

Production and Characterization of Recombinant Human and Murine TNF

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Summary

Here we describe the methods for the expression of human and murine soluble tumor necrosis factor (hTNF and mTNF) in *Escherichia coli* cells and for their extraction, purification, and characterization. The expression and purification procedure takes about 2 wk. Human and murine TNF can be purified from crude extracts with high yields (>50 mg from 1 L of fermentation) by hydrophobic-interaction chromatography, ion-exchange chromatography, and gel-filtration chromatography. The purity and the identity of the final products can be checked by SDS-PAGE, ELISA, Western blot, analytical gel-filtration chromatography, mass spectrometry, and lipopolysaccharide assay. The biological activity of both human and murine TNF is assessed by in vitro cytolytic assays using murine L-M cells. Purified hTNF and mTNF can be used for in vitro and in vivo studies in animal models.

Key Words: TNF; plasmids; *E. coli* expression; purification; characterization; cytolytic assay.

1. Introduction

Tumor necrosis factor (TNF) is a cytokine produced by several cell types (macrophages, subsets of T cells, B cells, mast cells, eosinophils, endothelial cells, cardiomyocytes, and so on). It is expressed as a 26-kDa integral transmembrane precursor protein from which a 17-kDa mature TNF protein is released by proteolytic cleavage (**1**). Soluble, bioactive TNF is a homotrimeric protein that slowly dissociates into inactive, monomeric subunits at picomolar levels (**2**). Biological activities are induced upon interaction of trimeric TNF with two distinct cell surface receptors (p55-TNFR and p75-TNFR).

Although limited amounts of TNF can be obtained from natural sources, relatively large quantities of bioactive, soluble TNF, for structural and functional studies, can be easily obtained by recombinant DNA technology. For instance, human and murine TNF cDNA has been successfully expressed in *Escherichia coli* (3,4) and has been recovered as soluble homotrimeric protein with high yields (5). Several mutants of TNF have also been successfully expressed in *E. coli* (5,6). Here we describe the methods for the expression of human and murine soluble TNF (hTNF and mTNF) in *E. coli*, for their extraction, purification, and characterization. Purified TNF, obtained according to this method, can be used for in vitro and in vivo studies in animal models.

2. Materials

2.1. cDNA Cloning and Expression

1. Enzymes for DNA manipulations.
2. Oligonucleotide primers.
3. RAW 264.7 cell line (clone TIB-71, ATCC).
4. *Salmonella minnesota* lipopolysaccharide (Sigma-Aldrich).
5. *E. coli* strain BL21(DE3) and DH5 α (Novagen).
6. pET11b expression plasmid (Novagen).
7. Electrophoresis equipment for DNA analysis.
8. Ampicillin.
9. Autoclaved Luria broth (LB): freshly prepared by mixing 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0, with 10 *N* NaOH.
10. Glycerol.
11. IPTG (isopropyl- β -D-thio-galactopyranoside).
12. Disposable 0.45- μ m and 0.2- μ m filters.
13. 2.5-L and 100-mL flasks for bacteria culture.
14. Extraction buffer: 20 mM Tris-HCl, 2 mM EDTA, pH 8.0.
15. Endonuclease.
16. Sonicator.
17. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.

2.2. Protein Purification

1. Chromatography equipment.
2. Phenyl-Sepharose (low sub) Fast Flow (Amersham Biosciences Europe GmbH).
3. DEAE-Sepharose (low sub) Fast Flow (Amersham Biosciences Europe GmbH).
4. HR Sephacryl S-300 (Amersham Biosciences Europe GmbH).
5. Superdex 75 HR column (Amersham Biosciences Europe GmbH).
6. Dialysis tubing (cut-off, 12,000 Da).
7. TA buffer: 100 mM Tris-HCl, 1M (NH₄)₂SO₄, 5% methanol, pH 8.0, ice cold.
8. 2X TA buffer: 200 mM Tris-HCl, 2M (NH₄)₂SO₄, 10% methanol, pH 8.0, ice cold.

