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Receptor-like properties of the 26 kDa transmembrane form of TNF

Abstract

Most members of the TNF family of proteins exist as transmembrane proteins with relatively long intracellular domains, and a number of them are involved in the ill-defined phenomenon of "reverse signaling". We have identified a putative nuclear localization signal in the cytoplasmic domain of TNF which proved to be functional in two assays. Western analysis identified an approximately 10 kDa peptide corresponding to the transmembrane and cytoplasmic domains of TNF after the proteolytic liberation of the 17 kDa, soluble form of TNF. This 10 kDa peptide was enriched in internal membranes and nuclear fractions of disrupted cells. Immune electron-microscopic studies proved its localization in transport

vesicles and the nucleus. The nuclear transport of the intracellular segment of TNF resembles the signaling process through the Notch-type of receptors. Indeed, the presence of the 10 kDa peptide seems to influence the expression of another inflammatory cytokine, interleukin-1 beta. These findings suggest that the transmembrane form of TNF has receptor-like properties and its interaction with the receptors initiates a bidirectional signaling.

Full text

ABBREVIATIONS

ICD: intracellular domain

IL-1: interleukin-1 beta

NLS: nuclear localization signal

PE: phycoerythrine

TNF: tumor necrosis factor alpha

INTRODUCTION

Tumor necrosis factor alpha (TNF) is described as a soluble, anti-tumor, pro-inflammatory cytokine, and a key modulator of the activity of the immune system [1, 2]. Depending on the type and developmental stage of target cells, TNF can induce activation, proliferation, differentiation or self-destruction of cells [3]. TNF is produced by many types of immune-related cells (monocytes, activated T and B cells, hematopoietic progenitors, microglia, Kupffer and mesangial cells, etc. [4]), some of them undergoing subsequent differentiation steps. Since high levels of circulating TNF cause very severe systemic effects [5], TNF synthesis is strictly regulated both at the transcriptional and translational level [6], and even the proteolytic processing and liberation of the soluble cytokine is controlled [7].

The primary translational product of the TNF gene is a type II transmembrane protein, which is fully active in biological assays [8], as both the cell-associated

and soluble forms of TNF bind to cell surface receptors inducing characteristic signaling events [9]. TNF and members of the TNF family of proteins exist as integral membrane proteins [10], although some are, also produced in soluble form. The same is true for TNF receptors and related proteins belonging to the TNF/NGF receptor family [9]. Several experiments have suggested that the transmembrane form of TNF alters the properties of the cells which produce it [11, 12]. This phenomenon termed "reverse signaling" has also been observed in the case of several TNF family members: TNF-alpha [13, 14] and ligands of CD30 [15], CD40 [16], Fas [17], 4-1BB [18].

Previously we have shown that the cytoplasmic domain of the 26 kD form was serine-phosphorylated [19] by a casein kinase and clustering of the protein elicited dephosphorylation and subsequent Ca^{2+} mobilization [20]. Sequence searches identified a putative nuclear localization signal (NLS) in the N terminal, intracellular domain of TNF. We hypothesized, that the transmembrane form of TNF is not only a ligand, but might be a itself receptor .

MATERIALS AND METHODS

Cell lines and tissue culturing

HeLa and transformed HeLa cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Sigma-Aldrich Kft, Budapest). HeLa-A11 and HeLa-M9 are genetically modified HeLa cell lines which constitutively express the wild type and a mutant form of human proTNF [19]. The P388D mouse myelomonocytoid leukemia cell line was grown in RPMI 1640 plus 5% FCS.

Peptides and reagents

TNF peptide A (EALPKKTGGPQGSRRGLYLC), containing the putative nuclear localization sequence and peptide B (MSTESMIRDVELAEEALP-KKTGGPQGSRRGLYLC), corresponding to the intracellular domain of TNF were synthesized on an Advanced ChemTech Model 200 peptide synthesizer according to the manufacturer's recommendations, using well-established solid-phase methods [21]. The peptide was purified by reverse phase high performance liquid chromatography on a Delta Pak C18300 A (Waters) column.

Phycoerythrin was the kind gift of Drs. M. Debreczeny and B. Szalontay (Institute of Biophysics, Biological Research Center, Szeged, Hungary). Anti-FLAG antibody (anti-FLAG-M5) was purchased from Sigma (Sigma-Aldrich KFT, Budapest).

