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Noncleavable Transmembrane Mouse Tumor Necrosis Factor-α (TNFα) Mediates Effects Distinct from Those of Wild-type TNFα *in Vitro* and *in Vivo**

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Tumor necrosis factor-α (TNFα) exists in two biologically active forms, a 26-kDa transmembrane form and a proteolytically cleaved and secreted form. We sequentially inactivated all three known cleavage sites of mouse TNFα by mutating the corresponding DNA sequences. A murine T cell hybridoma transfected with the nonsecretable mutant TNFα efficiently lysed L929 target cells in a cell contact-dependent manner and induced expression of vascular cell adhesion molecule-1 on mouse endothelioma cells. A genomic mouse TNFα clone encoding this mutant was subsequently introduced as a transgene into TNFα$^{-/-}$ lymphotoxin-α$^{-/-}$ mice. The 3′ AU-rich regulatory elements of the TNF locus were maintained in the transgene to assure adequate gene regulation. Transmembrane TNFα transgenic mice were fully protected from endotoxic shock, and no TNFα bioactivity was detectable in the serum after stimulation with lipopolysaccharide. Activated CD4 T cells from these animals, however, lysed L929 cells in a cell contact-dependent way. After administration of lipopolysaccharide, transmembrane TNFα transgenic mice produced significantly higher levels of interleukin-12 than wild-type mice or TNF-deficient mice. This indicates that transmembrane TNFα may greatly affect the course of a cellular immune responses *in vivo* and exerts quantitatively and qualitatively distinct functions from secreted TNFα *in vitro* and *in vivo*.

Tumor necrosis factor-α (TNFα)[1] is a pleiotropic cytokine produced by a wide variety of cell types of mostly hematopoietic, but also of nonhematopoietic, origin (for review, see Ref. 1). TNFα is instrumental in the immune elimination of various infectious agents such as *Candida albicans* (2), *Listeria monocytogenes* (3), or mycobacteria (4) and exerts potent proinflammatory effects, *e.g.* by inducing the expression of adhesion molecules such as VCAM-1, intercellular adhesion molecule 1 (ICAM-1), or E-selectin on endothelial cells and other cell types (5, 6). Aberrant production of TNFα, however, has been also implicated in the pathogenesis of various diseases, such as rheumatoid arthritis, insulin-dependent diabetes-mellitus, sialoadenitis, and inflammatory bowel disease, in particular Crohn's disease (7–11).

TNFα mediates its effects by binding to either TNF receptor 1 (TNFR1) or TNFR2. As revealed by mice deficient for either TNFR1 (12) or TNFR2 (13), these two receptors can mediate distinct effects (14). TNFα is synthesized as a 26-kDa precursor that is also expressed as a type II transmembrane molecule. The 26-kDa transmembrane molecule can be cleaved by membrane bound metalloprotease(s), including the TNFα converting enzyme (TACE) (ADAM-17) into 17-kDa secreted monomers that form biologically active homotrimers (15–19). By deleting the DNA sequence encoding the first 12 amino acids of the processed human 17-kDa TNFα monomer, Kriegler *et al.* (19) generated a nonsecretable 26-kDa transmembrane TNFα Δ1–12 mutant (tm TNFα). This human tm TNFα mutant is capable of lysing TNFα-sensitive target cells (20). Using transfectants overexpressing this human transmembrane TNFα, Grell *et al.* (21) were able to demonstrate in a series of elegant *in vitro* experiments that human transmembrane TNFα is signaling mainly through TNFR2. Indications for distinct roles of transmembrane and secreted TNFα *in vivo* have been recently obtained in transgenic mice and by the use of inhibitors of TACE inhibitors. Mice overexpressing murine tm TNFα are prone to developing arthritis (22) and concanavalin A-induced inflammatory liver disease (23). The inhibition of proteolytic TNFα cleavage by TACE inhibitors, however, protected mice completely from endotoxic shock (24). These results clearly indicate that the pleiotropic effects of TNFα *in vivo* may be further subdivided into effects mediated primarily by secreted, trimeric TNFα, and tm TNFα.