9. TB buffer: 100 mM Tris-HCl, pH 8.0, 70% ethylene glycol, 5% methanol, ice cold.
10. TA1 buffer: 20 mM Tris-HCl, pH 8.0, ice cold.
11. TB 2 buffer: 20 mM Tris-HCl, pH 8.0, 1M NaCl, ice cold.
12. PBS buffer: 150 mM NaCl, 50 mM NaH₂PO₄, pH 7.2, cooled at 4°C.

All solutions must be prepared with sterile and endotoxin-free water.

2.3. TNF Cytolytic Assay

1. L-M murine fibroblast cell line (clone CCL-1.2; ATCC).
2. 96-well flat-bottom plate for cell culture.
3. DMEM medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin-B, and 10% fetal bovine serum (complete medium).
4. Actinomycin D: prepare a stock solution at 200 µg/mL in DMEM complete medium and store at -20°C.
5. MTT solution: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, freshly prepared at 5 mg/mL in PBS.
6. Dimethyl sulfoxide.

2.4. Quantification of mTNF by ELISA

1. PVC microtiterplates (Becton Dickinson, cod. 3912).
2. Rat anti-mTNF, mAb V1q (kindly supplied by Dr. D. Mannel, University of Regensburg, Germany).
3. Nonfat dry milk powder.
4. Bovine serum albumin (BSA).
5. Tween-20.
6. Normal goat serum (NGS).
7. Assay buffer: 3% nonfat dry milk powder, 1% BSA in PBS containing 1% NGS.
8. Rabbit anti-mTNF, IgGs.
9. Goat anti-rabbit IgG-HRP.
10. O-phenylenediamine tablets (5 mg/tablet).
11. 36% hydrogen peroxide solution.

3. Methods

The methods described below outline (1) the preparation of cDNA coding for hTNF and mTNF, (2) the construction of expression plasmids, (3) the expression of hTNF and mTNF in *E. coli* cells, (4) the purification, and (5) the characterization of both proteins.

3.1. Preparation of cDNA Coding for mTNF and hTNF

DNA manipulations were performed by standard recombinant DNA methods.

The cDNA coding for murine TNF was prepared by RT-PCR, starting from a poly-A⁺ mRNA obtained from 4×10^7 murine macrophage cells (RAW 264.7 cell line) stimulated for 16 h with 100 ng/mL of lipopolysaccharide, using the following primers:

5' TGCCTCACATATATGCTCAGATCATCTTCTC 3' (5' *NdeI* primer);

5' CTGGATCCTCACAGAGCAATGACTCCAAAG 3' (3' *BamHI* primer).

The primers were designed to amplify the cDNA sequence coding for mature murine TNF₁₋₁₅₆.

The 5' *NdeI* primer contains the *NdeI* restriction site (underlined), necessary for cloning, and the translation starting codon ATG (bold). The 3' *BamHI* primer, corresponding to the antisense strand, contains the *BamHI* restriction site (underlined) and the stop codon.

The cDNA coding for mouse Met-TNF₁₋₁₅₆ and human Met-TNF₁₋₁₅₇ (generated using the same strategy) were 476 bp and 479 bp long, respectively, after digestion with *NdeI-BamHI* enzymes.

3.2. Construction of Expression Plasmids

The following subsections describe the expression vectors and the cloning strategy exploited for the production of hTNF and mTNF.

3.2.1. pET11b Expression Vector

The pET System is a system developed for cloning and expression of recombinant proteins in *E. coli* (7). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression can be induced by the addition of IPTG. The pET11b plasmid translation vector contains the highly efficient ribosome-binding site from phage T7 major capsid protein. Sequences inserted into the cloning site can be expressed as T7-Tag fusion proteins to facilitate detection, purification, or solubilization of the target protein. However, expression of the recombinant protein lacking the T7-Tag can be accomplished by removing the coding T7-Tag region by digestion with *NdeI* and *BamHI*. The pET11b vector contains an ampicillin-resistance gene for selection.

3.2.2. Cloning Strategy

The pET11b plasmid was digested with *NdeI-BamHI* enzymes to remove the sequence coding for the T7-Tag, dephosphorylated, and ligated with the TNF cDNAs obtained as described in **Subheading 3.1**. Both ligation products were used to transform *E. coli* DH5 α cells by standard methods (8). Transformed *E. coli* DH5 α cells were plated on LB plates containing ampicillin (100 μ g/mL) and incubated overnight at 37°C. Single colonies were selected and

grown overnight in LB ampicillin. The plasmids, named hTNF-pET11 and mTNF-pET11, were then isolated, analyzed by agarose gel electrophoresis after digestion with *NdeI* and *BamHI* and sequenced (see **Note 1**).