DNA constructs, oligonucleotides, transformation

The double-stranded oligonucleotide encoding the FLAG epitope was cloned into the N terminal of the mouse proTNF cDNA in $pcDNA_3$ eukaryotic expression vector (Invitrogen). The cDNA coding for the soluble form of the p55 TNF receptor was a kind gift from Dr. David Wallach, (Weizmann Institute, Rehovot). CAT reporter gene constructs controlled by IL-1 beta promoter fragments XT-CAT (- 3,757 to + 12) and P_2T-CAT (- 3,132 to + 12) [22], were generous gifts from Dr. Philip E. Auron (Harvard Medical School, Boston). pCAT3-ctrl plasmid was purchased from Promega. Two TNF constructs were made coding for only the N-terminal intracellular domain (ICD), or the whole peptide (AF) left in the membrane after the processing of TNF (intracellular *plus* the transmembrane domains with a short sequence of the extracellular domain), respectively. The TNF sequences were inserted into the $pcDNA_3$ vector (Invitrogen).

Transformation of HeLa cells was carried out according to standard procedures using calcium phosphate co-precipitation or liposomes. For promoter assay transient transfections, HeLa cells were plated 7.5×10^5 cells/well onto a 6-well plate one day before the transfection. The transforming mixture contained the following: 1 ml serum-free OptiMEM (GIBCO); 1 mug of IL-1 beta promoter construct XT-CAT or P_2T-CAT; 1 mug of $pcDNA_3$ vector, or $pcDNA_3$-AF, or $pcDNA_3$-ICD; and 0.5 mug of the pGL3-ctrl (Promega) firefly luciferase positive control vector; 15 mug Pfx-2 liposome (Invitrogen). After 5 hours of transfection, the medium was changed for culture medium, and the cells were incubated for 48 hours.

In vitro *nuclear transport in digitonin-permeabilized cells*

Phycoerythrin has a subunit structure of a_4b_2 with a native molecular weight of 132 kD. Transport substrate was prepared as described in [23].

Subconfluent HeLa cultures grown on cover slips were permeabilized with digitonin as described previously [23, 24]. The cover slips were then placed on a

0.05 ml drop of transport mixture and incubated for 30 min at the indicated temperature, in a humidified chamber. The transport mixture contained 1 mg/ml BSA and 500 ng of peptide-conjugated transport substrate dissolved in transport buffer [24]. The transport mixture was supplemented either with 1 mM ATP (Sigma), 5 mM creatine phosphate (Sigma) and 20 U/ml creatine phosphokinase (Sigma) or with 5 mg/ml HeLa cytosolic protein extract, as indicated. HeLa cytosolic protein extract was prepared as described by Adam *et al.* [24].

Evaluation was as described in [23]. We used an Olympos Vanox-S AH2 microscope equipped with SPlan 10NH and DPlan Apo100 UVPL objectives.

Anti-ICD antibody and Western blotting

The polyclonal anti-ICD antiserum was developed in rabbits against the mixture of TNF peptide A and B cross-linked to BSA. The antibody was affinity purified and its titer was measured in an ELISA.

After washing the cells three times with ice-cold phosphate-buffered saline (PBS), cells were either suspended in SDS-PAGE sample buffer (for electrophoresis) or in lysis buffer (for preparation of subcellular fractionations). Protein samples were separated by tris-tricine SDS-PAGE [25], and transferred onto nitrocellulose membrane (Amersham). Immunoblots were made according to standard protocols with a minor modification: the recognition of the antigen by the first antibody was carried out in a high salt buffer (500 mM NaCl in PBS, 0.1% Tween), in order to lower the background. Anti-TNF peptide A and B antibodies were diluted 2,000 times, the second antibody was horseradish peroxidase-labelled anti-rabbit serum (Sigma). The bands were visualized by luminol chemiluminescence.

Subcellular fractions

The cells were washed three times with ice-cold phosphate-buffered saline (PBS), then suspended in the lysis buffer (10 mM Tris pH 8.0, 3 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1% aprotinin). After Dounce homogenization, the undisrupted cells were centrifuged at 2,000 rpm for 5 min, then the nuclear/membrane fractions were centrifuged at 15,000 rpm for 20 min.

The pellet was resuspended in SDS-PAGE sample buffer and separated by SDS-PAGE.

Immunocytochemistry

After smooth mechanical scraping, the harvested cells were washed with PBS and fixed in 0.1 M sodium cacodylate-buffered fixative containing 3.0% (w/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde, 1.0% (w/v) sucrose and 3 mM $CaCl_2$ at 4° C for 2 hours. Fixed samples were rinsed in cacodylate buffer overnight, post-fixed with 0.5% (w/v) OsO_4, en bloc stained with 2.5% (w/v) uranyl acetate, dehydrated with graded ethanol and then embedded in Araldite (Durcupan, Fluca). Immunogold labeling was performed on 80-90 nm ultrathin sections with a slightly modified three-step biotin, anti-biotin-gold conjugate method described previously [26]. For the ultrastructural localization of TNF, the rabbit polyclonal antibody (anti-ICD) was used at a dilution of 1/100. The dilution of the monoclonal anti-FLAG-M5 was 1/20. Control sections were treated identically, except for the omission of the primary or biotin-conjugated secondary antibody. Immunogold-labelled sections were counterstained with lead citrate prior to ultrastructural analysis with a JEOL 100-CX electron microscope.