To further dissect the functions exerted by secreted and transmembrane TNFα and to assess the possible use of TACE inhibitors in modulating the potentially deleterious effects of secreted TNFα while maintaining the tm TNFα-mediated functions, we generated tm TNFα tg mice. To this end, the three known proteolytic cleavage sites of mouse TNFα were first inactivated by mutating the corresponding DNA sequences. The functionality of these mutants was first tested by transfecting NIH-3T3 fibroblasts and a mouse T cell hybridoma. Identical mutations were subsequently introduced into a genomic clone of mouse TNFα. The resulting transmembrane TNFα transgene is controlled by the mouse TNFα promotor, and the 3′ AU-rich elements of the genomic TNFα locus are preserved to assure adequate gene regulation *in vivo* (25, 26). In contrast to wt TNF mice, these tm TNFα tg mice are pro-

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[1] The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; LT, lymphotoxin; PCR, polymerase chain reaction; TACE, TNFα converting enzyme; tm, transmembrane; TNFR, TNF receptor; wt, wild-type; VCAM, vascular cell adhesion molecule.

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tected from LPS + D-galactosamine-induced mortality, and no TNFα bioactivity is found in the serum of these challenged mice. Interestingly, in the absence of secreted TNFα, *in vivo* stimulation of tm TNFα tg mice with LPS (100 μg/mouse) leads to significantly increased IL-12 serum levels when compared with wt TNF mice, thus demonstrating quantitatively distinct effects mediated by transmembrane TNFα *in vivo* in the absence and presence of secreted TNFα.

EXPERIMENTAL PROCEDURES

Generation of a cDNA Encoding a Noncleavable Mouse TNFα Mutant—Primers used to construct the various TNFα mutants were purchased from Microsynth (Balgad, Switzerland), and their sequences were as follows: to generate Δ[Leu^{-2} to Leu^{+1}], ttgagaagatgatctggtctgggccataga; to generate Δ[Leu^{-12} to Leu^{-10}], ggccatagaactgatgccatttgggaactt; to mutate Lys^{+11} to Glu^{+11}, gggctacaggctcgtcactcg; for PCR-mediated 5′ and 3′ linker addition contributing the *Xho*I and *Not*I cloning sites for subsequent cloning, gtcgactcgaggatccacatctccctccag and taccgcggccgctgcagagtgggggctggg.

To construct the mouse TNFα cDNA mutants, the 1108-base pair *Eco*RI fragment of the TNFα cDNA (kindly provided by Genentech Inc, South San Francisco, CA) was subcloned into the *Eco*RI site of M13mp18. DNA sequences encoding amino acids Leu^{-2} to Leu^{+1}, Leu^{-12} to Leu^{-10}, and Leu^{-2} to Leu^{+1}/Leu^{-12} to Leu^{-10} were deleted using the U-DNA mutagenesis kit from Roche Molecular Biochemicals, and the resulting *Eco*RI fragments were isolated and cloned into the *Eco*RI site of vector pBluescript KS+. Subsequently, 5′ and 3′ untranslated regions were trimmed off by PCR-mediated linker addition using Deep Vent polymerase (New England Biolabs). The resulting TNFα cDNA genes wt TNFα, L1 TNFα (Δ[Leu^{-2} to Leu^{+1}]), L2 TNFα (Δ[Leu^{-12} to Leu^{-10}]), and L3 TNFα (Δ[Leu^{-2} to Leu^{+1}/Leu^{-12} to Leu^{-10}]) were cloned into the *Xho*I/*Not*I site of the eukaryotic expression vector BCMGS-Neo (27) under the control of the constitutive cytomegalovirus promoter. Using the U-DNA Mutagenesis Phasmid set (Roche Molecular Biochemicals), an additional TNFα mutant L6 was constructed by mutating Lys^{+11} of mutant L3 to Glu^{+11}. Sequences of TNFα cDNA genes were verified by the dideoxy chain termination method using Sequenase® 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions for sequencing double strand DNA.

Cell Cultures—Adherent cell lines L929 and NIH-3T3 (obtained from Giuseppe Bertoni, Veterinary Hospital, Bern, Switzerland) as well as the nonadherent By155.16 T cell hybridoma (28); kindly provided by G. Hollönder, Boston, MA) were grown in Iscove's modified Dulbecco's medium supplemented with 5% fetal calf serum and 4 mM L-glutamine. By155.16 and NIH-3T3 transfectants were selected and maintained at 500 μg/ml and 750 g/ml G418 sulfate (Life Technologies, Inc.), respectively.

