrophages and lymphocytes, but is also expressed in endothelial cells and other cell types. TNF represents a major mediator of inflammatory, immunological, and pathophysiological reactions (reviewed by Fiers, 1991; Adolf et al., 1994). Kriegler et al. (1988) have demonstrated that the presumed atypical leader sequence of TNF in fact represents a transmembrane domain. Accordingly, two distinct species of the molecule exist: the 26 kDa membrane-expressed form of TNF (mTNF) and the soluble 17 kDa cytokine (sTNF), which is derived from proteolytic cleavage of the 26 kDa membrane form. Despite this precursor/product relationship of the two TNF forms, it was soon demonstrated that mTNF is, in principle, bioactive and confers, in situations of juxtracrine intercellular signaling, typical TNF responses such as cytotoxicity (Perez et al., 1990) or B lymphocyte activation (Aversa et al., 1993).

Two distinct membrane receptors for TNF of apparent molecular weight 55–60 kDa ($TNFR_{60}$) and 70–80 kDa ($TNFR_{80}$) have been identified and molecularly cloned. Both receptors have significant homologies in their extracellular domains with repeat cysteine-rich sequences, defining them as members of a novel, large receptor family (reviewed by Smith et al., 1994). Most cell lines and primary tissues coexpress both receptor types, although expression of $TNFR_{60}$ and $TNFR_{80}$ is controlled by distinct mechanisms. Typically, $TNFR_{60}$ is constitutively expressed at a rather low level, whereas the level of $TNFR_{80}$ expression is subject to both transcriptional and posttranscriptional regulation induced by external stimuli (Thoma et al., 1990; Brockhaus et al., 1990).

Recently, different $TNFR_{60}$- and $TNFR_{80}$-associated proteins have been characterized and molecularly cloned (Hsu et al., 1995; Rothe et al., 1994), suggestive of an independent role of both receptors in separate cellular responses. This prediction, however, contrasts with the current model of the apparent dominance of $TNFR_{60}$ in TNF signaling including accumulation of *c-fos*, interleukin-6 (IL-6), and manganese superoxide dismutase mRNA, synthesis of prostaglandin E_2, IL-2 receptor, and MHC class I and II antigen expression, growth inhibition, and cytotoxicity (reviewed by Adolf et al., 1994). Furthermore, it has been demonstrated in various in vitro and in vivo models that $TNFR_{60}$ induces cellular TNF responses independent of $TNFR_{80}$ stimulation (Engelmann et al., 1990; Pfeffer et al., 1993).

The contribution of $TNFR_{80}$ to cellular responses induced by sTNF appeared to be, by and large, of a supportive or modulating nature, with two distinct functional properties. First, the proteolytically cleaved extracellular domain may buffer excessive sTNF and, as a consequence, might be effective as a TNF inhibitor (Porteau and Hieblot, 1994). Second, $TNFR_{80}$-bound ligand may be passed over to $TNFR_{60}$ to enhance $TNFR_{60}$ signaling, a process termed ligand passing that is favored by the distinct kinetics of ligand association and dissociation of the two receptors (Tartaglia et al., 1993b). Aside from these features, only a few examples have been described in which a $TNFR_{60}$-independent, $TNFR_{80}$-mediated cellular response was induced by sTNF, namely granulocyte/macrophage colony-stimulating factor (GM-CSF) expression in a T cell hybridoma (Vandenabeele et al., 1992) and proliferation of thymocytes (Tartaglia et al., 1991, 1993a). In addition, independent activation of intracellular signaling cascades by $TNFR_{80}$ was readily demonstrated in a

number of experimental models when $TNFR_{80}$ overexpression systems were studied or $TNFR_{80}$-specific antibodies were used (Tartaglia et al., 1991; Heller et al., 1992; Vandenabeele et al., 1992; Gehr et al., 1992; Grell et al., 1993). The physiological significance of these findings, however, remained uncertain.

The present work provides a new basis for the comprehension of TNF–$TNFR_{80}$ interaction and function. In particular, we have discovered that $TNFR_{80}$ can be strongly stimulated by mTNF rather than by sTNF, suggesting that mTNF is the prime physiological activator of $TNFR_{80}$. As mTNF also signals via $TNFR_{60}$, the resulting cooperativity of both receptors leads to cellular responses much stronger than those achievable with sTNF alone. Moreover, we show that upon appropriate activation of $TNFR_{80}$, a phenotypic switch of the cellular response pattern to TNF can be observed, such that, as an example, cells fully resistant to the cytotoxic action of sTNF become highly susceptible and are killed upon contact with mTNF.

Results

Cellular Responses to sTNF Are Dominated by TNFR60

To clarify the functional role of $TNFR_{80}$ in TNF responses, we used mutants of the TNF molecule (muteins) that have previously been demonstrated to interact specifically with only one of the two TNFRs and, hence, represent efficient receptor-selective tools (Loetscher et al., 1993). The ability of each of the two TNFRs to mediate an independent cellular signal was studied in three different cell types predominantly expressing $TNFR_{80}$ that had been shown to be involved in TNF signaling by inhibition with receptor-specific antagonistic antibodies (Scheurich et al., 1992; Grell et al., 1993). These studies had indicated an auxiliary function of $TNFR_{80}$ in $TNFR_{60}$-controlled cellular responsiveness, although at that time the mechanism remained unresolved.

In accordance with these earlier studies, a dominant role of $TNFR_{60}$ was also revealed using the receptor-specific TNF muteins. In particular, the $TNFR_{60}$-specific mutein induced cytotoxicity in the cell line KYM-1, enhanced interferon-γ (IFNγ)-induced HLA-DR expression in the cell line Colo205, and up-regulated HLA-DR expression in activated human peripheral blood T cells (Figures 1A–1C). The magnitude of these responses was comparable to that of wild-type TNF, although somewhat higher concentrations of the mutein were needed. In contrast, the $TNFR_{80}$-specific mutein was unable to induce a response in Colo205 cells and in activated T cells (Figures 1B and 1C), and only a small cytotoxic effect was observed in KYM-1 cells at high TNF mutein concentrations up to 10 nM (Figure 1A).

Antibodies Are Superior to sTNF in Stimulation of TNFR80

The predominant role of $TNFR_{60}$ in the aforementioned TNF responses was confirmed using agonistic antibodies. In all three systems, the $TNFR_{60}$-specific agonistic monoclonal antibody (MAb) Htr-1 could induce comparable responses (Figures 1D–1F). However, in contrast with the complete lack or inefficient stimulation of cells by the $TNFR_{80}$-specific TNF mutein, $TNFR_{80}$-specific agonistic antibodies could efficiently induce cytotoxicity in KYM-1 cells (Grell et al., 1994; Figure 1D) and enhance HLA-DR expression in activated T cells (Figure 1E), indicating a superior efficacy of antibodies in $TNFR_{80}$ stimulation as compared with sTNF.