Ribonuclease protection assay (RPA)

Total cellular RNA was prepared from P388D mouse myeloid cells after LPS induction by the guanidium isothyocianate method according to standard protocols, and the integrity of the RNA samples was checked by running 5 mug RNA on formaldehyde gels.

RNase protection assay (RPA) was carried out with the ML-11 multiple probe set (kind gift of Dr. M.V. Hobbs, Scripps Research Institute, La Jolla, CA, USA) using 10 mug of total cellular RNA essentially as described earlier [27]. In each gel, 1/10 part of the total amount of probe and a commonly used mixed liver sample from LPS treated mice were included as molecular weight marker and positive control, respectively. RPA gels were visualized and quantified using a Phosphorimager 445SI (Molecular Dynamics).

CAT assay

Forty-eight hours after transfection, HeLa cells were washed twice with PBS, scraped with a rubber policeman and collected by centrifugation (2,000 rpm, 5 min): 80% of the cells were resuspended in 250 mM Tris (pH 7.5). After three of freeze-thaw cycles, lysates were heat inactivated at 65° C for 10 min and centrifuged at 4° C for 10 min. The CAT assays were carried out using 129 mul of the supernatant and 20 mul of 3.5 mM acetyl coenzyme A (Sigma), and 25 nCi of [^{14}C] chloramphenicol (Amersham Pharmacia). The 3 hours reaction product was extracted with ethyl acetate (Sigma) then applied on thin-layer chromatography plates (TLC, Merck) and developed in a 95% chloroform, 5% methanol solvent. The CAT activities were quantified by using a Phosphorimager 445SI (Molecular Dynamics) ImageQuant software.

Firefly luciferase activity was used to normalize transfection efficiencies among experiments. Twenty percent of the transformed cells were lysed by Cell Culture Lysis Reagent (Promega), and the luciferase assay was carried out with firefly luciferase substrate (Promega).

RESULTS

The NLS sequence is functional

First we investigated the nuclear localization function of a segment of the N-terminal intracellular domain (ICD) of the 26 kDa form of TNF. The corresponding peptide EALP**KKTGGPQGSRR**GLYLC (the putative NLS in bold) was chemically synthesized and conjugated to phycoerythrin (PE), a large, fluorescent protein. The presumed nuclear localization signal made up the first 15 amino acids of this peptide, while the 5 C-terminal amino acids were added to promote the accessibility of NLS-binding proteins. Phycoerythrin has a hexameric structure. The average number of conjugated peptides was estimated to be between 6-7 NLS/PE subunit (according to SDS-polyacrylamide gel analysis, not shown).

The nuclear transport of the phycoerythrin-peptide conjugate (PE-peptide) was studied in digitonin-permeabilized HeLa cells. As shown in Figure 1A, phycoerythrin was unable to enter the nucleus by passive diffusion due to its large size (132 kD), and its transport was not facilitated by the addition of ATP and HeLa cytosol. The PE-peptide conjugate however, was very efficiently transported to the nucleus of digitonin-permeabilized HeLa cells at 30° C in the presence of ATP and HeLa cytosolic protein extract (Figure 1E), indicating that

the peptide sequence conjugated to PE can function as an NLS. The nuclear transport of PE-peptide conjugate is absolutely energy dependent and completely blocked at zero degree centigrade (Figure 1B).

If the transport reaction was incubated at 30° C, in the presence of ATP but without exogenously added HeLa cytosol, there was a minimal level of nuclear (mainly nucleolar) accumulation of the transport substrate (Figure 1C). This residual nuclear transport may be due to an incomplete extraction of cytosolic proteins involved in the nuclear transport during the permeabilization step.

The PE-peptide conjugate was targeted to the nuclear membrane if the experiment was performed in the presence of HeLa cytosol, but in the absence of ATP, indicating that membrane assosiation is ATP-independent and requires only the presence of cytosolic proteins. The translocation step, however, was blocked in the absence of ATP (Figure 1D).

Wheat germ agglutinin (WGA) is a strong inhibitor of nuclear transport [28]. As shown in Figure 1F, WGA strongly inhibited the nuclear translocation of PE-peptide conjugate, while the perinuclear accumulation was not affected.

Size and transport of the processed peptide

The 26 kDa form of TNF is processed by TACE, a metalloproteinase [reviewed in 29]. Cleavage of the precursor yields the 17 kDa soluble cytokine molecule, leaving the transmembrane and intracellular domains (76 and 79 amino acid residues in human and mouse TNF, respectively) cell-bound. We raised polyclonal antibodies against a synthetic peptide corresponding to amino acids 1-33 in order to follow the fate of the cleavage product.