DNA Transfection—Transfection of NIH-3T3 and By155.16 cells with various TNFα expression constructs was accomplished by electroporation. Typically, log phase NIH-3T3 cells were intensively washed in sucrose buffer, and 5×10^6 cells in 0.8 ml of ice-cold sucrose buffer were electroporated with 5 μg of plasmid in a 0.4-cm-gap cuvette (Bio-Rad) at 1000 V and 1 uF, applying four pulses separated by 1-min intervals on ice. Electroporated cells remained for an additional 10 min on ice before being transferred into supplemented Iscove's modified Dulbecco's medium. Three days later, the selection of transfectants was performed using G418 sulfate at 750 μg/ml. Resistant colonies were expanded in supplemented Iscove's modified Dulbecco's medium in the presence of G418 sulfate.

Electroporation of By155.16 cells was performed as described above except that only three pulses were applied. One day after electroporation, 500 μg/ml G418 sulfate was added to the medium to select for transfectants. TNFα-expressing By155.16 cells were finally enriched by sorting brightly fluorescent cells on a FACS Vantage using serum from mice transgenic for soluble TNFR p55-human Fcγ3 fusion protein (9) as a primary reagent, and FITC-conjugated $F(ab')_2$ rabbit anti-human IgG as a secondary reagent (Dako, Copenhagen, Denmark) for detection.

TNFα Bioassay—Biologically active TNFα in the supernatant was detected using the TNF-sensitive L929 fibroblast bioassay as described (29). As a specificity control, a neutralizing polyclonal rabbit anti-mouse TNFα antibody (IP-400; Genzyme) was added to the L929-containing wells together with cell culture supernatants or TNFα producing cells.

Isolation of TNFα by Ammonium Sulfate Precipitation—Where required, purification of TNFα from the medium was accomplished by ammonium sulfate precipitation (30). Total proteins in culture supernatants were precipitated at 85% saturation of ammonium sulfate, and the protein pellet was extracted twice with 40% saturated ammonium sulfate and 50 mM Tris, pH 7.8. Finally, TNFα in the remaining protein pellet was redissolved in 50 mM Tris, pH 7.8, and used for Western blot analysis. Using this purification scheme we were able to quantitatively isolate TNF from the medium and to reduce more than 80% of bovine serum albumin added to supplement the culture medium.

Extraction of Membrane-associated TNF—Membrane-associated TNF was prepared by lysing TNF transfectants in 0.5% Triton X-100, 300 mM NaCl, and 50 mM Tris-HCl, pH 7.6, in the presence of a mixture of protease inhibitors including 1 mM benzamidine, Trasylol (]1/200] vol), 1 μg/ml leupeptin, 1 mM Pefabloc (Roche Molecular Biochemicals), and 1.8 mg/ml iodoacetamide. At this point, samples were analyzed by Western blotting.

Western Blot Analysis—Protein samples were concentrated by chloroform/methanol extraction (31) and electrophoretically separated on 12% polyacrylamide gels under reducing conditions. Electroblotting to 0.2 μm nitrocellulose membranes (Schleicher & Schuell, Inc.) was carried out using a Trans-Blot electrophoretic blotting system (Idea Scientific Co.). After blocking the filters overnight in 3% bovine serum albumin, the immunoblots were developed with rabbit anti-murine TNFα antibody (Genzyme IP-400) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dako). 3,3′-Diaminobenzidine was used as the chromogenic substrate (Sigma).

Co-culture of bEnd.5 Endothelioma Cells with TNFα-transfected By155.16 T Cells—bEnd.5 endothelioma cells (32) (kindly provided by Dr. Y. Reiss, Max-PIank-Institut, Bad Nauheim, Germany) (passages 16–18) were cultured in six-well plates. 1×10^5 By155.16 T hybridoma cells transfected with BCMGS-Neo (mock control), BCMGS-wt TNFα, or BCMGS-L6 (tm TNFα) were added to semiconfluent bEnd.5 cells and co-cultured for additional 24 h. Stainings for VCAM-1 expression were done on ice in the culture wells: after extensive washing and removal of By155.16 transfectants, cells were first incubated with G4.2 (anti-CD16/32) hybridoma supernatant followed by anti-VCAM-1 monoclonal antibody (Pharmingen) and anti-rat-PE (Southern Biotechnology Associates, Inc). Stained bEnd.5 cells were detached by incubation with 5 mM EDTA (without Mg^{2+} and Ca^{2+}) at 4 °C and were subsequently analyzed on a FACScan using Cellquest software (Becton Dickinson) for data acquisition and analysis.