In the cell line Colo205, selective $TNFR_{80}$ triggering by specific antibodies could not enhance HLA-DR expression (Figure 1F), making questionable the signaling capability of $TNFR_{80}$ in these cells. To analyze whether antibody

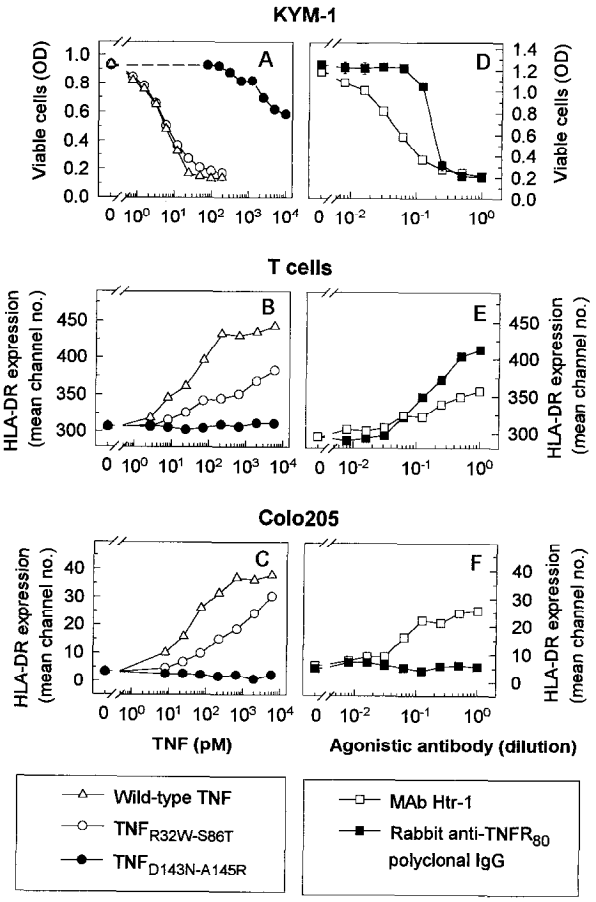


Figure 1. Disparity between TNF and Receptor-Specific Agonistic Antibodies to Induce Cellular Responses via $TNFR_{80}$

The induction of cytotoxicity in KYM-1 cells (A and D), enhancement of HLA-DR antigen expression in activated peripheral blood T lymphocytes (B and E), and enhancement of IFNγ-induced HLA-DR expression in Colo205 cells (C and F) was determined as described in the Experimental Procedures.

(A–C) Cells were treated with serial dilution of recombinant human TNF, a $TNFR_{60}$-specific TNF mutant ($TNF_{R32W-S86T}$), and a $TNFR_{80}$-specific TNF mutant ($TNF_{D143N-A145R}$).

(D–F) Cells were stimulated with the agonistic MAb Htr-1 (10^0 = hybridoma supernatant 1:100) and $TNFR_{80}$-specific purified rabbit IgG (10^0 = 40 μg/ml). Matched control antibodies showed no effect in any of the cellular systems. The results are representative for at least three independent experiments.

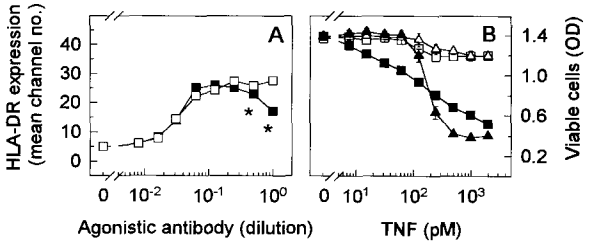


Figure 2. Antibody-Mediated Hyperstimulation of $TNFR_{80}$ Changes the Cellular Response Pattern of Colo205 Cells

(A) Antibody stimulation of $TNFR_{80}$ does not enhance $TNFR_{60}$-mediated up-regulation of HLA-DR expression. Colo205 cells were treated for 40 hr with $TNFR_{60}$-specific MAb Htr-1 (10^0 = hybridoma supernatant 1:100) in the absence (open squares) or presence (closed squares) of agonistic $TNFR_{80}$-specific rabbit IgG (10 μg/ml). The assay was performed in the presence of 30 pg/ml IFNγ, and expression of HLA-DR was assessed by direct flow cytometric analysis. Shown is the specific fluorescence mean channel number after subtraction of values determined by a matched control antibody. Asterisks indicate cultures with >50% dead cells after 40 hr as determined by vital exclusion dye.

(B) Induction of cytotoxicity by costimulation with TNF and $TNFR_{80}$-specific antibodies. Colo205 cells were incubated in triplicates for 40 hr with serial dilutions of TNF in the absence (open triangles) or presence of $TNFR_{60}$-specific MAb Htr-1 (hybridoma supernatant, 1:100, open squares), $TNFR_{80}$-specific rabbit serum (1:200, closed squares), and the $TNFR_{80}$-specific MAb 80M2 (5 μg/ml, closed triangles).

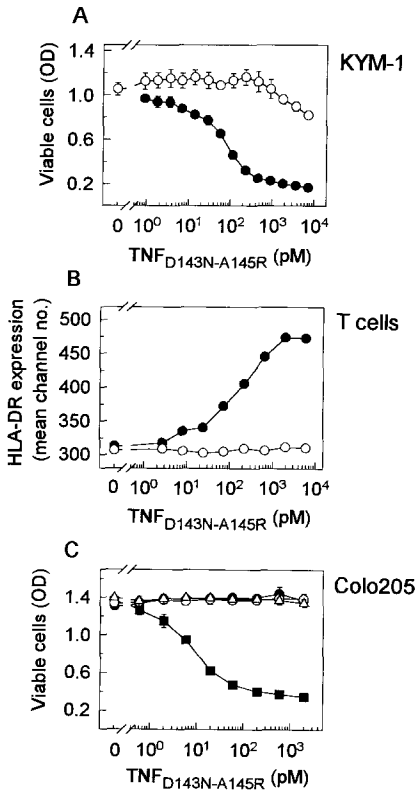


Figure 3. $TNFR_{80}$-Dependent Responses to sTNF Are Substantially Enhanced by MAb 80M2

Assay systems to determine induction of cytotoxicity in KYM-1 cells (A) and Colo205 cells (C) and enhancement of HLA-DR antigen expression in activated peripheral T lymphocytes (B) were performed as described in the Experimental Procedures. Cells were treated with a $TNFR_{80}$-specific TNF mutein in the absence (open circles) or presence of MAb 80M2 (5 μg/ml, closed circles). Strong responses were obtained upon costimulation with the MAb 80M2 in KYM-1 and T cells, whereas in Colo205 cells an additional costimulation of $TNFR_{60}$ with a $TNFR_{60}$-specific TNF mutein (TNFR32W-S86T, 50 ng/ml) was necessary to induce cytotoxicity (C, closed squares). A combination of both TNF muteins in Colo205 cells in the absence of MAb 80M2 was ineffective (open triangles).

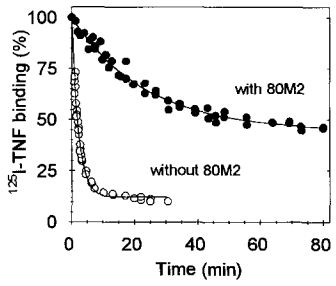
costimulation of $TNFR_{80}$ in the presence of $TNFR_{60}$ stimulation could lead to cooperation with the $TNFR_{60}$-mediated response, Colo205 cells were simultaneously treated with both $TNFR_{80}$-specific and $TNFR_{60}$-specific agonistic antibodies. This type of stimulation did not lead to an enhancement of HLA-DR expression triggered by $TNFR_{60}$ (Figure 2A), but surprisingly induced a strong cytotoxic effect in Colo205 cells (Figure 2B). Obviously, antibody costimulation of both TNFRs in Colo205 cells had changed the cellular response pattern from an immunostimulatory type (i.e., up-regulation of HLA-DR antigens) to one that induces cytotoxicity. This is remarkable because cytotoxicity in Colo205 cells could not be induced by sTNF alone, even with high concentrations (Figure 2B). The observed change in response pattern was apparently caused by an antibody-mediated hyperstimulation of $TNFR_{80}$, as Colo205 cells were efficiently killed by TNF in a dose-dependent manner in the presence of agonistic $TNFR_{80}$-specific antibodies or the nonagonistic $TNFR_{80}$-specific MAb 80M2. By contrast, costimulation of the cells with TNF and various different $TNFR_{60}$-specific, agonistic MAbs, Htr-1 (Figure 2B), Htr-9, and Htr-2 (data not shown), proved to be ineffective in this regard.

