Continuous Autotropic Signaling by Membrane-expressed Tumor Necrosis Factor*

(Received for publication, October 15, 1998, and in revised form, April 2, 1999)

Elvira Haas, Matthias Grell, Harald Wajant, and Peter Scheurich‡
From the Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany

Tumor necrosis factor (TNF) exists in two bioactive forms, the membrane-integrated form and the proteolytically derived soluble cytokine. Cells that produce TNF are often responsive to TNF, allowing autocrine/juxtacrine feedback loops. However, whether the membrane form of TNF is involved in such regulatory circuits is unclear. Here we demonstrate that HeLa cells, expressing a permanently membrane-integrated mutant form of TNF, constitutively express TNF-TNF receptor complexes at their cell surface. These cells show a permanent activation of the transcription factor NF-κB, exert constitutive p38 mitogen-activated protein kinase activity, and produce high amounts of interleukin-6. In parallel, transmembrane TNF-expressing HeLa cells display high sensitivity to cycloheximide or interferon-γ, similar to untransfected cells treated with these agents in combination with sTNF. Moreover, cycloheximide-induced apoptosis in transmembrane TNF transfectants can be blocked by the caspase inhibitor zVAD-fmk and does not necessarily need cell to cell contact, indicating a critical role of constitutive autotropic signaling of TNF-TNF receptor complexes. These data demonstrate that autotropic signaling loops of membrane TNF can exist, which may be of importance for cells that express both TNF and TNF receptors, such as T lymphocytes, macrophages, and endothelial cells.

Tumor necrosis factor (TNF) is the prototype cytokine of a ligand family whose members are typically expressed as type II transmembrane proteins (1). Action of a metalloproteinase (2, 3) leads to proteolytical release of soluble TNF (sTNF). Both TNF forms are bioactive and bind to two membrane receptors of 55–60 kDa (TNF-R1) and 75–80 kDa (TNF-R2). TNF-R1 is constitutively expressed in nearly all tissues and represents the main mediator of cellular TNF responses (for review, see Ref. 4). TNF-R2 is more restricted in expression, e.g. to lymphoid tissue, is tightly regulated in its expression, and modulates cellular responses to sTNF or transmembrane TNF in a cooperative manner with TNF-R1. We have recently shown that TNF-R2, in contrast to TNF-R1, needs stimulation by the membrane form of the cytokine for full activation (5). The low efficiency of sTNF for stimulation of TNF-R2 in comparison to TNF-R1 has been explained by the transient sTNF-TNF-R2 interaction at physiological temperatures (6).

The cellular response pattern to TNF stimulation is extremely broad and cell type-dependent. For example, TNF can induce apoptosis and initialize a variety of other signals, including induction of cytokine production, enhancement of adhesion molecule expression, and growth stimulation (for review, see Ref. 4). These cellular responses are caused by different intracellular signaling pathways induced by TNF-R1. One major signaling pathway of TNF-R1 leads to the activation of aspartate-directed proteinases (caspases) via the death domain proteins TNF receptor-associated death domain protein and Fas-associated death domain protein (7). Another major signaling pathway leads to the activation of the transcription factor NF-κB, N-terminal c-Jun kinase, and p38 kinase. NF-κB is believed to be activated via TNF receptor-associated protein 2 (TRAF2), a member of the TRAF family, and/or the death domain-containing protein kinase RIP (receptor interacting protein) (8, 9). Activation of NF-κB results in the production of cytokines such as interleukin (IL)-6 (10) but also induces protective mechanisms by stimulation of the expression of regulatory proteins with potential antiapoptotic activity, such as TRAF1, TRAF2, and the inhibitor of apoptosis (IAP) proteins c-IAP1 and c-IAP2 (11). In addition to this antiapoptotic activity, TRAF2 is also critically involved in a positive, proapoptotic TNF receptor cooperation, not dependent on de novo gene induction (12, 13).