3.3. Expression of mTNF and hTNF in *E. coli*

The entire process (expression and purification) takes about 10 d. The methods described below outline (1) the preparation of glycerol stocks, and (2) the fermentation process. The protocol refers to 1 L of fermentation.

3.3.1. Preparation of Glycerol Stocks

1. Transform BL21(DE3) *E. coli* strain with hTNF-pET11 or mTNF-pET11 using standard protocols (8).
2. Plate cells on LB plates containing ampicillin (100 µg/mL) and incubate overnight at 37°C.
3. Select single colonies and grow them in 20 mL of LB containing 100 µg/mL ampicillin until the optical density (OD) at 600 nm is about 0.6 units.
4. Add 4 mL of autoclaved glycerol (20% final concentration), mix well, and prepare several cryovials.
5. Quickly freeze the cryogenic vials in liquid nitrogen and transfer at -80°C (see **Note 2**).

3.3.2. Preparation of Preinoculum

1. Streak transformed cells from one cryogenic vial and transfer to a 100-mL flask containing 20 mL of LB containing 100 µg/mL ampicillin. Incubate at 37°C under shaking (200 rpm) until the OD at 600 nm is about 1.0 unit (about 6 h).
2. Transfer the preinoculum into a 50-mL tube and store overnight at 4°C.
3. The next day, spin the product (3500g, 10 min, 4°C), discard the supernatant, and gently resuspend the pellet in 20 mL of LB containing 100 µg/mL ampicillin.

3.3.3. Cell Culture and Protein Expression

1. Fill two flasks (void volume, 2500 mL each) with 500 mL of LB containing 100 µg/mL ampicillin, and add an amount of preinoculum giving an OD at 600 nm of 0.03 units.
2. Incubate at 37°C under vigorous shaking (200 rpm) until the OD at 600 nm is about 0.7–0.8 units and induce protein expression by adding IPTG (final concentration, 1 mM). Let the cells grow for 3.5 h.
3. Centrifuge the culture (4000g, 20 min, 4°C).
4. Discard the supernatant and recover the bacterial pellet.
5. Freeze the bacterial pellet at -20°C (see **Note 3**).

STEP

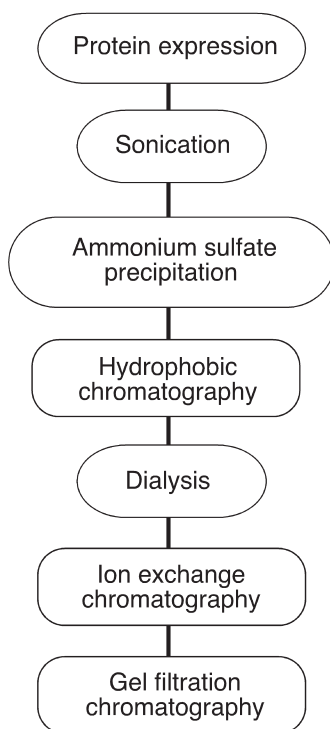


Fig. 1. Flow chart of hTNF and mTNF purification.

3.4. Purification of mTNF From *E. coli* Extracts

The flow chart of the purification method is shown in **Fig. 1**. The entire procedure takes about 7 d. The steps described in **Subheadings 3.4.1. and 3.4.2.** outline the procedure for extracting mTNF from *E. coli* cells as a soluble protein. The steps described in **Subheadings 3.4.3. to 3.4.6.** outline the procedure for mTNF purification from soluble crude extracts. During the extraction and purification process each fraction is stored on ice. All centrifugation steps are performed at 15,000g for 20 min at 4°C.

3.4.1. Disruption of Bacteria by Sonication

1. Resuspend the bacterial pellet with 50 mL of extraction buffer and add 40 U/mL endonuclease.

2. Lyse the bacterial suspension by sonication (*see Note 4*).
3. Centrifuge the sonicated material, recover the soluble fraction and discard the pellet (*see Note 5*).