The Hyperstimulating $TNFR_{80}$-Specific MAb 80M2 Stabilizes the Ligand-Receptor Complex

To examine the mechanism by which the nonagonistic MAb 80M2 causes a hyperstimulation of $TNFR_{80}$ in the presence of sTNF, we tested this MAb together with the $TNFR_{80}$-specific mutein. In all three cellular systems, costimulation of the cells induced a response similar to that obtained with the agonistic $TNFR_{80}$-specific antibodies. In particular, the presence of MAb 80M2 potentiated the $TNFR_{80}$-specific mutein-induced cytotoxicity in KYM-1 cells (Figure 3A) and rendered activated T cells sensitive to $TNFR_{80}$-specific mutein-induced HLA-DR expression (Figure 3B). In accordance with the results using agonistic antibodies (Figure 2B), only the adequate stimulation of both TNFRs caused cytotoxicity in Colo205 cells: a combination of the $TNFR_{60}$-specific and $TNFR_{80}$-specific muteins exerted a strong cytotoxic effect only in the presence of the MAb 80M2 (Figure 3C).

Because MAb 80M2 is per se neither agonistic nor antagonistic (Grell et al., 1993), we analyzed whether MAb 80M2 would affect the binding properties of sTNF to $TNFR_{80}$. Kinetic studies with iodinated TNF at 37°C revealed that preincubation of cells with 80M2 led to a strong reduction in the dissociation rate constant (K_{off}, Figures 4A and 4B), whereas the association rate constant (K_{on}) was almost unchanged (Figure 4B). Accordingly, MAb

A



B

	control	+ 80M2
K_{on} [M^{-1} min^{-1}]	1.2×10^{9}	1.7×10^{9}
K_{off} [min^{-1}]	4.3×10^{-1}	3.6×10^{-2}
K_d [M]	3.6×10^{-10}	2.1×10^{-11}
$t_{1/2diss.}$ [min]	1.6	19.4

Figure 4. Inhibition of Ligand Dissociation by the Antibody 80M2

(A) Time course of TNF dissociation from $TNFR_{80}$ at 37°C. KYM-1 cells were incubated for 1 hr with 0.5 nM [^{125}I]TNF at 4°C in the presence (closed circles) or absence of 80M2 (open circles), and subsequently, dissociation of radioiodinated ligand was determined at 37°C in the presence 50 nM unlabeled TNF. 100% [^{125}I]TNF binding equalled to 6,617 cpm (control) and 13,322 cpm (with 80M2). Nonspecific binding in the presence of a 100-fold excess of unlabeled TNF was determined at several timepoints, and revealed values were <50 cpm.

(B) Comparison of [^{125}I]TNF binding data in the absence or presence of MAb 80M2. Dissociation rate constants and half-life time values of bound [^{125}I]TNF were calculated as described in the Experimental Procedures. In addition, the association rate constants derived from association kinetics experiments are shown as well as the calculated values for the dissociation constants ($K_d = K_{off}/K_{on}$). Mean values of two independent experiments are presented. Similar differences caused by MAb 80M2 in TNF dissociation but not TNF association rate constants were found using Colo205 cells (data not shown).

80M2 raises the affinity of $TNFR_{80}$ for sTNF more than 10-fold as revealed from the calculated dissociation constant (K_d) values (Figure 4B). These data suggest that the altered signaling capability of $TNFR_{80}$ induced by TNF in the presence of MAb 80M2 is linked to the change in ligand binding characteristics.

The Transmembrane Form of TNF Strongly Activates $TNFR_{80}$

The finding that prolonged binding of TNF to $TNFR_{80}$ caused by MAb 80M2 is associated with signal capability of this receptor prompted us to look for potential physiological correlates. In particular, we asked whether this experimental setting simply mimics the biological effects of the membrane-anchored form of TNF, which has been shown to possess bioactivity (Perez et al., 1990). To that end, we transfected Chinese hamster ovary (CHO) cells with the DNA coding for wild-type human TNF. The resulting cell clones expressed mTNF, as revealed by cytofluorometric analysis (data not shown) and immunofluorescence microscopy (Figure 5E). In fact, Colo205 cells were efficiently killed when cocultivated with TNF-transfected CHO cells (Figure 5B), but not with control CHO cells in the presence of sTNF (Figure 5A). To ensure that the observed cytotoxicity is mediated by the membrane-expressed cytokine alone, without any effect of sTNF, we generated an uncleavable TNF deletion mutant by site-directed mutagenesis as described previously (Perez et al., 1990). Similarly, CHO cells transfected with this mutant expressed mTNF (Figure 5F) and lysed Colo205 cells very efficiently (Figure 5C). No sTNF was detectable in the supernatant of such cocultures, as revealed by highly sensitive bioassays (data not shown). The capability of mTNF-expressing CHO clones to induce cytotoxicity in Colo205 cells does not require strong overexpression of the mTNF molecule, because independently isolated transfectants, expressing mTNF at various levels between about 1000 and 30000 molecules per cell, as estimated from fluorescence-activated cell sorting (FACS) analysis, induced cytotoxicity in Colo205 cells (data not shown). Similar results were obtained using an additional, independently generated cell line, NIH $3T3_{\Delta(1-12)TNF}$, also expressing the uncleavable mutant form of TNF (Perez et al., 1990), as well as with transiently transfected COS1 cells using the same DNA construct (data not shown). The specificity of these effects was demonstrated further using neutralizing anti-TNF antibodies, which completely abrogated the cytotoxic effects (Figure 5G). Accordingly, mTNF possesses the capability to activate TNFRs of Colo205 cells in a similar way as the MAb 80M2 in the presence of sTNF. The induction of cytotoxicity in Colo205 cells was inhibited by antagonistic antibodies directed against either of the TNFRs (Figure 5G), indicating that both $TNFR_{60}$ and $TNFR_{80}$ are involved in mTNF signaling in this particular cellular response.

Next, we probed into whether the phenomenon of hyperresponsiveness due to $TNFR_{80}$ activation could also be observed in thymocytes, a cellular system in which sTNF effects have been demonstrated to be initiated by $TNFR_{80}$ independently of $TNFR_{60}$ (Tartaglia et al., 1991, 1993a). To analyze TNF responses attributed to $TNFR_{80}$ such as proliferation and GM-CSF production (Tartaglia et al., 1991; Vandenabeele et al., 1992), we took advantage of the fact that human TNF does not bind to murine $TNFR_{80}$. Therefore, we used mouse thymocytes from transgenic mice generated to express selectively the human $TNFR_{80}$ under the control of the CD2 promoter in the T cell compartment (E. D., unpublished data). Thymocytes of these mice constitutively expressed the human $TNFR_{80}$ in the range of 1000–2000 molecules/cell as determined by FACS and saturation binding analyses using human $TNFR_{80}$-specific antibodies and iodinated human TNF, respectively (data not shown). In contrast with thymocytes of nontransgenic littermates (data not shown), human $TNFR_{80}$ transgenic thymocytes responded well to the stimulation with human sTNF in all parameters of thymocyte activation analyzed, i.e., cell proliferation, up-regulation of IL-2 receptor α chain (CD25), and GM-CSF production (Figures 6A–6C). Stimulation of transgenic thymocytes