A11 and M9 clones of genetically modified HeLa cells expressing the human TNF gene at very high levels, were used to produce subcellular fractions enriched in plasma membrane, inner membranes and nuclei. The tris-tricine buffer system [25] was used for electrophoresis to achieve excellent resolution in the low molecular mass range. Western analysis of TNF producing cell extracts and subcellular fractions detected the intact 26 kDa form of TNF and 3-4 close bands with apparent electrophoretic mobilities corresponding to 9-11 kDa (Figure 2A). The size of these polypeptides indicated that the transmembrane and

intracellular domains were not separated by proteolytic cleavage. No immunoreactive proteins were detected in untransformed, control HeLa cells.

Subcellular fractions rich in plasma membrane vesicles contained greater amounts of the 26 kDa form, while the 9-11 kDa bands were more abundant in fractions rich in nuclei and inner membranes (Figure 2B). No smaller peptides in the 3-5 kDa range (corresponding to the synthetic peptides) were detected by the anti-TNF cytoplasmic domain serum in these gels. The presence of several 9-11 kDa bands could have indicated the presence of differently modified forms or cleavage of the precursor at different locations (as reported earlier by Cseh and Beutler [30]).

Subcellular localization of the cell-associated fragment of TNF

The exact subcellular localization of the immunoreactive peptide was determined by immunoelectron microscopic investigations. TNF-over-producing cells (A11) were labelled by the immunogold technique, which detects mostly accumulated antigens, not single molecules. The ultrastructural analysis of the gold-labelled sections of these cells indicated that the cell-bound cleavage product of the processed TNF was localized in the nuclei and in cytoplasmic multivesicular bodies (MVB) (Figure 3). Clusters of gold particles were seen close to nuclear pores (Figure 3C). Control HeLa cells did not show any immunogold labelling in similar experiments.

A recent paper reported that the majority of the 26 kDa form of TNF is not present on the cell surface but in Golgi membranes [31]. Therefore, vesicular transport of TNF from the trans-Golgi network to the plasma membrane might be responsible for part of the labeling in our MVB.

As the antibody reacted with the cytoplasmic domain of TNF, we had only indirect information about the fate of the transmembrane domain. Probably the hydrophobic, membrane-spanning sequence was not degraded during the vesicular transport. Experiments using leupeptin, a specific inhibitor of lysosomal protein degradation did not change the localization of the peptide and the electrophoretic mobility of the immunoreactive material, proving that proteolytic processing of the 9-11 kDa peptide(s) was not a prerequisite for nuclear import. Western analysis of the low molecular mass peptides

(corresponding to the cytoplasmic tail) could not detect any immunoreactive peptides of smaller size.

To rule out possible artefacts (immunogold labeling of cross-reactive proteins in the nuclei), we produced a modified TNF gene tagged with a FLAG sequence built into the cytoplasmic tail of TNF. Cells expressing the corresponding protein at relatively high levels were labeled with anti-FLAG antibody using the immunogold method. The results proved the validity of the previous experiments (Figure 3D).

The effect of the cell-associated fragment of TNF on activity of cytokine genes

We performed a pilot experiment to see if the 10 kDa, cell-associated fragment of TNF had any influence on the expression of cytokine genes. P388D mouse myeloid cells were activated with LPS treatment to upregulate TNF synthesis. Cultures of TNF-producing cells were exposed to anti-TNF antibodies, soluble TNF receptor (human recombinant p55, the clone was provided by Dr. D. Wallach, Rehovot) or a plasma membrane fraction of mouse L929 cells (L929 cells express mouse TNF receptor p55). Control cells were untreated or treated with LPS alone.

RNA was extracted from the cells and a ribonuclease protection assay was performed (detecting the presence of ten different cytokine mRNAs). Ligation of mouse TNF molecules by receptors or anti-mouse TNF antibody elicited an increase in interleukin-1beta mRNA levels without influencing the expression of other cytokines (Figure 4). No increase in IL-1 was observed in samples treated with an antibody specific for human TNF.

In the next experiments, CAT constructs controlled by SV40 and IL-1beta promoters were introduced into HeLa and TNF-overproducing HeLa (A11) cells. As shown in Figure 5, the ratios of CAT activities controlled by IL-1 and SV40 promoters, respectively, were 3-7 fold higher in A11 cells than in HeLa cells. The A11 clone contains relatively high levels of the 10 kDa peptide (detected by the immunogold technique, see Figure 3) as the result of TNF overproduction.