TNF is produced by many different cell types including macrophages, T lymphocytes, and endothelial cells (for review, see Ref. 4). These cells often also express TNF receptors and thus display TNF responsiveness. Auto/juxtacrine-acting TNF has been implicated in monocyte-mediated cytotoxicity (14), primary T cell activation (15),2 and induction of endothelial tissue factor production by cross-linking of adhesion molecules (16). A critical involvement of transmembrane TNF signaling has been revealed in immunological reactions such as anti-leishmanial defense in macrophages, T cell/B cell interaction, and tumor cell killing by infiltrating lymphocytes (17, 18, 19). Moreover, the transgenic expression of a noncleavable and, thus, permanently membrane-anchored TNF mutein in mice is sufficient to induce a pathological phenotype similar to rheumatoid arthritis (20), underlining the high biological potential of local TNF signaling. Whether and to what extent a juxtathotropic signaling loop of transmembrane TNF plays a role in these models, i.e. whether cell surface-expressed TNF acts predominantly on neighboring cells (juxtathotropic) or in a true autotropic signaling mode, is unresolved.

To investigate the selective effects of transmembrane TNF

* This research was supported by Deutsche Forschungsgemeinschaft Grants Sch 349/5-1, Wa 1025/3-1, and Gr 1307/3-2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany. Tel.: 49 711 685 6987; Fax: 49 711 685 7484; E-mail: Peter.Scheurich@po.uni-stuttgart.de.
1 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; sTNF, soluble TNF; tTNF, transmembrane TNF; TRAF, TNF receptor-associated factor; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; IFN-γ, interferon-γ; IAP, inhibitor of apoptosis; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; MAP kinase, mitogen-activated protein kinase; mAb, monoclonal antibody.

2 M. Grell and P. Scheurich, unpublished observations.

This paper is available online at http://www.jbc.org
expression on a TNF-sensitive cell, we have generated HeLa transfectants expressing a noncleavable, constitutively membrane-bound, biologically active derivative of human TNF (He-LanTNF). We show that these cells display a phenotype similar to TNF-stimulated untransfected HeLa cells. Accordingly, TNF-mediated signaling pathways involving the transcription factor NF-κB and the p38 MAP kinase are constitutively stimulated, and high amounts of IL-6 are produced in a TNF-dependent manner. In addition, HeLaanTNF cells undergo apoptosis in the presence of cycloheximide (CHX) or interferon-γ (IFN-γ), similar to Hela cells treated with a combination of TNF and one of these reagents.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Reagents—Recombinant human TNF (specific activity 2 × 10^6 units/mg) was kindly provided by I-M. von Bredow, Knoll AG, Ludwigshafen, Germany. The TNF-R1-specific mAb H389 has been described elsewhere (21). The TNF-specific mAb T1 was provided by Dr. Boettiger, University of Stuttgart, Germany. The anti-human TNF mAb 357–101-4 was kindly provided by P. Meager (National Institute for Biological Standards and Control; Herts, United Kingdom). The fluorescein isothiocyanate-labeled goat anti-mouse IgG + IgM Ab was obtained from Dianova, Hamburg, Germany. The construct coding for a noncleavable form of human TNF (TNFα(D1–12)) was inserted into the mammalian expression vector pZEO (Invitrogen, Groningen, The Netherlands). The caspase inhibitor zVAD-fmk was purchased from Bachem AG (Bubendorf, Switzerland). All other reagents were obtained from Sigma if not otherwise stated.

**Cells—**HeLa cells and 293 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum and 2 mM l-glutamine. Liposome-mediated transfections were performed using PFPX (Invitrogen, Groningen, The Netherlands) or SuperFect (Qiagen, Hilden, Germany) according to the protocols of the manufacturers. Stable transfectants were generated by selection with 400 μg/ml Zeocin (Invitrogen, Groningen, The Netherlands), and populations of transgene-positive cells were sorted using immunofluorescent staining with TNF-specific Abs and a FACStarplus cell sorter (Becton and Dickinson, San Jose, CA).