Generation of a Murine Noncleavable tmTNFα Transgene—The genomic mutant encoding noncleavable transmembrane TNFα was constructed by the overlapping extension method (33) employing the following primers (Microsynth, Balgach, Switzerland): muTNF1 (encompassing *Nar*I at position 4448 of clone Y00467), catacccaaggcgccacatctccc; muTNF2 (deletion of Leu^{-12} to Leu^{-10}), gtctgggccatagaactgatgccatttgggaac; muTNF3 (deletion of Leu^{-2} to Leu^{+1}, partially overlapping muTNF2), atcagttctatggcccagaccagtaagtgttccc; muTNF4 (mutation of Lys^{+11} to Glu^{+11}), cgtgggctacaggctcgtcactcg; muTNF5 (complementary to muTNF4), cgagtgacgagcctgtagcccacg; and muTNF6 (encompassing *Ava*I at position 5658 of clone Y00467), gctatgaggtcccgggtggccccc.

An Asp^{718}/*Sal*I fragment of a murine genomic clone of LTα/TNFα locus (34) was subcloned into pBluescript KS+ via the flanking Asp^{718} and *Sal*I sites. This plasmid, pTNF A/S, was used as template for the generation of mutated fragments A, B, and C by PCR amplification using primer pairs muTNF1/muTNF2, muTNF3/muTNF4, and muTNF5/muTNF6, respectively. Prior to further use, PCR fragments were treated with mung bean nuclease and purified by gel excision and phenol extraction. Due to their complementary ends, fragments B and C were then annealed and linked in a conventional PCR containing primers muTNF3 and muTNF6. Fragment A was subsequently linked by PCR to the fragment BC using the primers muTNF1 and muTNF6. The resulting *Nar*I/*Ava*I PCR fragment carrying the deletions and point mutation was finally used to replace the *Nar*I/*Ava*I wild-type fragment in pTNF A/S, thus yielding pmutgenTNF12. Reintroduction of a 1.5-kilobase pair *Ava*I fragment from pTNF A/S into pmutgenTNF12 at the respective site delivered pmutgenTNF13, a full-size mutant TNFα gene, which should lead to uncleavable TNFα expression. All mutations were verified by DNA sequencing using Sequenase® 2.0 (United States Biochemical Corp.) according to the manufacturer's instructions.

Generation of tm TNFα Transgenic Mice—A 4.842-kilobase Asp^{718}/*Sal*I fragment from pmutgenTNF13 containing the mutated genomic clone of TNFα was isolated and used to generate transgenic mice. The microinjection of about 1–2 pl buffer into the male pronucleus of TNFα/LTα double deficient C57Bl/6 × 129 mice ($TNF^{-/-}$ mice) (35) was performed by standard procedures with a final DNA concentration of 2 ng/μl. Detection of transgenic mice was carried out by PCR on mouse tail DNA using the primer pair muTNFtg1 (aagttcccaaatggcatcag) and muTNFtg2 (ctacgacgtgggctacag), allowing the amplification of the transgene only.