Following this observation, we studied the effect of N-terminal fragments of TNF on IL-1beta promoter activity. In each experiment we transformed HeLa cells with

3 DNA constructs. The plasmid pGL3-control (carrying the *luc* gene) was used to calculate the efficiency of transformation. A second plasmid in which synthesis of (different sized N-terminal) fragments of TNF was driven by the constitutive CMV promoter. Finally, a DNA construct coding for the CAT (chloramphenicol-acetyl-transferase) enzyme under the control of (parts of) the IL-1beta promoter.

We used two constructs carrying TNF fragments (one coded for the intracellular domain, the other corresponded to the 10 kDa, cell-associated fragment). A third plasmid, without any TNF sequence, was used as control. CAT enzyme expression was regulated by different segments of the interleukin-1 beta gene promoter (- 3,757-+ 12 and - 3,132-+ 12 [22], generous gifts of Philip E. Auron, Harvard Medical School).

The experiments clearly showed that the presence of both TNF fragments significantly increased the activity of both IL-1 promoter sequences. The shorter IL-1 promoter fragment was more responsive to the presence of TNF fragments and optimal activation was seen in cells transformed with the shorter IL-1 promoter construct and the plasmid coding for the 10 kDa TNF peptide (Figure 5).

DISCUSSION

Members of the TNF family of proteins are expressed as integral membrane proteins on the surface of lymphoid/myeloid cells [32]. They control the proliferation, differentiation, activation and/or programmed cell death of other leukocytes, hematopoietic progenitors (and other healthy and tumor cells) [33].

The transmembrane form of TNF and some related proteins (CD30L, CD40L, FasL), frequently interact with their receptors during cell-to-cell interactions inducing phenotypic changes in *both* cell types. This phenomenon, called reverse signaling is not well understood.

According to our hypothesis, the transmembrane form of TNF is a functional receptor and its signaling process can influence the expression of (cytokine) genes. The intracellular domain of TNF is unusually long to be a mere signal sequence. It is casein kinase-phosphorylated on a serine residue in resting cells. Receptor-mediated clustering of TNF molecules induces dephosphorylation of

the molecule by specific phosphatases and leads to Ca^{2+} mobilization in the cells [19, 20].

The transmembrane form of TNF is processed by TACE, a metalloproteinase. Although the enzyme cleaves both free and receptor-bound TNF molecules [34], the liberated, cell-associated peptides are different, as the result of the dephosphorylation of the receptor-bound molecule.

In this paper, we prove by *in vitro* and *in vivo* experiments, that the 10 kDa peptide generated by the proteolytic processing of the 26 kDa form of TNF carries a functional NLS sequence. The nuclear transport we observed with PE-peptide conjugate fulfilled all the criteria of a specific, selective nuclear import. The peptide sequence KKTGGPQGSRR matches the (K/R)2Xn(K/R)2 subtype of non-typical NLSs, classified by Boulikas [35].

In vivo experiments using an antibody against (the dephosphorylated form of) the intracellular domain proved that the NLS directed the nuclear import of the cleavage product corresponding to the transmembrane plus intracellular domains of TNF. The processed peptide was detected by the immunogold technique inside the nucleus, close to nuclear pores and in association with transport vesicles. Unexpectedly, degradation of the hydrophobic transmembrane domain did not take place in these vesicles. We could not detect any immunoreactive peptides smaller than 9-11 kDa, the original cleavage product. Inhibition of proteolysis within the vesicular compartment by leupeptin did not change the localization or size of the peptide.

In genetically modified cells, the expression of the N-terminal peptide of TNF altered the IL-1 beta mRNA levels. In cells transfected with DNA construct(s) coding for N terminal peptide(s) of TNF, the promoter sequence of IL-1 beta showed increased activity. The 10 kDa fragment is unlikely to have a DNA binding domain, but might change the activity of genes by interacting with transcriptional factor(s).

This mechanism resembles the signaling of the *Notch*-type of receptors mediating cell-fate decisions [36]. The intracellular domain of Notch receptors is released upon ligand binding as a result of proteolytic processing steps and the peptide migrates to the nucleus, carrying a nuclear factor, and activates the transcription of target genes [36, 37]. It is interesting to note that the TNF-

converting enzyme (TACE), which liberates TNF from its precursor also plays a role in the processing of Notch [38].

As TNF is a pleiotropic cytokine involved in development, differentiation and immune processes, it is difficult to predict the primary function of reverse signaling. Our first observation on the upregulation of the IL-1beta gene was made in P388D, a myeloid cell line ready to produce inflammatory cytokines. Recent papers reported development of LPS resistance in myeloid cells and E-selectin expression in T lymphocytes as the result of reverse signaling [13, 14]. It is conceivable that reverse signaling may alter the activity of different genes in diverse cell types.