**Cell Death Assays—**HeLa cells (1.5 × 10^6/well) were seeded into 96-well microtiter plates overnight. The next day CHX was titrated in the presence or absence of additional reagents in a final volume of 150 μl. After 18 h of culture, supernatants were discarded, and cells were washed once with PBS followed by crystal violet staining (20% methanol, 0.5% crystal violet) for 15 min. The cells were washed with H2O and air-dried. The dye was resolved with methanol for 15 min, and the absorbance was measured with an ELISA plate reader (Dynatech, Guernsey, UK). For cytotoxicity assays using IFN-γ, HeLa cells (4 × 10^5) were seeded into 96-well microtiter plates overnight. Next day the cells were treated with IFN-γ (20 ng/ml) in the absence or presence of TNF (50 ng/ml) in a final volume of 150 μl. After 3 additional days of culture, crystal violet staining was performed as described above.

**Analysis of p38 MAP Kinase Phosphorylation—**Cells (2.5 × 10^6) were seeded overnight in 100-mm culture dishes. The next day the cells were stimulated as indicated, washed once with PBS, lysed in 120 μl of SDS loading buffer, boiled for 10 min, and electrophoresed on a 12% SDS-polyacrylamide gel (20 μl/lane) under reducing conditions. After transfer onto nitrocellulose membranes, Western blots were performed according to the instructions of the manufacturer (Bio Labs, New England).

**Western Blotting—**For preparation of cytosolic extracts, 3 × 10^6 cells were seeded in 100-mm cell culture dishes and cultured overnight. After stimulation with the indicated reagents for 4 h, the cells were washed once with cold PBS, harvested, and resuspended in 200 μl of buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). After 20 min of shaking and subsequent centrifugation, the lysates containing the nuclear proteins in the supernatant were used for the electrophoretic mobility shift assay after protein determination with bovine serum albumin as the standard (Bio-Rad). High performance liquid chromatography-purified purified NF-κB-specific oligonucleotides (5′-ATCGG-GACTTTCCGGCTTGGCATTTCCGCG-3′) obtained from MWG-Biotech (Ebersberg, Germany) were end-labeled with γ-32P]ATP using T4 polynucleotide kinase. EMSAs were performed using 10 μg of nuclear extracts with 5 μg of poly(dI-dC) in a binding buffer (5 mM HEPES, pH 7.8, 5 mM MgCl2, 50 μM KCl, 0.2 mM EDTA, 5 mM dithiothreitol, 10% glycerol). The double-stranded, end-labeled, purified oligonucleotide probe (2 × 10^7–5 × 10^8 cpm) was added to the reaction mixture for 15 min at room temperature. The samples were then separated by native polyacrylamide gel electrophoresis in low ionic strength buffer.

**Luciferase Reporter Assay—**HeLa cells (1.0 × 10^6) were seeded in 96-well microtiter plates overnight and subsequently transfected with a minimal promoter containing a three-NF-κB element-driven Firefly luciferase reporter plasmid (23), kindly provided by Marius Ueffing (Gesellschaft für Strahlenforschung, Munich, Germany) using the SuperFect reagent (Qiagen). After 1 day of recovery, the cells were treated as indicated, washed once in PBS, and harvested in 50 μl of lysis buffer (Promega, Madison, WI). 45-μl aliquots of cell lysates were mixed with 10 μl of luciferase assay reagent (Promega), and the luciferase activity was determined using a Lumat LB9501 (Berthold, Wildbad, Germany). For normalization of transfection efficiency, a Renilla (control) luciferase-encoding plasmid (pRL-TK, Promega) was co-transfected, and luciferase activity was determined with a second luciferase substrate after quenching the light reaction of the first luciferase substrate in a single step following the the manufacturer's instructions (dual-luciferase reporter assay system, Promega).

**RESULTS**

Coexpression of TNF-R1 and tmTNF in TNFfL(C1–12)-transfected HeLa Cells—Transfection of cells with an expression construct for human TNF in which the codons for the amino acids 1 to 12 of the soluble TNF have been deleted results in the expression of the bioactive transmembrane TNFα(D1–12) (tmTNF) without production of detectable amounts of bioactive sTNF (22, 5). To characterize possible autostimulatory effects of the
membrane-expressed form of TNF, we transfected tmTNF into HeLa cells, a cell line that reacts to TNF with a variety of cellular responses. Three independent pools of transfected cells (HeLa-tmTNF) were established by cell sorting. High levels of cell surface-expressed TNF could be readily detected by flow cytometry (Fig. 1A). Virtually no cell surface-expressed TNF-R1 was found on HeLa-tmTNF cells in contrast to untransfected control vector-transfected HeLa cells using the TNF-R1-specific mAb H398 (Fig. 1C and data not shown).