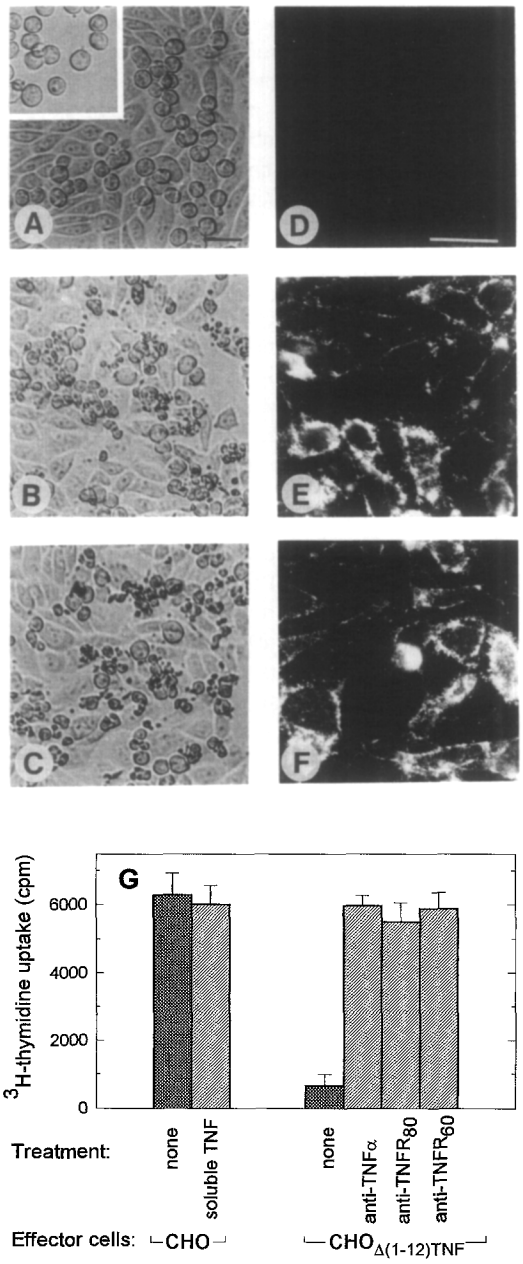


Figure 5. The Transmembrane Form of TNF Induces Cytotoxicity in Colo205 Cells

Control CHO cells (A and D) or CHO cells transfected with the wild-type human TNF DNA (CHO_{wtTNF}) (B and E) or the DNA for an uncleavable mutant transmembrane form of TNF ($CHO_{\Delta(1-12)TNF}$) (C and F) were cultured to form monolayers. Membrane expression of TNF on CHO cells (D), CHO_{wtTNF} cells (E), and $CHO_{\Delta(1-12)TNF}$ cells (F) was visualized by fluorescence microscopy using a FITC-conjugated anti-membrane TNF antibody (R&D Systems). For cell-to-cell killing experiments, Colo205 cells were added to the cultures in the absence (B and C) or presence (A) of sTNF (1 nM), and photographs were taken after 24 hr. Colo205 cells do not spread out on the culture vessel (inset of A) or CHO monolayer and are easily distinguishable from the adherent CHO cells. Scale bars represent 50 μm.

(G) Both TNFRs are essential for mTNF-induced cytotoxicity in Colo205 cells. Irradiated CHO cells or $CHO_{\Delta(1-12)TNF}$ cells were cocultured for 30 hr with Colo205 cells in the absence or presence of 0.2 nM TNF, 30 μg/ml neutralizing anti-TNF MAb, 50 μg/ml antagonistic Fab fragments of polyclonal $TNFR_{80}$-specific antibodies, or 30 μg/ml antagonistic $TNFR_{80}$-specific MAb H398. [^{3}H]thymidine incorporation was determined as described in the Experimental Procedures. [^{3}H]thymidine background uptake of irradiated CHO cells or $CHO_{\Delta(1-12)TNF}$ cells alone is subtracted in the figure (3331 and 3725 cpm, respectively).

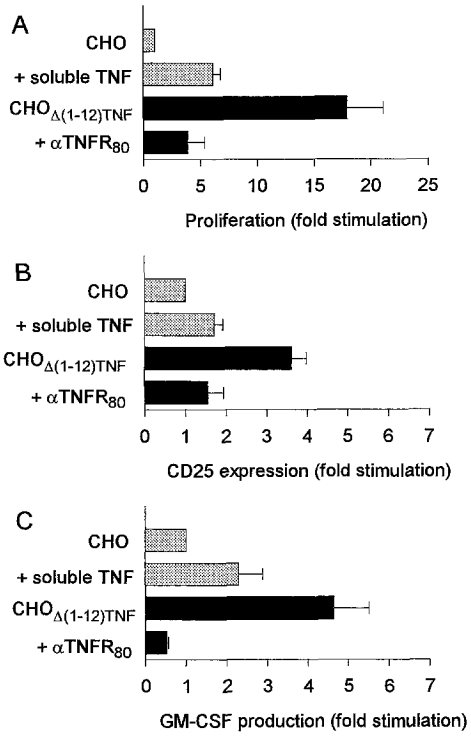


Figure 6. Stimulation of Thymocytes by the Transmembrane Form of TNF

Thymocytes of human $TNFR_{80}$-transgenic mice were isolated and cultured in the presence of 0.5 μg/ml concanavalin A on a monolayer of control CHO cells without or with titrated concentrations of human sTNF (stippled bars). The results from groups with maximal response to sTNF are shown (0.5–1 nM). In parallel, cocultures were set up with thymocytes and $CHO_{\Delta(1-12)TNF}$ cells (closed bars) alone or in the presence of antagonistic $TNFR_{80}$-specific antibodies. After 40 hr, proliferative responses were determined by [^{3}H]thymidine incorporation (A), expression of murine CD25 was analyzed by cytofluorometry (B), and murine GM-CSF secretion was determined from culture supernatants by ELISA (C) as described in the Experimental Procedures. Data of experimental groups are presented as stimulation of untreated controls (means ± SEM of three independent experiments). The respective values of untreated control groups in individual experiments were 2758, 2346, and 4103 cpm per 10^6 cells (proliferation assay), 34, 49, and 11 arbitrary fluorescence units (CD25 expression, mean channel number) and 35, 40, and 23 pg/ml (GM-CSF production).

with mTNF in all three assays led to a stronger response than with saturating concentrations of sTNF (17.9-fold versus 6.1-fold stimulation of proliferation, Figure 6A; 3.6-fold versus 1.7-fold stimulation of CD25 expression, Figure 6B; 4.7-fold versus 2.3-fold stimulation of GM-CSF production, Figure 6C). $TNFR_{80}$ transgene dependence in mediating these responses was verified by inhibition with $TNFR_{80}$-specific antagonistic antibodies (Figures 6A–6C).

Further evidence for the physiological importance of the superiority of mTNF over sTNF in the triggering of $TNFR_{80}$ comes from experiments using cells from normal human tissues. First, we analyzed activated human peripheral blood T lymphocytes for which TNF has been demonstrated to act as a costimulatory molecule (Scheurich et al., 1987). Coculture of activated T cells with CHO cells expressing the noncleavable membrane form of TNF resulted in a significantly stronger up-regulation of HLA-DR expression than coculture with control CHO cells in the presence of saturating concentrations of sTNF (Figure 7A). The critical involvement of $TNFR_{80}$ in this hyperstimulation was demonstrated by the fact that costimulation with MAb 80M2 greatly enhanced the response by the $TNFR_{80}$-specific mutein (Figure 3B), or by wild-type TNF resulting in an approximately 3-fold increase over the maximal response induced by saturating TNF concentrations in the presence of 80M2 versus TNF alone (289% ± 103%, eight different blood donors; data not shown).

Second, we compared the reactivity of human umbilical vein endothelial cells (HUVECs) toward sTNF and mTNF for the following reasons: these cells, comprising the lining of the vasculature, play a crucial role in local inflammatory processes that are, in part, controlled by TNF (Pober and Cotran, 1990); recent data have implicated both TNFRs in the induction of prothrombotic tissue factor expression (Schmidt et al., 1995b), which is a key event in coagulation both in the course of septic shock and in local tissue necrosis; a role of $TNFR_{80}$ in TNF-induced skin necrosis is apparent from experiments employing $TNFR_{80}$ gene knockout mice (Erickson et al., 1994). In a direct comparison, the coculture of HUVECs with mTNF-expressing cells for 3 hr led to a significantly higher tissue factor expression than could be achieved even with saturating concentrations of sTNF (Figure 7B). The involvement of both receptors in this mTNF response is evident from the efficient inhibition by antagonistic antibodies directed against each of the two receptors (Figure 7B).