$TNF^{-/-}$ animals suffer from structural and functional defects. These animals fail to develop follicular dendritic cell networks, germinal centers, normal maturation of the humoral immune response is inhibited, differentiation and maturation of hematopoietic progenitors are also altered [39,40]. These observations and the fact that most members of the TNF family participate in cell fate decision events suggest that reverse signaling might also play roles in development and differentiation.

CONCLUSION

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Figures

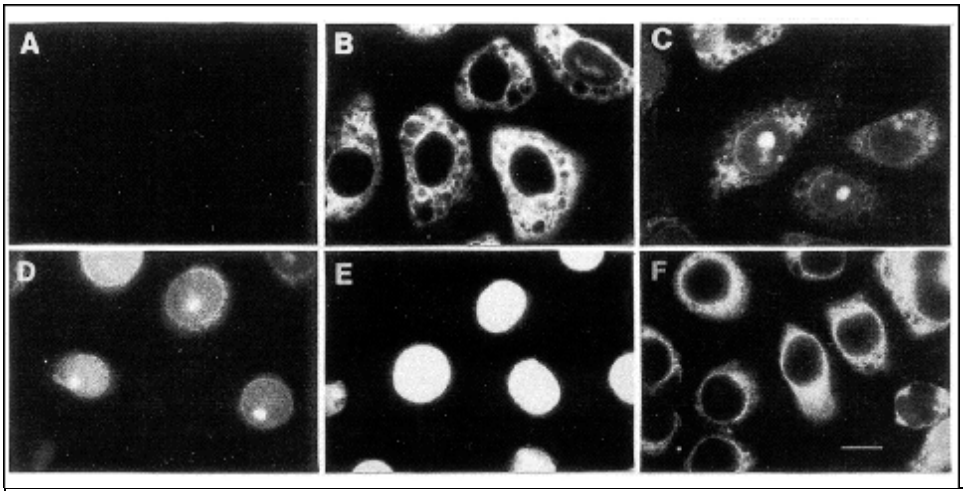


Figure 1
Specificity of the nuclear transport of PE-peptide conjugate.

A: Permeabilized HeLa cells were incubated at 30° C with unconjugated PE in the presence of ATP and HeLa cytosol. **B:** Permeabilized HeLa cells were incubated at 0° C with PE-peptide conjugate in the presence of ATP and HeLa cytosol. **C:** Permeabilized HeLa cells were incubated at 30° C with PE-peptide conjugate in the presence of ATP without HeLa cytosol. **D:** Permeabilized HeLa cells were incubated at 30° C with PE-peptide conjugate in the presence of HeLa cytosol without ATP. **E:** Permeabilized HeLa cells were incubated at 30° C with PE-peptide conjugate in the presence of ATP and HeLa cytosol. **F:** Permeabilized HeLa cells were incubated at 30° C with PE-peptide conjugate in the presence of ATP, HeLa cytosol and 0.1 mg/ml wheatgerm agglutinin. Bar is 10 mum.

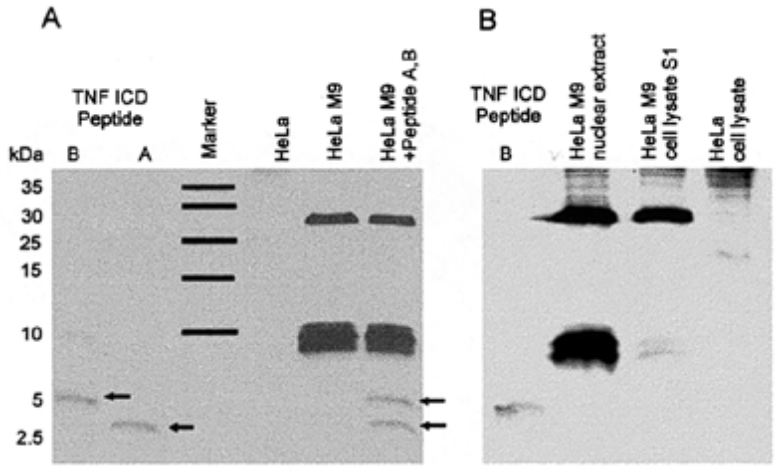


Figure 2
Size and subcellular localization of the ICD of TNF.

A polyclonal antibody raised against the intracellular domain of TNF (amino acids 1-33, see Materials and Methods) was used to detect the cell-associated cleavage product of TNF in subcellular fractions. Panel A shows the presence of the 26 kDa transmembrane form of TNF and several bands in the 9-11 kDa range in TNF-producing M9 HeLa cells. No smaller cleavage products (similar in size to peptides A and B) corresponding to the intracellular domain were detected. (The amounts of peptides A and B were kept very low in the electrophoresis so as not to exhaust the antibody molecules during blotting). As can be seen on panel B, the 10 kDa immunoreactive peptides are enriched in the nuclear fraction of Dounce homogenized cells, while a large proportion of the 26 kDa transmembrane form is found in the nuclei-free cell lysate (S1).