The lack of detectable amounts of TNF-R1 on HeLa-tmTNF cells could represent down-regulation of cell surface-expressed TNF-R1 by endogenous tmTNF and/or formation of cell surface-expressed tmTNF-TNF-R1 complexes in which the H398 binding epitope is masked. In fact, after treatment of HeLa-tmTNF cells with an acidic buffer, pH 3.0, to disrupt putative tmTNF-TNF-R1 complexes, H398 specifically bound to HeLa-tmTNF (Fig. 1B) in amounts comparable with untreated (Fig. 1C) or pH 3.0-treated HeLa cells (Fig. 1D). Equilibrium binding studies performed with radiolabeled TNF at 0 °C confirmed the absence of free TNF-R1 and the presence of tmTNF-TNF-R1 complexes at the cell surface of HeLa-tmTNF cells (Fig. 1E). No specific binding of the label was obtained with untreated cells, whereas specific 125I-TNF binding capacity of pH 3.0-treated HeLa-tmTNF cells was about 50% that of parental HeLa cells. The presence of cell surface-expressed tmTNF-TNF-R1 complexes was further substantiated by ligand association studies at 37 °C. Under these conditions a rapid and specific association of 125I-TNF to otherwise untreated HeLa-tmTNF cells could be observed (Fig. 1F). The association rate was similar to that observed with HeLa cells (Fig. 1F).

Constitutive IL-6 Production, Activation of NF-κB, and p38 MAP Kinase in HeLa-tmTNF Cells—The permanent presence of tmTNF-TNF-R1 complexes at the cell surface implies that continuous TNF signaling in HeLa-tmTNF cells may occur. A typical cellular response induced by TNF-R1 triggering is the stimulation of IL-6 gene expression. In fact, HeLa-tmTNF cells continuously produced high amounts of this cytokine, comparable with that of HeLa cells stimulated with sTNF (Fig. 2A). Addition of exogenous sTNF did not further enhance IL-6 production. Incubation of HeLa-tmTNF cells with TNF-specific neutralizing antibodies inhibited the constitutive IL-6 production, although the inhibition was only partial at high concentrations of the antibody used (Fig. 2B). This is in agreement with our own findings that neutralization of the effects of transmembrane TNF versus sTNF generally requires much higher TNF-specific antibody concentrations.3 These data further argue for permanent signaling of cell surface-expressed tmTNF-TNF-R1 complexes in HeLa-tmTNF cells.

Because IL-6 production has been shown to be critically dependent on the transcription factor NF-κB (10), we used a transient reporter gene assay in which gene expression is under the control of a NF-κB minimal promoter to investigate NF-κB activation in HeLa-tmTNF cells. The data revealed a significant, constitutive NF-κB activation (Fig. 3A) that could be confirmed by EMSA, demonstrating a permanent nuclear translocation of NF-κB in all three HeLa-tmTNF cell pools (Fig. 3B). NF-κB activation in HeLa-tmTNF cells was lower as compared with TNF-treated HeLa cells, but treatment with exogenous sTNF did not further enhance NF-κB activation (Fig. 3, A and B). A permanently activated status of NF-κB, driven by endogenous tmTNF, was confirmed in 2 distinct 293-cell populations stably transfected with TNFα (1–12) (Fig. 3C, inset) showing a strong constitutive NF-κB activation comparable with sTNF-treated 293 cells (Fig. 3C).

Next, we looked for activation of the p38 kinase pathway, which has been demonstrated to be also critically involved in TNF-induced IL-6 production (24). Western blot analyses with antibodies specific for phosphorylated p38 kinase revealed an activation of this signaling pathway in HeLa-tmTNF cells (Fig. 3D).