3.4.2. Ammonium Sulfate Precipitation

1. Add the amount of ammonium sulfate necessary to obtain 30% saturation to the soluble fraction obtained in **Subheading 3.4.1.** and incubate for 1 h at 4°C under stirring.
2. Centrifuge and recover the soluble fraction; discard the pellet.
3. Add ammonium sulfate to the soluble fraction (65% saturation) and incubate overnight at 4°C under stirring.
4. The next day, centrifuge and discard the soluble fraction. Gently resuspend the pellet by adding 15 mL of sterile water, then slowly add 15 mL of 2X TA buffer and incubate on ice for 10 min.
5. Centrifuge and discard the pellet. Filter the soluble fraction (SM-HIC) using a 0.45 µm filter.

3.4.3. Hydrophobic Chromatography

1. Load the product (SM-HIC) onto a 16 × 250 mm phenyl-sepharose column, equilibrated with TA buffer, using a peristaltic pump (4 mL/min).
2. Wash the column with 160 mL TA and elute the protein using the following gradient: 20–70% TB (301 mL); 70–100% TB (1 mL); 100% TB (150 mL); flow rate 3 mL/min.
3. Collect fractions of 10 mL and analyze them by SDS-PAGE. Pool fractions, trying to discard those fractions containing impurities in high proportion (Pool HIC). Store the pooled fractions at 4°C until the next step.

3.4.4. Dialysis

1. Dialyze the product (Pool HIC) against 23 volumes of 2 mM EDTA, 20 mM Tris-HCl, pH 8.0 (2 h, 4°C).
2. Repeat this step twice and dialyze the product for 60 h at 4°C. Filter the product (Pool HIC-f) using a 0.22-µm filter (*see Note 6*).

3.4.5. Ion-Exchange Chromatography

1. Load the product (Pool-HIC-f) onto a 16 × 320 mm DEAE-sepharose column, equilibrated with TA1 buffer, using a peristaltic pump (2 mL/min).
2. Wash the column with 510 mL TA1 and elute the protein using the following gradient: 0–50% TB1 (180 mL); 50–100% TB1 (2 mL); 100% TB1 (92 mL); flow rate 2 mL/min.
3. Collect fractions of 5 mL and analyze them by SDS-PAGE. Pool fractions, trying to discard impurities (Pool DEAE). Store the pooled fractions at 4°C until the next step.

Table 1
Purification of mTNF From *E. coli* Extract (1 L of Fermentation)

Fractions	Volume (mL)	TNF antigen ^a (mg)	Protein ^b (mg)	Purity antigen/protein (%)	Yield (%)
Crude extract	55	180	450	40	100
Hydrophobic interaction chromatography	155	123	190	65	68
Ion-exchange chromatography	15	61.4	66	93	34.1
Gel-filtration chromatography	50	51	52	98	28.3

^aBy ELISA.

^bBy BCA protein assay kit (Pierce).

3.4.6. Gel Filtration Chromatography

1. Gel-filter the product (Pool DEAE) through a 50 × 600 mm HR Sephacryl S-300 column, pre-equilibrated with PBS.
2. Elute the protein with PBS (flow rate 2 mL/min), collect the first 400 mL of effluent in a bottle and then collect 40 fractions of 12.5 mL each.
3. Analyze each fraction by SDS-PAGE and pool the fractions with the highest protein content and purity.
4. Filter the pooled fractions (Pool GF) under a sterile hood using 0.22-μm filters, aliquot, and store at −20°C (see **Note 7**).

3.5. Purification of hTNF From *E. coli* Extracts

We have successfully used the same protocol for the purification of hTNF, with minor modification: In **Subheading 3.4.2**, we use 35% saturated ammonium sulfate concentration instead of 30%.

3.6. Biochemical and Biological Characterization of hTNF and mTNF

The protein content and the immunoreactivity of the chromatographic fractions and the final products can be determined by commercial protein detection and TNF-ELISA kits. **Table 1** shows the yield of each step of mTNF purification. Typically, more than 50 mg of purified material can be recovered from 1 L of fermentation.