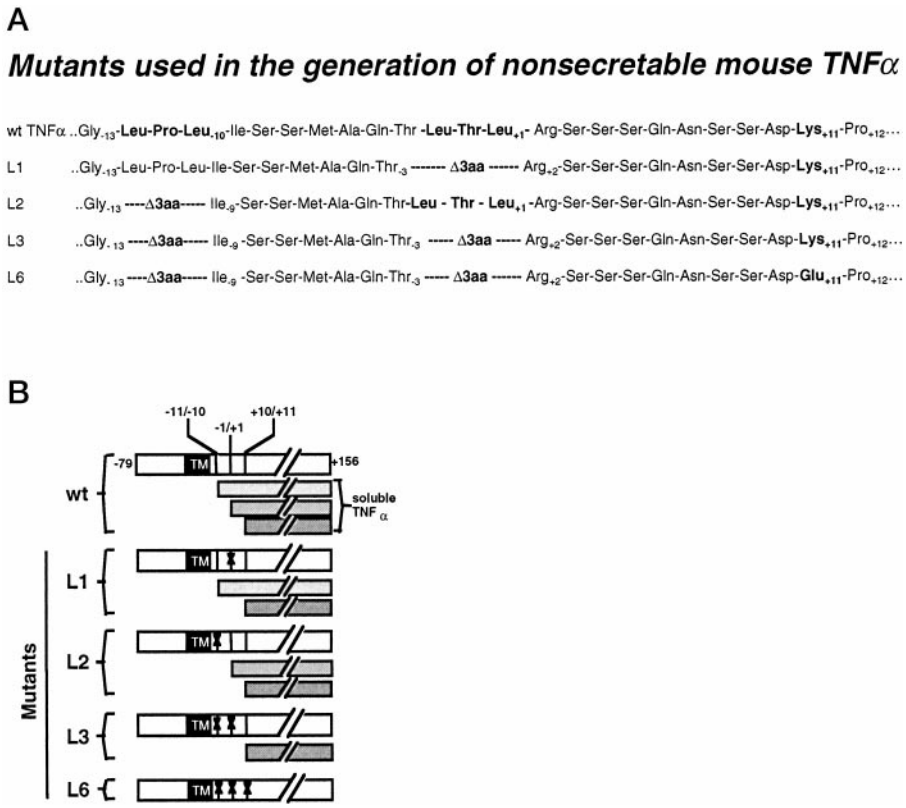

FIG. 1. *A,* mutants used in the generation of nonsecretable mouse TNFα. *B,* schematic depiction of TNFα cleavage mutants with the expected secreted TNFα fragments.

Administration of LPS + D-Galactosamine—B6 × 129 mice, TNF $^{-/-}$ B6 × 129 mice, and tm TNFα tg TNF$^{-/-}$ B6 × 129 mice were challenged with intraperitoneal administration of 10 μg of LPS + 20 mg of D-galactosamine. Mice were observed for 72 h. Mice were sacrificed when they became moribund or at the end of the observation period, *i.e.* 72 h after LPS + D-galactosamine administration.

In Situ Hybridization—A 1108-base pair cDNA fragment of the murine TNFα gene (positions 1–1108; obtained from Genentech Inc., San Francisco, CA) was subcloned into pGEM-2. After linearization of the plasmid, sense and antisense RNA probes were prepared using the appropriate RNA-polymerase as described previously (36). *In situ* hybridizations of paraformaldehyde-fixed cryostat sections were performed as described previously (36).

Generation of CD4 T Cell Blasts from ex Vivo Isolated Splenocytes—After osmotic lysis of erythrocytes, splenocytes were incubated with 0.5–1 μg of biotinylated anti-CD8α and anti-B220 monoclonal antibody per 10^6 cells for 15 min on ice. Avidin magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used as second step reagents for the negative selection of CD4 T cells. The CD4 T cell-enriched negative fraction (90% purity) was resuspended at 2×10^6 cells/ml in RPMI medium + 10% fetal calf serum in the presence of 3×10^6 cells/ml of irradiated antigen-presenting cells (2000 rad) and was incubated with 2 μg/ml concanavalin A for 48 h. After 2 days, the cells were washed and incubated for additional 72 h in the presence of 100 units/ml IL-2. The CD4 T cell blasts were then purified on a Ficoll gradient, and 2×10^6 cells/ml were incubated for 4 h in the presence of 50 units/ml IL-2, 20 μg/ml phorbol 12-myristate 13-acetate, and 200 μg/ml ionomycin and subsequently co-cultured with L929 cells.

Determination of IL-12 and TNFα Production upon Stimulation in Vivo with LPS—Mice were sacrificed 3 and 6 h after intraperitoneal administration of 100 μg of LPS, and serum was collected for subsequent determination of IL-12 p70 levels. IL-12 p70 was detected by enzyme-linked immunosorbent assay in 96-well microtiter plates using the anti-IL-12 p70 monoclonal antibody 9A5 as a coating antibody and biotinylated and IL-12 p40 monoclonal antibody C17.8 as a detecting antibody. The standard curve was constructed using recombinant mouse IL-12 p70 (all reagents from Pharmingen). For statistical analysis and calculation of *P* values, the Student *t* test was performed using InStat version 2.03 software (GraphPad Inc). For the detection of TNFα in the serum, mice were sacrificed 1.5 h after administration of 100 μg of LPS, intraperitoneal and the serum was collected for subsequent assessment of bioactive TNFα using L929 indicator cells.