HeLa-tmTNF Cells Are Sensitive to Apoptosis by the Addition of CHX or IFN-γ—HeLa cells are relatively resistant to the cytotoxic effects of TNF. However, in the presence of the protein synthesis inhibitor CHX, TNF induces apoptosis, indicative for protective mechanisms dependent on de novo protein synthesis (25). We therefore asked whether HeLa-tmTNF cells are sensitive to the action of the protein synthesis inhibitor CHX. In fact, HeLa-tmTNF cells were highly susceptible, as the ED50 for CHX-

\(^3\) M. Grell, unpublished observations.
induced cytotoxicity was about 1 μg/ml, whereas the majority of HeLa cells survived CHX concentrations as high as 60 μg/ml (Fig. 4A). A combination of sTNF and CHX did not further enhance cytotoxicity in HeLa tmTNF cells, whereas HeLa cells showed the expected strong cytotoxic response (not shown). In HeLa tmTNF cells, CHX-induced cytotoxicity could be blocked by the broad spectrum caspase inhibitor zVAD-fmk (Fig. 4A). TNF is known to induce cell death synergistically with IFN-γ (26). Fig. 3 shows that the HeLa tmTNF cells can be killed by the sole addition of IFN-γ, whereas HeLa cells are resistant to this treatment. To confirm that the cytotoxic effects in HeLa tmTNF cells do reflect apoptosis, we investigated the central apoptosis executioner caspase-3, which is known to be activated by proteolytic cleavage (27). HeLa tmTNF cells showed an almost complete caspase-3 activation after treatment with CHX only (Fig. 4C), like HeLa cells upon combined CHX and sTNF treatment. Together, these data demonstrate that expression of tmTNF in HeLa cells initiates the apoptotic signal cascade in a similar way, like sTNF in parental HeLa cells, which is in both cases counterbalanced by CHX/IFN-γ-sensitive factors.

Transmembrane TNF Can Act at the Single Cell Level in an Autotropic Manner—In the experimental settings described above, cells had been cultured as confluent monolayers, which allows in principle both stimulation of TNF-R1 on the neighboring cell (juxtatropic) as well as on the TNF-expressing cell (autotropic). To investigate whether tmTNF is able to stimulate TNF-R1 of the very same cell, i.e. in an autotropic manner, HeLa tmTNF cells were seeded at a statistical density of 1 cell/well and then treated with CHX for 18 h. After diluting off the CHX, the percentage of surviving cells was determined by analysis of growing colonies after 14 days of culture. CHX exerted a strong cytotoxic effect on HeLa tmTNF cells but not on HeLa cells (Fig. 5), similar to the experiments using confluent cultures (Fig. 4A). The addition of zVAD-fmk inhibited CHX-induced cytotoxicity (Fig. 5), confirming that CHX treatment induces cell death in HeLa tmTNF cells in a caspase-dependent manner. More important, the results strongly suggest that tmTNF can stimulate TNF-R1 in an autotropic manner, i.e. by ligand/receptor interaction at the single cell level.

FIG. 2. Constitutive IL-6 production in HeLa tmTNF cells. A, the concentration of IL-6 in the supernatants of the indicated cells cultured in the presence or absence of TNF (10 ng/ml) for 6 h was determined by an IL-6-specific ELISA. The results of a representative experiment are shown (n = 3). B, effects of neutralization of TNF on the constitutive IL-6 production of HeLa tmTNF cells. Cells were cultured for 5 days with the indicated concentrations of a neutralizing TNF-specific antibody (mAb 357-101-4) or control mouse IgG and washed, and IL-6 production was determined after a further 6-h culture in the presence of the indicated antibodies.