The purity and the identity of the final products can be estimated using the following analytical procedures: (1) SDS-PAGE, (2) Western blot, (3) analytical gel filtration, and (4) mass spectrometry. The results of SDS-PAGE of purified mTNF and hTNF, under reducing and nonreducing conditions are shown in **Fig. 2**. A single band of 17–18 KDa, with purity greater than 98%, should be observed. The hydrodynamic volumes, estimated by analytical gel filtration,

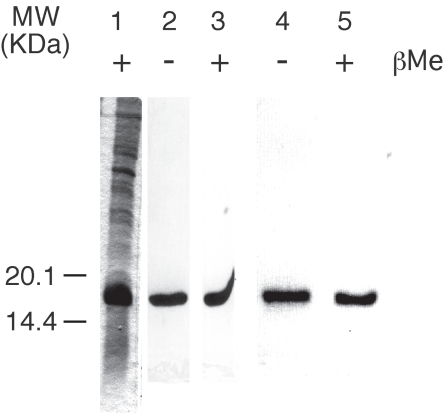


Fig. 2. SDS-PAGE analysis of mTNF and hTNF. Lane 1: Soluble crude extract of mTNF after sonication; Lanes 2 and 3: purified mTNF; Lanes 4 and 5: purified hTNF. The samples were analyzed under reducing (+) and nonreducing (–) conditions on 12.5% SDS-PAGE stained with Coomassie R250. MW: molecular markers.

correspond to proteins of 40 KDa (**Fig. 3**). The discrepancy between the theoretical molecular weight (about 50 KDa) and the observed hydrodynamic behavior (40 KDa) is likely because TNF is a compact trimer (**9**) with a small hydrodynamic volume (**2**). The expected molecular masses of hTNF_{1–157} and mTNF_{1–156} subunits are 17,350.7 Da and 17,386.7 Da, respectively. Electrospray ionization mass spectrometry (ESI-MS) analysis of hTNF shows a heterogeneous product consisting of a major peak of 17,349 Da, corresponding to mature TNF_{1–157}, and a minor peak of 17,481 Da, corresponding to the mass expected for human Met-TNF_{1–157}. Typically, in our preparations this product is about 20% of total (**Fig. 4**). The molecular mass of mTNF, by ESI-MS, is 17,386 Da, corresponding to the mass expected for murine Met-TNF_{1–156}. Many in vitro and in vivo applications of TNF require low endotoxin contamination. The amount of lipopolysaccharide in our preparations is typically approx 0.1 U/μg of purified protein, as measured by quantitative chromogenic *Limulus* amebocyte lysate (LAL) test. The in vitro cytotoxic activities of hTNF and mTNF are about 5 × 10⁷ and 8 × 10⁷ U/mg of protein, respectively, as determined by standard cytolytic assay with murine L-M mouse fibroblasts (see **Subheading 3.7.**).

3.7. Cytolytic Assay

Here, we describe a cytolytic assay for the quantitative detection of TNF, based on murine fibroblast L-M cells, and a dose-response curve in the range of 3–3000 pg/mL (**Fig. 5**). L-M cell are cultured in DMEM medium supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin,

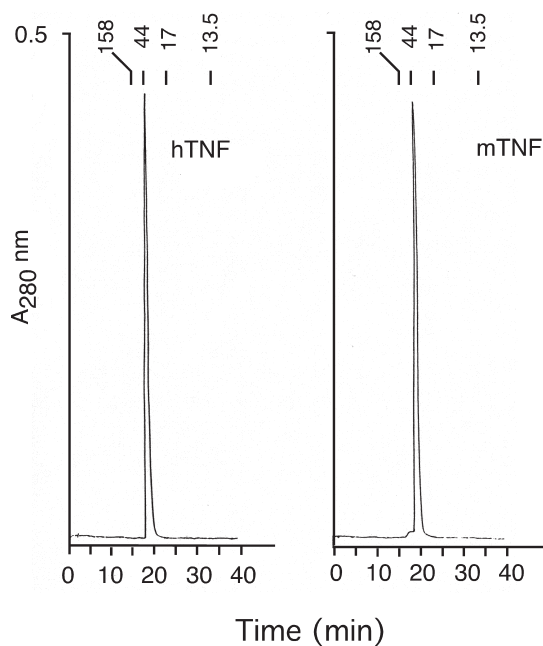


Fig. 3. Analytical gel filtration chromatography of hTNF and mTNF (Superdex 75 HR column). The column was equilibrated with PBS and calibrated with the following proteins: thyroglobulin (670 KDa), IgG (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa) and B₁₂ vitamin (1.35 KDa).