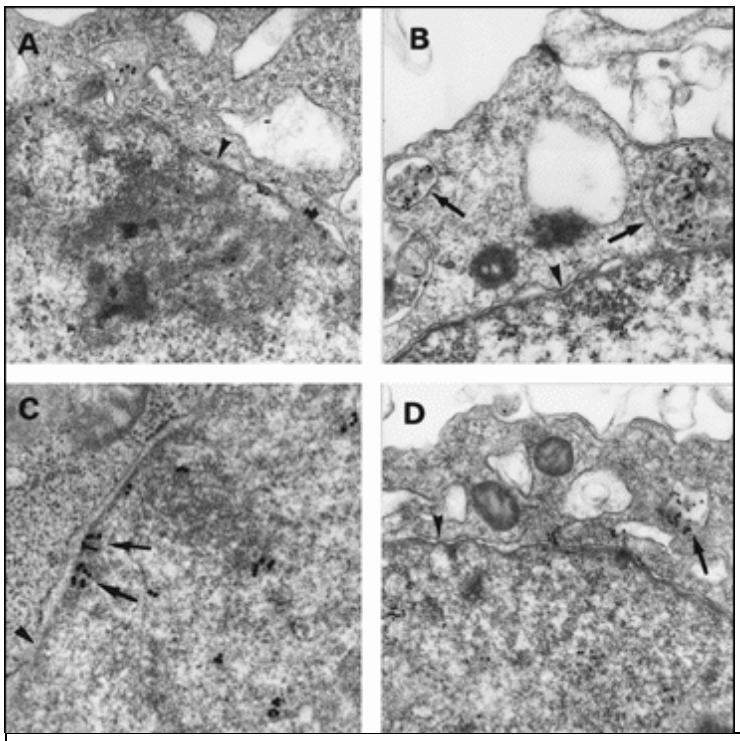


Figure 3
Subcellular localization of the intracellular domain of TNF.

Immunogold labeling was used for the determination of the intracellular localization of the 10 kDa fragment in HeLa-A11 cell sections. **A**, **B** shows the presence of the peptide in the nucleus and in multilamellar transport vesicles (arrows). In **C**, one can see clusters of gold particles close to nuclear pores (arrows). To rule out detection of cross-reacting proteins, FLAG epitope was inserted into the intracellular domain of TNF and anti-FLAG antibody was used for immunogold detection of the FLAG-labeled TNF fragment in transformed HeLa cells **(D)**. The arrows show transport vesicles, arrowhead indicates the outer nuclear membrane. No gold particles were observed in control HeLa cells (not shown).