FIG. 3. Constitutive activation of NF-κB and p38 MAP kinase in tmTNF-expressing cells. A, activation of a NF-κB-dependent reporter gene was determined 24 h after transient transfection of the indicated cells with a NF-κB-driven luciferase reporter plasmid in the absence or presence of soluble TNF (20 ng/ml) for 6 h. Luciferase activity was determined, and transfection efficiency was normalized by cotransfection of a control luciferase reporter plasmid. B, activation of NF-κB in HeLa tmTNF cells and HeLa cells was analyzed by EMSA using nuclear extracts of untreated or TNF-treated (30 min; 20 ng/ml) cells with a 32P-labeled NF-κB-specific oligonucleotide probe. C, pools of 293 cells stable-transfected with TNFΔD (1–12) and untransfected 293 cells were analyzed for NF-κB activation by luciferase reporter gene assays as described in A. Expression of tmTNF was controlled by immunofluorescent staining and fluorescence-activated cell sorter analysis using the TNF-specific mAb T1 (inset). D, activation of p38 MAP kinase in HeLa tmTNF cells and HeLa cells was determined by Western blotting with anti-phospho-p38 MAP kinase antibody (p38 (top panel)) and with anti-p38 MAP kinase antibody (p38 (bottom panel)) using cell lysates of untreated or TNF-treated (20 ng/ml, 15 min) cells.

FIG. 4. A, IL-6 production in HeLa tmTNF cells. B, IL-6 production in HeLa tmTNF cells after treatment with TNF (10 ng/ml) for 6 h. C, IL-6 production in HeLa tmTNF cells after treatment with TNF (10 ng/ml) for 6 h. D, IL-6 production in HeLa tmTNF cells after treatment with TNF (10 ng/ml) for 6 h.
brane TNF. Inhibition of protein synthesis in HeLa<sub>tmTNF</sub> cells induces cytotoxicity in a caspase-dependent manner. <i>A</i>, cell viability of untreated or zVAD-fmk-treated (20 μM) HeLa<sub>tmTNF</sub> cells and HeLa cells was determined in the presence of titrated concentrations of CHX after 18 h by crystal violet staining. <i>B</i>, cell viability of indicated cells was determined after treatment with IFN-γ (20 ng/ml) in the presence or absence of TNF (50 ng/ml) for 3 days by crystal violet staining. The results given show the mean values ± S.D. from three independent experiments, each performed in duplicate in percent of viable cells (100% = untreated controls). C, caspase-3 degradation in HeLa<sub>tmTNF</sub> cells and HeLa cells was determined by Western blotting after treatment with CHX and TNF as indicated for 4 h.

![Image](50x367 to 297x474)

![Image](50x348)

than cellular unresponsiveness. As a model, we have chosen the human cell line HeLa, which expresses about 3,000 molecules of TNF-R1/cell but only negligible amounts of TNF-R2 (28, 12). HeLa cells are largely unresponsive to the cytotoxic activity of TNF per se but can be rendered sensitive to treatment with e.g. CHX or IFN-γ. This cellular response pattern resembles that of normal tissue cells, which are typically resistant to the cytotoxic action of TNF unless metabolically stressed by e.g. virus infection (29). In addition, HeLa cells are well known to respond to TNF with activation of the transcription factor NF-xB and production of IL-6 (12). To study membrane TNF action under conditions where effects by soluble TNF can be avoided, TNF was constitutively expressed as a permanently membrane-anchored mutein, human Δ(1–12) TNF (tmTNF) (22). In the mouse system, the homologue Δ(1–12) deletion mutant of mouse TNF has a reduced bioactivity (30) and only partly down-regulates TNF receptor expression in L929 cells (31). It is therefore important to mention that the Δ(1–12) deletion mutant of human TNF shows full bioactivity on human cells. We have recently demonstrated that the naturally occurring transmembrane form of TNF as well as the Δ(1–12) deletion mutant derived thereof have superior bioactivity on TNF-R2 when compared with sTNF (5). When acting on TNF-R1, both molecules possess a bioactivity indistinguishable from sTNF.4