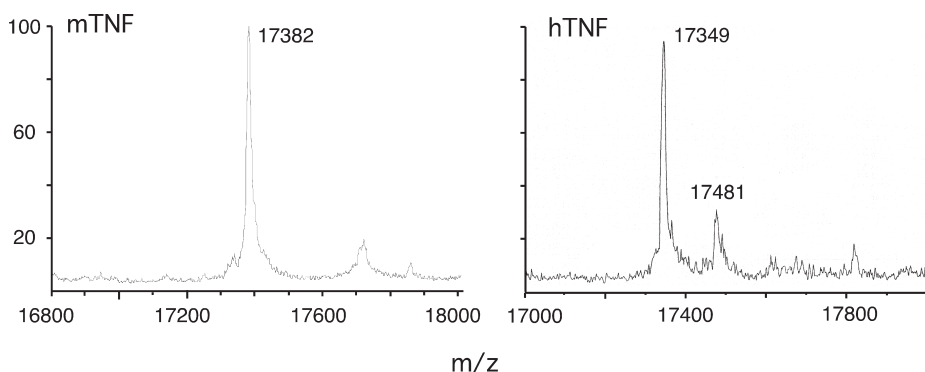


Fig. 4. Analysis of purified hTNF and mTNF by electrospray ionization mass spectrometry (ESI-MS).

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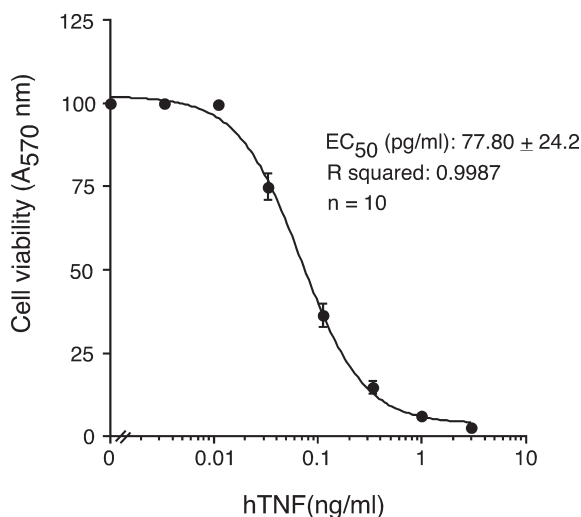


Fig. 5. Calibration curve of the hTNF cytolytic assay. L-M cells (30,000 cell/well) were incubated with 2 $\mu\text{g/mL}$ of actinomycin D and increasing amount of hTNF. Similar results can be obtained with mTNF.

0.25 $\mu\text{g/mL}$ amphotericin-B and 10% fetal bovine serum (*see Note 8*). The cells are detached with a trypsin-EDTA solution and expanded three times per week. The protocol described below takes about 3 d to be performed and can be used for both hTNF and mTNF. Other, more sensitive bioassays have been described using different cell lines (*10–13*).

1. Plate 30,000 cells/well (100 μL /well, 96-well flat-bottom plate) in DMEM medium and incubate overnight at 37°C under 5% CO_2 .
2. Prepare various solutions of standard TNF (3–3000 pg/mL , final concentration) and samples in “complete medium” (*see Note 9*).
3. Add to each well 50 μL of actinomycin-D (2 $\mu\text{g/mL}$ final concentration) and 50 μL of TNF solutions, then incubate 24 h at 37°C under 5% CO_2 .
4. Add to each well 10 μL of MTT solution and incubate 3 h at 37°C under 5% CO_2 (*see Note 10*).
5. Remove the supernatant using a Pasteur pipet connected to a vacuum pump, and add 200 μL of dimethyl sulfoxide to dissolve the formazan crystals.
6. Measure the absorbance at 540 nm or at 570 nm.