Based on the data presented, a model of the functional role of the two TNFRs is proposed that takes into account the differential interplay of the soluble versus the membrane-expressed form of the ligand with each of the two receptors (Figure 8). Typically, $TNFR_{60}$ is the main mediator of a wide array of effects induced by sTNF. Binding of sTNF accounts for TNF responses upon application of soluble, recombinant TNF or upon endogenous TNF production at a timepoint when TNF has been processed and released into the body fluids. An appealing feature of the novel model described here is the fact that mTNF is the physiological precursor of sTNF and thus could be an early and very effective stimulus because it could induce independent signal pathways at each of the TNFRs. This signal is dependent on cell-to-cell contact and is thus locally restricted. Depending on whether the juxtaposition of mTNF and TNFRs involves just one or both receptor types on the same cell, the quality and quantity of the induced cellular response may therefore differ strikingly.

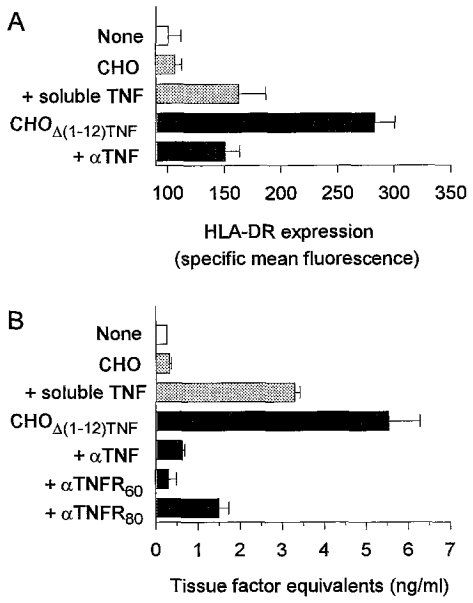


Figure 7. Stimulation of Normal T Lymphocytes and Endothelial Cells by mTNF

(A) PHA-activated peripheral blood T lymphocytes were cultured for 40 hr without any further additions (open bar), or on a monolayer of control CHO cells (stippled bars) without or with sTNF. sTNF was titrated, and data of the TNF concentration (2 nM) resulting in the maximal response are shown. In parallel, T cells were cultured on a monolayer of $CHO_{\Delta(1-12)TNF}$ cells (closed bars) without or with antagonistic antibodies as indicated. HLA-DR antigen expression of the T cells was determined by direct cytofluorometry.

(B) HUVECs were left untreated (open bar), or stimulated for 3 hr by the addition of CHO cells (stippled bars) in the absence or presence of saturating concentrations of sTNF (2nM), or by the addition of $CHO_{\Delta(1-12)TNF}$ cells (closed bars) in the absence or presence of the indicated antagonistic antibodies. Subsequently, tissue factor production was determined as described in the Experimental Procedures. Presented values are the means of triplicates.

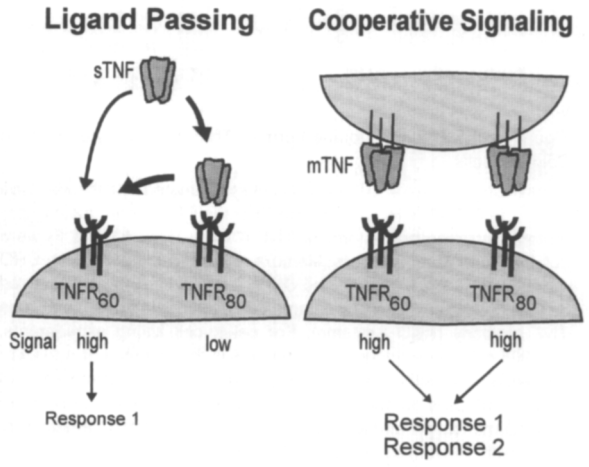


Figure 8. Model of the Functional Role of $TNFR_{80}$

The model proposes the major functional roles of $TNFR_{80}$ in response to sTNF (left) and mTNF (right).

Discussion

Based on numerous in vitro and in vivo studies, a predominant role of $TNFR_{60}$ in mediating TNF responses became apparent, whereas the contribution of the coexpressed $TNFR_{80}$ remained, by and large, unclear. With the data presented here showing that $TNFR_{80}$ defines the quality and quantity of cellular responses to mTNF, we provide evidence that the functional significance of $TNFR_{80}$ has been grossly underestimated. We propose that mTNF rather than sTNF is the prime physiological activator of $TNFR_{80}$, which implies that $TNFR_{80}$ controls the local response pattern in the microenvironment of tissues reactive to external stimuli with endogenous TNF expression.

Retrospectively, earlier assessments of the functional role of the two TNFR were biased toward a $TNFR_{60}$ dominance because of the use of exogenous or induction of endogenous sTNF. Although under these conditions antagonistic $TNFR_{80}$-specific antibodies partially blocked TNF responses, such as activation of NF-κB, upregulation of adhesion molecules, and HLA-DR expression, $TNFR_{80}$-selective agonists failed to induce the very same responses (Shalaby et al., 1990; Scheurich et al., 1992; Mackay et al., 1993). Recently, the participation of $TNFR_{80}$ in TNF responses has been attributed to the ability of $TNFR_{80}$ to raise the virtual TNF concentration in the proximity of $TNFR_{60}$ and, therefore, to enhance signaling of the latter receptor (the model of ligand passing by Tartaglia et al., 1993b). Such an accessory function of $TNFR_{80}$ could be envisaged due to its higher affinity for TNF compared with $TNFR_{60}$ and is, in particular, based on its fast ligand association and dissociation kinetics. Therefore, ligand passing is expected to be most effective at low concentrations of sTNF. According to this model and in agreement with experimental data obtained in the majority of in vitro systems, TNF responses appear to be limited by ligand interaction with $TNFR_{60}$ without significant generation of an intracellular signal by $TNFR_{80}$.

The availability and application of novel, $TNFR_{80}$-specific antibodies with agonistic activity shed new light onto this question by showing that $TNFR_{80}$ can directly induce cellular responses independent of $TNFR_{60}$ (Tartaglia et al., 1991; Gehr et al., 1992; Grell et al., 1993). We have here directly compared TNF muteins that have exclusive specificity for either $TNFR_{60}$ or $TNFR_{80}$ with TNFR-specific agonistic antibodies and show that antibody stimulation is a more potent stimulus for $TNFR_{80}$ than sTNF (Figure 1). The inefficiency of the $TNFR_{80}$-specific TNF mutein to induce cytotoxicity in KYM-1 cells and the complete inability to elicit a response in activated human T cells could not be attributed to the lack of binding, as the mutein exerts only a slightly reduced affinity to $TNFR_{80}$ when compared with wild-type TNF (Loetscher et al. 1993; data not shown) and has been applied up to saturating concentrations. However, in both cell types, the principal signaling capability of $TNFR_{80}$ could be demonstrated using either a $TNFR_{80}$-specific agonistic polyclonal immunoglobulin G (IgG) from rabbit (Figures 1D and 1E) or a $TNFR_{80}$-specific monoclonal antibody MR2-1 (data not shown). This perceived superiority of antibody triggering of $TNFR_{80}$ over TNF is in accordance with an earlier report showing a stronger stimulation of human thymocyte proliferation with agonistic antibodies compared with TNF (Tartaglia et al., 1993a).

The mechanism by which antibodies can act as $TNFR_{80}$ agonists is most likely to be based on the fact that dimerization of $TNFR_{80}$ is an obligatory step for induction of signal cascades (Grell et al., 1993). Obviously, similarily to TNF, antibodies can readily dimerize $TNFR_{80}$ because of their bivalency and flexibility in antigen binding. The difference between sTNF and agonistic antibodies in the potency to induce a cellular response could be related to the different binding kinetics. Whereas the dissociation kinetics of sTNF from $TNFR_{80}$ is extremely rapid at 37°C (Figure 4), the dissociation kinetics of antibodies from $TNFR_{80}$ were several orders of magnitude slower (data not shown). The underlying molecular mechanisms of this differential $TNFR_{80}$ activation by sTNF versus agonistic antibodies is, at present, not understood. Considering that the association/dissociation steps of intracellular signaling molecules, like the recently defined TNF receptor–associated proteins (TRAFs, Rothe et al., 1994), could be response limiting in $TNFR_{80}$ signaling, it is feasible that an increased stability of receptor complex formation by antibody binding is responsible for the qualitative shift in $TNFR_{80}$ signaling observed.