HeLa cells transfected with human Δ(1–12) TNF (HeLa<sub>tmTNF</sub>) expressed cell surface TNF in high amounts and did not release bioactive soluble TNF into the culture supernatants (data not shown). They further expressed considerable amounts of tmTNF-TNF-R1 complexes at the cell surface. This is indicated by the facts that free TNF-R1 was detectable only after a pH 3.0 treatment of the cells (Fig. 1) and that constitutive IL-6 production could be inhibited by TNF-specific antibodies (Fig. 2B). More important, the latter data also indicate that cell surface-expressed tmTNF-TNF-R1 complexes are functional with respect to signal transduction, although it cannot be excluded that in addition, signaling from intracellular complexes might occur. Accordingly, HeLa<sub>tmTNF</sub> cells show a phenotype similar to sTNF-stimulated HeLa cells. The transcription factor NF-xB (Fig. 3, A and B) and the p38 MAP kinase (Fig. 3D) are constitutively activated, and consequently, the cells produce high amounts of IL-6 (Fig. 2A). In addition, HeLa<sub>tmTNF</sub> cells undergo apoptosis in the presence of CHX (Fig. 4A) or IFN-γ (Fig. 4B). Together, these data strongly support the hypothesis that in HeLa<sub>tmTNF</sub> cells, TNF signaling pathways are permanently activated by the action of endogenous tmTNF. Analysis at the single cell level (Fig. 5) indicates that tmTNF action can occur in a truly autotropic manner. Closer analysis of the constitutive activation pattern of HeLa<sub>tmTNF</sub> cells revealed a differential regulation of the signaling pathways; NF-xB seems to be only partly activated in HeLa<sub>tmTNF</sub> cells, whereas IL-6 production and the kinetics of induction of apoptosis in the presence of CHX were quantitative similar to sTNF-treated HeLa cells (Fig. 2A and data not shown). These differences might reflect differential regulation of responses to the permanent tmTNF stimulation in HeLa<sub>tmTNF</sub> cells versus short term sTNF treatment of HeLa cells. Long term sTNF-stimulated HeLa cells show in fact a signifi-

4 M. Grell, manuscript in preparation.
cant NF-κB down-regulation (data not shown). Using the TNF-sensitive mouse cell line L929, Decoster et al. (31) have recently demonstrated that expression of tmTNF resulted in a total TNF receptor downmodulation paralleled by full TNF unresponsiveness. Obviously, induction of unresponsiveness induced by the autotropic action of TNF was dependent on the transmembrane localization of the cytokine, as a secretable TNF mutein was ineffective in this regard (31). These data are clearly at variance to our results. However, we believe that the TNF-induced cellular stimulation described here is not cell-specific but rather reflects a physiological relevant response pattern. First, identical results have been obtained using two distinct cell lines, HeLa and 293 (Fig. 3, A and C). Second, recent data confirm an autocrine, TNF-mediated permanent stimulation of NF-κB in HuT-78 cells (32). Third, it is unlikely that full TNF unresponsiveness is the typical cellular response pattern for normal, TNF-R1-positive tissue cells that are also capable of producing TNF. For example, human umbilical cord vein endothelial cells coexpress both TNF receptors and produce tissue factor upon adhesion molecule crosslinking via autocrine/juxtacrine TNF action (16).

With regard to a potential cellular TNF response in vivo, we suggest that the response pattern observed here could be of physiological relevance. We show that cells capable of producing TNF are principally able to autostimulate themselves via a tmTNF-mediated autotropic signaling pathways and do not enter the apoptotic pathway. In the case of a prolonged tmTNF-mediated stimulus, as mimicked here by constitutive expression plasmids. The expert technical assistance of Guidrun Zimmermann, Gisela Schubert, and Nathalie Peters was gratefully acknowledged.