3.8. mTNF Quantification by ELISA

1. Coat PVC microtiterplates with mAb V1q (10 $\mu\text{g/mL}$ in PBS), 50 μL /well, and incubate overnight at 4°C.
2. After washing three times by emptying and filling with PBS, incubate the plate with 3% nonfat dry milk powder, 1% BSA in PBS containing 0.05% Tween-20, 200 μL /well, for 2 h at 37°C, and then wash again three times with PBS.

3. Fill the wells with 50 μL of mTNF standard solutions (0.010–10 ng/mL), or samples diluted in “assay buffer” (see **Subheading 2.4.**), and incubate for 2 h at room temperature.
4. Wash the plate eight times by emptying and filling with PBS containing 0.05% Tween-20 (PBS-TW).
5. Add to each well 50 μL of anti-mTNF IgGs (10 $\mu\text{g/mL}$ final concentration) diluted in “assay buffer” and incubate for 1.5 h at room temperature.
6. After washing eight times with PBS-TW, add to each well 50 μL of goat anti-rabbit IgG-HRP diluted 1:1000 in “assay buffer” and incubate for 45 min at room temperature.
7. Wash again with PBS-TW and fill each well with 75 μL of *o*-phenylenediamine solution (1 tablet in 7.5 mL of distilled water containing 10 μL of 36% hydrogen peroxide) and incubate for 30 min.
8. Block the chromogenic reaction by adding 10% sulfuric acid (75 μL /well). The absorbance at 490 nm of each well is then measured using an ELISA plate reader.

A dose-response curve in the 20–1000 pg/mL range can be obtained with this protocol.

4. Notes

1. Another very efficient method for screening recombinant clones exploits the cytolytic activity of TNF (see **Subheading 3.7.**). Given that TNF cDNA is not toxic to BL21(DE3), the product of ligation can be directly used to transform these cells. The clones obtained can then be inoculated in 100 μL of LB containing 100 $\mu\text{g/mL}$ ampicillin, in a 96-well plate, and incubated at 37°C under shaking (make a master plate of the picked colonies). When the culture is slight turbid, the plate is centrifuged (820g for 10 min), the supernatant is discarded, and the bacterial pellet is resuspended with 100 μL /well of LB containing 100 $\mu\text{g/mL}$ ampicillin and 1 mM of IPTG. After 3 h of induction at 37°C under shaking, the plate is centrifuged (2500g for 10 min), the supernatant is eliminated, and the bacteria are lysed by adding 30 μL /well of B-PER Reagent for 10 min (Pierce). Then add 70 μL of PBS to each well and centrifuge (2500g for 10 min) to remove the bacterial debris. Analyze the supernatant (containing the soluble fraction protein) by cytolytic assay. The clones able to kill the cells are isolated and sequenced. With this protocol hundreds of clones can be screened in 2 d.
2. Glycerol stocks of BL21(DE3) containing TNF-coding plasmids are very stable (up to 5 yr).
3. Alternatively, the frozen pellet can be stored at –80°C for several days (up to 3 mo).
4. During the sonication process, store the sample on ice to avoid overheating. To assess the bacterial lysis during sonication, remove 10 μL of the sonicated sample, centrifuge 5 min at 13,000g, dilute the supernatant 1:100 with extraction buffer, and measure the absorbance at 280 nm. Repeat the sonication step until the absor-

bance at 280 nm is stable. Typically, 7 cycles are sufficient to obtain a final absorbance at 280 nm of about 100 units.

5. The crude extract can be stored -80°C for several days (up to 3 mo).
6. During dialysis the sample volume may increase one-third. Thus, to avoid the rupture of tubing, leave some air between sample and clamp.
7. Purified TNF can be stored at -80°C for several years. Also, diluted TNF (1–5 $\mu\text{g/mL}$) can be stored for several years, with minor loss of activity, provided it is diluted in the presence of carrier proteins. For instance, TNF can be stored in DMEM containing 5–10% of serum at -20°C . Storing highly diluted TNF solution (less than 10 ng/mL) at $>4^{\circ}\text{C}$ should be avoided, as it may dissociate to inactive monomers (2).
8. L-M cells must be mycoplasma free. Infection causes marked loss of assay detectability.
9. Diluted TNF solutions should be added to the plate within 1 h (see also **Note 7**).
10. Remove any particulate by filtration through a 0.22- μm filter.

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