Experimental evidence for a causal relationship between ligand binding kinetics and bioactivity comes from our experiments performed with the MAb 80M2. This antibody possesses the striking feature of being able to hyperstimulate $TNFR_{80}$ in the presence of the natural ligand, although it is, on its own, neither agonistic nor antagonistic. Here, we could clearly show that this MAb stabilizes TNF binding to $TNFR_{80}$ (Figure 4). In cellular systems, however, in which $TNFR_{80}$ has no independent signaling capability and plays solely a role as a ligand-passing molecule for $TNFR_{60}$, one would expect that 80M2-mediated stabilization of TNF binding to $TNFR_{80}$ annuls this particular accessory effect of $TNFR_{80}$. In fact, we have observed an inhibitory effect of MAb 80M2 when low concentrations of TNF were used to induce superoxide anion release in human neutrophils (data not shown), which is exclusively mediated by $TNFR_{60}$ (Menegazzi et al., 1994), and cytotoxicity in human HeLa cells transfected with $TNFR_{80}$, in which cytotoxicity is mainly induced by $TNFR_{60}$ (data not shown). Similar observations of stabilization of TNF binding to $TNFR_{80}$ and interference of $TNFR_{80}$-specific antibodies with the mechanism of ligand passing have recently been reported (Bigda et al., 1994).

Why is a $TNFR_{80}$-linked signaling apparatus of high potency present in various cells that can be readily activated by an experimental stimulus (antibody) but not by the natural ligand TNF? We have resolved this question here by showing that mTNF, but not sTNF, is a strong activator of $TNFR_{80}$. This is intelligible because in a situation of cell-to-cell contact a juxtaposition of mTNF and TNFRs can occur that allows formation of ligand–receptor complexes of greater stability. It is conceivable that such an

interaction is qualitatively distinct from the transient interaction of $TNFR_{80}$ with sTNF. This interpretation is nicely illustrated by the results obtained with a carcinoma cell line, Colo205, that responds in a strikingly different manner to sTNF and mTNF, which produce up-regulation of HLA-DR expression and induction of cytotoxicity, respectively (Figures 1 and 2).

Activation of $TNFR_{80}$ by mTNF is not restricted to tumor cells. Rather, this finding holds true for all cellular systems analyzed in which $TNFR_{80}$ does not respond or only weakly responds to the soluble ligand, but is highly sensitive to antibody stimulation. Experiments using mouse thymocytes expressing the human $TNFR_{80}$ (Figure 6), human peripheral blood T lymphocytes, and human endothelial cells (Figure 7) indicate that mTNF might also play a crucial role in TNF responses of normal tissues as an important physiological activator of $TNFR_{80}$. In fact, previous studies have proposed a role for mTNF, rather than sTNF, in immunological reactions such as antileishmanial defense in macrophages, T cell–B cell interaction, and tumor cell killing mediated by infiltrating lymphocytes (Birkland et al., 1992; Aversa et al., 1993; Lopez-Cepero et al., 1994). According to our data, the action of mTNF, locally restricted by cell-to-cell contact, would be both qualitatively and quantitatively different from the more promiscuous effects of sTNF because of the simultaneous and efficient activation of both TNFRs (Figure 8).

The physiological relevance of $TNFR_{80}$ function is illustrated from very recent data obtained with $TNFR_{80}$ gene knockout mice. Although no apparent gross alterations in phenotype and composition of the immune cells were noted, and only a slight reduction in sensitivity against high dosage LPS shock was found, it had been surprising to find a dramatic decrease in sensitivity against tissue necrosis caused by repeated subcutaneous injections of high doses of murine TNF (Erickson et al., 1994). In a similar experimental model, TNF-induced skin necrosis in normal mice could be completely blocked by the in vivo administration of antagonistic $TNFR_{80}$-specific antibodies (Sheehan et al., 1995). The absolute $TNFR_{80}$ dependence noted in these studies clearly supports an active signaling rather than a ligand-passing function of $TNFR_{80}$. Although in both models tissue necrosis is induced by sTNF, these data are not contradictory to a superior activating function of mTNF, the induction of which has not been assessed in these studies. Here, the demonstrated superiority of mTNF in the stimulation of tissue factor expression in HUVECs suggests that under physiological conditions mTNF interaction with $TNFR_{80}$, in combination with $TNFR_{60}$ stimulation, might play an important role in inflammatory reactions leading to hemorrhagic necrosis. In fact, hemorrhagic necrosis may be initiated by leucocyte–endothelial interactions resulting in a conversion of the endothelium into a procoagulant state with subsequent thrombotic events and vascular leakage. We have shown in independent studies that the initial event is a juxtacrine, TNF-dependent process (Schmidt el al., 1995a) that can be induced by mTNF expressed on either endothelial cells or lymphocytes and that depends on both $TNFR_{60}$ and $TNFR_{80}$ signaling (Schmidt et al., 1995b).

Finally, the aforementioned novel characteristics of $TNFR_{80}$ disclose what may be a general principle of generating diversity of cytokine-induced responses by differential receptor activation. We propose that our findings have implications for other members of this large ligand family and their respective receptors, because it is apparent that most other members of the ligand family exist as genuine membrane molecules, or as heteromeric molecules in membrane-associated forms. Aside from TNF, other soluble derivatives of this ligand family begin to emerge. For example, the soluble form of the Fas ligand has been shown to be released by activated peripheral T lymphocytes (Tanaka et al., 1995) and a murine thymoma cell line has been shown to secrete soluble CD40 ligand (Armitage et al., 1992). These soluble ligands are known to be bioactive, but a detailed characterization of their spectrum of bioactivities awaits further investigation. It is therefore possible that differences in signaling capacity of soluble versus membrane bound forms of the same ligand will soon be unravelled for other receptor ligand systems of this family.

Experimental Procedures

Cell Culture and Biological Reagents

The human colon carcinoma cell line Colo205 and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 10 mM glutamine, and 50 μg/ml each of streptomycin and penicillin (Biochrom, Berlin, Federal Republic of Germany). The human rhabdomyosarcoma cell line KYM-1 was supplied by M. Sekiguchi (University of Tokyo, Tokyo, Japan) and maintained in Click–RPMI medium (Biochrom) containing 10% FCS. The cell line $NIH3T3_{\Delta(1-12)TNF}$ was provided by Chiron Corporation (Emeryville, CA) and maintained in RPMI medium containing 10% FCS and 400 μg/ml G418. T lymphocytes were prepared from heparinized whole blood by Ficoll–Hypaque density sedimentation as described (Scheurich et al., 1987), stimulated with 1 μg/ml phytohemagglutinin (PHA_M, GIBCO BRL, Gaithersburg, MD), and expanded in Click–RPMI containing 10% FCS, 2-mercaptoethanol (10^{-5} M), and 10 U/ml human IL-2 (Biotest, Dreieichenhain, Federal Republic of Germany) until use. Recombinant human TNF (2×10^7 U/mg) was provided by Knoll AG (Ludwigshafen, Federal Republic of Germany). IFNγ was provided from Bender Company (Vienna, Austria). The generation and specificity of the MAb H398 (Thoma et al., 1990), MAbs Htr-1, Htr-9, Htr-2, and MAb Utr-1 (Brockhaus et al., 1990), MAb 80M2, and polyclonal rabbit anti-human $TNFR_{80}$-specific IgG (Grell et al., 1993) have all been described. The anti-human TNF MAb (357-101-4) was provided by A. Meager (National Institute for Biological Standards and Control, Potters Bar, England).