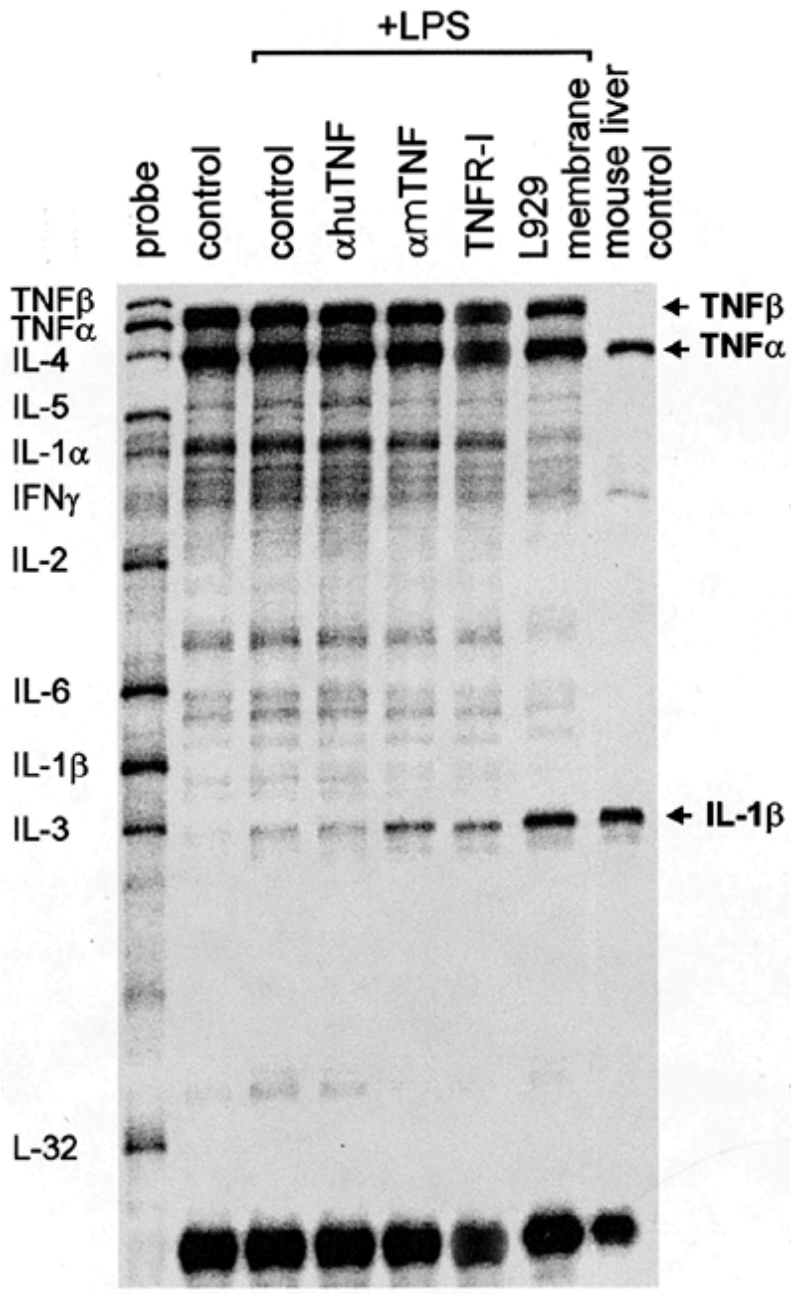


Figure 4
RNase protection assay for the detection of changes in the cytokine mRNA profiles of P388D cells.

P388D cells were activated by 1 mug/ml LPS for 180 min followed by different treatments (90 min). Levels of ten different mouse cytokines were followed in LPS-induced mouse myeloid cells. Clustering of TNF molecules was induced by anti-TNF antibodies or recombinant, soluble receptor molecules or purified plasma membrane fraction of TNF-sensitive, L929 mouse tumor cells. mRNA isolated

from the liver of LPS-treated mice was used as a positive control. (In RP assays, mobility of the probe differs from that of the detected molecule.) TNF clustering elicited a moderate increase in the IL-1beta mRNA level. Mouse TNF receptors present on L929 cell membranes seemed to be more effective in increasing the message than human (recombinant soluble) receptors. Anti-mouse TNF antibody was active, while specific anti-human TNF antibody had no effect.

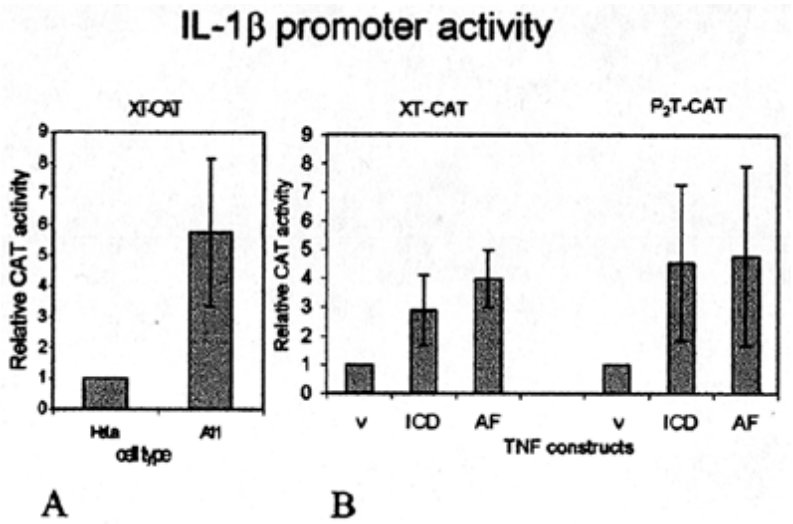


Figure 5
The positive regulatory effect of TNF (A) **or the 10 kDa peptide molecule** (B) **on IL-1-beta promoter activity.**

Transformed HeLa and TNF-over-producing A11 cells were infected with a CAT reporter gene under the control of the IL-1beta promoter construct. We detected a several-fold increase in the level of CAT activity in A11 cells. Immunogold assay detected the 10 kDa fragment in the nuclei of these cells **(A)**. IL-1-beta promoter activity was measured in HeLa cells transformed with constructs coding for two TNF fragments: the shorter one corresponded to the intracellular domain (ICD), the other coded for the 10 kDa peptide (AF). Both peptides were

produced constitutively. Cells transformed with the empty plasmid pcDNA$_3$ (v) served as control. CAT enzyme expression was regulated by the interleukin-1beta gene promoter (XT and P$_2$T refers to promoter sequences -3,757± 12 and - 3,132 ± 12, respectively). As shown, the presence of TNF fragments significantly increased the activity of both IL-1beta promoter sequences **(B)**. Figures represents results of four individual experiments.

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microsatellites, interleukin-10 promoter, multiplex-PCR, Caucasian, African.

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