REFERENCES

1. Wallach, D., Boldin, M., Varfolomeev, E., Beyaert, R., Vandenabeele, P., and Fiers, W. (1997) FEBS Lett. 410, 96–100
2. Mose, M. L., Jin, S. L., Cilla, M. E., Barkhart, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, P., Seaton, T., Su, J. L., Warner, J., Willard, D., and Becherer, J. D. (1997) Nature 385, 738–742
3. Blacic, R. A., Rauch, C. T., Kozlosky, C. J., Peschina, J. J., Slack, J. L., Woldson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Sririvasa, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Dalis, R., Fitzer, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) Nature 385, 729–733
4. Grell, M., and Scheurich, P. (1997) in Growth Factors and Cytokines in Health and Disease (LeRoith, D., and Bondy, C., eds) pp. 669–726, Jai Press Inc., Greenwich, CT
5. Grell, M., Doun, E., Wajant, H., Lohnden, M., Claus, M., Maxeiner, B., Georgopouloos, S., Lesslauer, W., Kollia, G., Pfizenmaier, K., and Scheurich, P. (1995) Cell 83, 793–802
6. Grell, M., Wajant, H., Zimmermann, G., and Scheurich, P. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 570–575
7. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
8. Liu, Z. G., Hsu, H., Goeddel, D. V., and Karin, M. (1996) Cell 87, 565–576
9. Kolbher, M. A., Grimm, S., Ishida, Y., Kuo, P., Stanger, B. Z., and Leder, P. (1998) Immunity 8, 297–303
10. Libermann, T. A., and Baltimore, D. (1990) Mol. Cell. Biol. 10, 2327–2334
11. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S. (1998) Science 281, 1680–1683
12. Weiss, T., Grell, M., Hessabi, B., Bourteille, S., Muller, G., Scheurich, P., and Wajant, H. (1997) J. Immunol. 158, 2398–2404
13. Weiss, T., Grell, M., Siemonoki, K., Mulchenbeck, F., Durrkop, H., Pfizenmaier, K., Scheurich, P., and Wajant, H. (1998) J. Immunol. 161, 3136–3142
14. Smith, D. M., Lackides, G. A., and Epstein, L. B. (1998) Cancer Res. 58, 3146–3153
15. Pintet-Munos, F. X., Mazana, J., and Fresno, M. (1994) J. Biol. Chem. 269, 24244–24429
16. Schmid, E. P., Binder, K., Grell, M., Scheurich, P., and Pfizenmaier, K. (1995) Blood 86, 1836–1841
17. Birkland, T. P., Syeep, J. P., and Wyler, D. J. (1992) J. Leukocyte Biol. 51, 296–299
18. Aversa, G., Punnonen, J., and de Vries, J. E. (1993) J. Exp. Med. 177, 1575–1585
19. Lopez-Cepero, M., Garcia-Sanz, J. A., Herbert, L., Riley, R., Handel, M. E., Podack, E. R., and Lopez, D. M. (1994) J. Immunol. 152, 3333–3341
20. Alexopoulou, L., Pasparakis, M., and Kollia, G. (1997) Eur. J. Immunol. 27, 2588–2592
21. Thoma, B., Grell, M., Pfizenmaier, K., and Scheurich, P. (1990) J. Exp. Med. 172, 1019–1023
22. Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooden, L., and Krieger, M. C. (1990) Cell 63, 251–258
23. Mitchell, T., and Sugden, B. (1995) J. Virol. 69, 2968–2976
24. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeli, P., Cohen, P., and Fiers, W. (1996) EMBO J. 15, 1914–1923
25. Wallach, D., Hoffmann, H., Engelmann, H., and Nohara, Y. (1988) J. Immunol. 140, 2994–2999
26. Scheurich, P., Uer, U., Ronke, M., and Pfizenmaier, K. (1986) Int. J. Cancer 38, 127–133
27. Quan, L. T., Tewari, M., O'Rourke, K., Dixit, V., Snipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J., and Salvesen, G. S. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1972–1976
28. Keller, B. A., Song, K., Pan, N., and Chang, D. J. (1992) Cell 70, 47–56
29. Wong, G. H., Taglialia, L. A., Lee, M. S., and Goeddel, D. V. (1992) J. Immunol. 149, 3350–3353
30. Decoster, E., Vanhaesebroeck, B., Vandenabeele, P., Grooten, J., and Fiers, W. (1995) J. Biol. Chem. 270, 18473–18478
31. Decoster, E., Vanhaesebroeck, B., Boone, E., Plaisance, S., Devois, K., Hageman, G., Grooten, J., and Fiers, W. (1998) J. Biol. Chem. 273, 3271–3277
32. Giri, D. K., and Aggarwal, B. B. (1998) J. Biol. Chem. 273, 14008–14014