Generation of CHO Clones Expressing Human TNF

For construction of the expression vectors for wild-type TNF and the uncleavable mutant of human TNF, an oligonucleotide (5′-ACCTACAACATGGGCTACTGCCTGGGCCAGAGGGCA-3′) was used to delete the DNA sequence encoding amino acids +1 to +12 of the mature, 17 kDa TNF form. This Δ1–12 mutation was constructed on a 2.8 kb EcoRI fragment of the human TNF gene by using the altered sites in vitro mutagenesis system (Promega, Madison, WI). A second mutagenesis step was performed on the same fragment to create a NcoI restriction site on the ATG translation initiation codon by using a 5′-CATGCTTTCAGTGCCCATGGTGTCCTTTCC-3′ oligonucleotide. Either the 2 kb wild-type TNF or the mutant 1.8 kb Δ(1–12)TNF NcoI–EcoRI fragment containing the complete coding region of the human TNF gene plus 173 bp of 3′ UTR sequences was inserted into the polylinker site of a pEF–BOS–GST expression vector (provided by E. Spanopoulou, Rockefeller University, New York, NY), a derivative of the pEF–BOS vector (Mizushima and Nagata, 1990) containing an

altered polylinker site in between an additional GST-encoding BglII–BamHI fragment of the pGEX–2T expression vector and the polyadenylation signal from the human G-CSF cDNA. The resulting plasmids were cotransfected by standard electroporation procedures with pMAMneo (Clontech, Palo Alto, CA) into CHO cells. G418-resistant cells were sorted for expression of mTNF by cytofluorometric analysis using the anti-TNFα-FITC MAb Fluorokine kit (R&D Systems, Minneapolis, MN), according to the recommendations of the manufacturer, and a FACStar plus (Becton Dickinson, San Jose, CA).

Up-Regulation of HLA-DR Antigen Expression

Colo205 cells or activated human T lymphocytes (day 6–8 after activation) were seeded into 24-well tissue culture plates (Greiner, Nürtingen, Federal Republic of Germany) at 3×10^5/well in a final volume of 0.5 ml of the respective medium (see above) containing 30 pg/ml IFNγ and 10 U/ml IL-2, respectively. After a 40 hr culture period with the different stimuli, cells were harvested, washed twice with phosphate-buffered saline containing 2% bovine serum albumine and 0.02% NaN_3, and suspended in the same buffer including a FITC-labeled anti-human HLA-DR monoclonal antibody (Dianova, Hamburg, Federal Republic of Germany) or an isotype-matched control antibody. Specific fluorescence was determined in duplicates by FACS analysis.

Generation of Transgenic Mice and Assays for Thymocyte Activation

Thymocytes from 5-week-old transgenic mice expressing a human $TNFR_{80}$ cDNA transgene in their T cell compartment have been employed to assess $TNFR_{80}$ function in a standard thymocyte comitogenic assay. Preparation of the gene constructs and generation of transgenic mice were performed essentially as published (Probert et al., 1993) and will be described in detail elsewhere (E. D. et al., unpublished data). T cell–targeted expression of the human $TNFR_{80}$ transgene was achieved by the use of a human CD2 minigene expression cassette (Lang et al., 1991). Thymocytes of transgenic mice or nontransgenic littermates were cultured in Click–RPMI medium (Biochrom) supplemented with 1% FCS, 0.5 μg/ml concanavalin A (Con A, Serva, Heidelberg, Federal Republic of Germany), and antibiotics in 24-well flat-bottomed culture plates (Greiner) at 1.2×10^6/0.45 ml on a monolayer of CHO control cells or $CHO_{\Delta(1-12)TNF}$ cells in the absence or presence of different concentrations of sTNF, or a combination of antagonistic $TNFR_{80}$-specific antibodies MAb Utr-1 (30 μg/ml) and polyclonal rabbit IgG Fab fragments (50 μg/ml). After 40 hr at 37°C, the concentration of murine GM-CSF in the culture supernatants of the individual groups was determined by ELISA (Biotrak, Amersham, Braunschweig, Federal Republic of Germany). Thereafter, thymocytes were harvested, adjusted to the same cell number, and cultured in triplicates in 96-well round-bottomed culture plates (Greiner) in medium without Con A in the presence of 10 μCi/ml [^{3}H]thymidine (82 Ci/mmol, Amersham). The purity of thymocytes in the individual assay groups was >99.5%, as controlled by FACS analysis. After 8 hr, cells were harvested onto glass fiber filters, and incorporated radioactivity was determined using a digital autoradiograph (Berthold, Wildbad, Federal Republic of Germany). Normal standard deviation was <15%. Specific IL-2 receptor α chain (CD25) surface expression of thymocytes was determined by FACS analysis as described above using FITC-conjugated rat anti-mouse CD25 and isotype-matched control antibodies (PharMingen, Hamburg, Federal Republic of Germany).

Tissue Factor Activity of HUVECs

HUVECs were prepared as described (Thornton et al., 1983) and cultured in EGM medium (PromoCell, Heidelberg, Federal Republic of Germany). Expression of tissue factor was assessed as described previously (Clauss et al., 1990). In brief, cultures with TNF or added cells (or with both) in MDCB 131 medium (GIBCO), containing 10 mM HEPES (pH 7.4), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 2.5 mg/ml fungizone (GIBCO) were incubated for 3 hr at 37°C. For studies with neutralizing antibodies (50–100 μg/ml), cells were preincubated for 30 min at room temperature before addition of cells, cytokines, or both. Assays were carried out with whole cells obtained in suspension following scraping from the dish. Citrated human plasma was added, and the clotting time was measured after recalcification. Tissue factor equivalents were calculated using a standard curve of purified human tissue factor (Clauss et al., 1990).

Cytotoxicity and Proliferation Assays

Assays were carried out essentially as described (Grell et al., 1993). In brief, KYM-1 cells (1×10^4/well) or Colo205 cells (1.5×10^4/well) were seeded into 96-well microtiter plates in 100 μl of the respective medium (see above) and allowed to grow overnight before addition of the different substances to a final volume of 200 μl. After 18 hr (KYM-1) or 40 hr (Colo205) of culture, metabolic activity was determined by the MTT method. For analysis of mTNF-mediated cytotoxicity in cell-to-cell killing experiments, the respective CHO clones were irradiated (5000 rad), washed extensively, and seeded into microtiter plates overnight (2×10^4 cells/well). Subsequently, Colo205 cells (1×10^4/well) were added, and treatment with the respective stimuli was performed in six replicate wells for 30 hr including [^{3}H]thymidine (Amersham; 0.5 μCi/well) during the last 6 hr. Cells were harvested onto glass fiber filters and lysed by distilled water, and cell-bound radioactivity at the filter was measured in a liquid scintillation counter.

Binding Assays

TNF was labeled with ^{125}I by the chloramine-T method, and binding assays were performed essentially as described (Dower et al., 1985). For dissociation kinetics, cells were incubated for 1 hr with 0.5 nM [^{125}I]TNF in culture medium in the presence or absence of MAb 80M2 (1 μg/ml) at 4°C. Subsequently, cells were suspended in the presence 50 nM unlabeled TNF at 37°C for several time periods, and cell-bound [^{125}I]TNF was determined after centrifugation of cells through a phthalate oil mixture. The timepoint at which the cells were resuspended was taken as the start of the reaction, and the corresponding value was set at 100%. For association kinetics, cells were preincubated with MAb 80M2 or medium and then incubated for different time periods with 0.5 nM [^{125}I]TNF at 37°C. Cell-bound radioactivity was determined as described above. The respective dissociation constant was computed from the ratio of the dissociation rate constant to the association rate constant calculated from the net association rate.

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References

Adolf, G.A., Grell, M., and Scheurich, P. (1994). Tumor necrosis factor. In Epidermal Growth Factors and Cytokines, T.A. Luger and T. Schwarz, eds. (New York: Marcel Dekker, Incorporated), pp. 63–88.

Armitage, R.J., Sato, T.A., MacDuff, B.M., Clifford, K.N., Alpet, A.R., Smith, C.A., and Fanslow, W.C. (1992). Identification of a source of biologically active CD40 ligand. Eur. J. Immunol. *22*, 2071–2076.

Aversa, G., Punnonen, J., and De Vries, J.E. (1993). The 26-kD transmembrane form of tumor necrosis factor α on activated $CD4^+$ T cell clones provides a costimulatory signal for human B cell activation. J. Exp. Med. *177*, 1575–1585.

Bigda, J., Beletsky, I., Brakebusch, C., Varfolomeev, Y., Engelmann, H., Bigda, J., Holtmann, H., and Wallach, D. (1994). Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. J. Exp. Med. *180*, 445–460.

Birkland, T.P., Sypek, J.P., and Wyler, D.J. (1992). Soluble TNF and membrane TNF expressed on $CD4^+$ T lymphocytes differ in their ability to activate macrophage antileishmanial defense. J. Leukoc. Biol. *51*, 296–299.

Brockhaus, M., Schoenfeld, H.J., Schlaeger, E.J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990). Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc. Natl. Acad. Sci. USA *87*, 3127–3131.

Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familletti, P.C., Pan, Y.-C.E., Olander, J.V., Connolly, D.T., and Stern, D. (1990). Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J. Exp. Med. *172*, 1535–1545.

Dower, S.K., Kronheim, S.R., March, C.J., Conlon, P.J., Hopp, T.P., Gillis, S., and Urdal, D.L. (1985). Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. J. Exp. Med. *162*, 501–515.

Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y.S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O., and Wallach, D. (1990). Antibodies to a soluble form of tumor necrosis factor receptor have TNF-like activity. J. Biol. Chem. *265*, 14497–14504.

Erickson, S.L., de Sauvage, F.J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K.C.F., Schreiber, R.D., Goeddel, D.V., and Moore, M.W. (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2–deficient mice. Nature *372*, 560–563.

Fiers, W. (1991). Tumor necrosis factor: characterization at the molecular, cellular and *in vivo* level. FEBS Lett. *285*, 199–212.

Gehr, G., Gentz, R., Brockhaus, M., Loetscher, H.R., and Lesslauer, W. (1992). Both tumor necrosis factor receptor types mediate proliferative signals in human mononuclear cell activation. J. Immunol. *149*, 911–917.

Grell, M., Scheurich, P., Meager, A., and Pfizenmaier, K. (1993). TR60 and TR80 tumor necrosis factor (TNF)-receptors can independently mediate cytolysis. Lymphokine Cytokine Res. *12*, 143–148.

Grell, M., Zimmermann, G., Hülser, D., Pfizenmaier, K., and Scheurich, P. (1994). TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. J. Immunol. *153*, 1963–1972.

Heller, R.A., Song, K., Fan, N., and Chang, D.J. (1992). The p70 tumor necrosis factor receptor mediates cytotoxicity. Cell *70*, 47–56.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1–associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495–504.

Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S.D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell *53*, 45–53.

Lang, G., Mamalaki, C., Greenberg, D., Yannoutsos, N., and Kioussis, D. (1991). Deletion analysis of the human CD2 gene locus control region in transgenic mice. Nucl. Acids Res. *19*, 5851–5856.

Loetscher, H., Stueber, D., Banner, D., Mackay, F., and Lesslauer, W. (1993). Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. J. Biol. Chem. *268*, 26350–26357.

Lopez-Cepero, M., Garcia-Sanz, J.A., Herbert, L., Riley, R., Handel, M.E., Podack, E.R., and Lopez, D.M. (1994). Soluble and membrane-bound TNF-α are involved in the cytotoxic activity of B cells from tumor-bearing mice against tumor targets. J. Immunol. *152*, 3333–3341.

Mackay, F., Loetscher, H., Stueber, D., Gehr, G., and Lesslauer, W. (1993). Tumor necrosis factor α (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J. Exp. Med. *177*, 1277–1286.

Menegazzi, R., Cramer, R., Patriarca, P., Scheurich, P., and Dri, P. (1994). Evidence that tumor necrosis factor α (TNFα)-induced activation of neutrophil respiratory burst on biological surfaces is mediated by the p55 TNF receptor. Blood *84*, 287–293.

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. Nucl. Acids Res. *18*, 5322–5324.

Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., and Kriegler, M. (1990). A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell *63*, 251–258.

Pfeffer, K., Matsuyama, T., Kündig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Krönke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Pober, J.S., and Cotran, R.S. (1990). Cytokines and endothelial cell biology. Physiol. Rev. *70*, 427–451.

Porteau, F., and Hieblot, C. (1994). Tumor necrosis factor induces a selective shedding of its p75 receptor from human neutrophils. J. Biol. Chem. *269*, 2834–2840.

Probert, L., Keffer, J., Corbella, P., Cazlaris, H., Patsavoudi, E., Stephens, S., Kaslaris, E., Kioussis, D., and Kollias, G. (1993). Wasting, ischemia, and lymphoid abnormalities in mice expressing T cell–targeted human tumor necrosis factor transgenes. J. Immunol. *151*, 1894–1906.

Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell *78*, 681–692.

Scheurich, P., Thoma, B., Ücer, U., and Pfizenmaier, K. (1987). Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cell responses. J. Immunol. *138*, 1786–1790.

Scheurich, P., Grell, M., Meager, A., and Pfizenmaier, K. (1992). Agonistic and antagonistic antibodies as a tool to study the functional role of human tumor necrosis factor receptors. In Tumor Necrosis Factor, Volume 4, W. Fiers and W.A. Buurman, eds. (Basel, Switzerland: Karger), pp. 52–57.

Schmidt, E., Müller, T.H., Budzinski, R.M., Binder, K., and Pfizenmaier, K. (1995a). Signalling by E-selectin and ICAM-1 induces endothelial tissue factor production via autocrine secretion of PAF and TNFα. J. Interferon Cytokine Res. *15*, 819–825.

Schmidt, E.F., Binder, K., Grell, M., Scheurich, P., and Pfizenmaier, K. (1995b). Both tumor necrosis factor receptors, $TNFR_{60}$ and $TNFR_{80}$, are involved in signaling endothelial tissue factor expression by juxtacrine tumor necrosis factor α. Blood *86*, 123–132.

Shalaby, M.R., Sundan, A., Loetscher, H., Brockhaus, M., Lesslauer, W., and Espevik, T. (1990). Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. J. Exp. Med. *172*, 1517–1520.

Sheehan, K.C.F., Pinckard, J.K., Arthur, C.D., Dehner, L.P., Goeddel., D.V., and Schreiber, R.D. (1995). Monoclonal antibodies specific for murine p55 and p75 tumor necrosis factor receptors: identification of a novel *in vivo* role for p75. J. Exp. Med. *181*, 607–617.

Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959–962.

Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995). Expression of the functional soluble form of human Fas ligand in activated lymphocytes. EMBO J. *14*, 1129–1135.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A., Jr., and Goeddel, D.V. (1991). The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc. Natl. Acad. Sci. USA *88*, 9292–9296.

Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendley, B.M., and Palladino, M.A., Jr. (1993a). Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J. Immunol. *151*, 4637–4641.

Tartaglia, L.A., Pennica, D., and Goeddel, D.V. (1993b). Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J. Biol. Chem. *268*, 18542–18548.

Thoma, B., Grell, M., Pfizenmaier, K., and Scheurich, P. (1990). Identification of a 60-kD tumor necrosis factor (TNF) receptor as the major signal transducing component in TNF responses. J. Exp. Med. *172*, 1019–1023.

Thornton, S., Mueller, S., and Levine, E. (1983). Human endothelial cells: use of heparin in cloning and long term serial cultivation. Science *222*, 623–626.

Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., and Fiers, W. (1992). Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. J. Exp. Med. *176*, 1015–1024.