RESULTS

Generation of a Mouse TNFα cDNA Encoding Nonsecretable TNFα—To assess the differential proteolytic cleavage of mouse TNFα, the three known proteolytic cleavage sites of mouse TNFα (17, 18) were sequentially deleted in a mouse TNFα cDNA clone. To this end, four different mutants of mouse TNFα cDNA were created, with inactivation of the cleavage sites at positions +1 (TNFα mutant L1); −10 (TNFα mutant L2); +1 and −10 (TNFα mutant L3) and +1, −10, and +11 (TNFα mutant L6) (Fig. 1). Mutant cDNAs were subsequently cloned into the BCMGS-Neo expression vector and transfected into NIH-3T3 fibroblasts or the T cell hybridoma By155.16 (28). As shown in Fig. 2*A,* in the L3 TNFα mutant, the inactivation of the two proteolytic cleavage site at −10 and at +1 by the deletion of three amino acids each leads to the expected reduction in the molecular weight of the TNFα precursor compared to nonmutated tmTNFα. When transfected with wt TNFα construct, NIH3T3 cells secrete three protein species with a molecular mass of around 17 kDa, as revealed by Western blot analysis (Fig. 2*B, lane 2*). Sequential deletions of the known cleavage sites display altered Western blot patterns. In particular, deletion of the cleavage site at +1 results in the loss of one band and the appearance of a higher mobility band (Fig. 2*B, lane 3*). On the other hand, deleting the cleavage site at −10 results in the disappearance of the slowest mobility band only (Fig. 2*B, lane 4*). However, when cleavage sites at +1 and −10 were deleted, only one band was visible on Western blots (Fig. 2*B, lane 5*). This TNFα species still exerts biological activity in a L929 bioassay.[2] Hence, we inactivated the third potential cleavage site of mouse TNFα at position +11 by a $Lys^{11} \rightarrow Glu$ substitution (18). By155.16 T cells transfected with the resulting mutant L6 do not secrete TNFα into the supernatant as shown in a Western blot analysis (Fig. 2*C, lane 4*) and in a L929

[2] M. Imboden and S. Trachsel, unpublished observations.

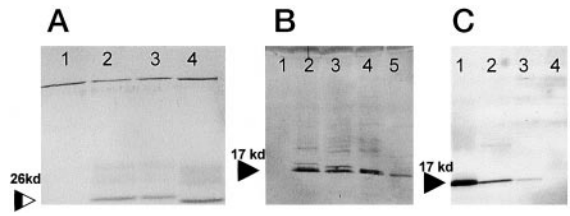

FIG. 2. *A,* Western blot analysis of membrane-associated TNFα from NIH-3T3 fibroblasts transfected with BCMGS-Neo (*lane 1*), BCMGS-wt TNFα (*lanes 2* and *3*; two different clones), and BCMGS-L3 mutant TNFα (*lane 4*). *B,* Western blot analysis of secreted TNFα from NIH-3T3 cells transfected with BCMGS-Neo (*lane 1*), BCMGS-wt TNFα (*lane 2*), BCMGS-L1 mutant TNFα (*lane 3*), BCMGS-L2 mutant TNFα (*lane 4*), and the BCMGS-L3 mutant TNFα (*lane 5*). *C,* Western blot analysis of secreted TNFα from the T cell hybridoma By155.16 transfected with BCMGS-wt TNFα (*lane 1*), BCMGS-L1 mutant TNFα (*lane 2*), BCMGS-L3 mutant TNFα (*lane 3*), and the BCMGS-L6 mutant TNFα (*lane 4*).

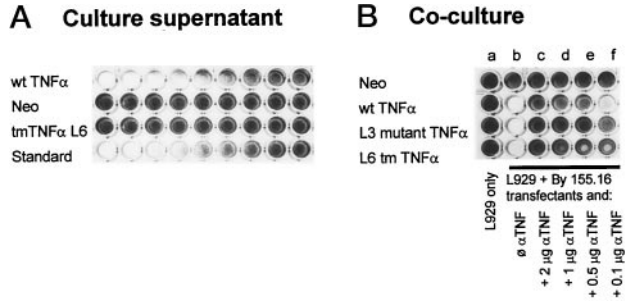

FIG. 3. **TNFα bioassay.** *A,* L929 cells were incubated with serial dilutions (1:2) of supernatant from the T cell hybridoma By155.16 transfected with wt TNFα, BCMGS-Neo, L6 mutant TNFα (supernatant concentrated 20-fold), or recombinant mouse TNFα (starting concentration, 3800 units/ml) as a standard. *B,* co-culture of L929 target cells with the T cell hybridoma transfected with BCMGS-Neo, wt TNFα, L3 mutant TNFα, and L6 mutant TNFα. Shown are L929 only (no By155.16 transfectants added) (*lane a*) and co-culture with By155.16 transfectants in the absence (*lane b*) and in the presence of 2 (*lane c*), 1 (*lane d*), 0.5 (*lane e*), and 0.1 μg (*lane f*) of TNFα neutralizing antibody.

bioassay (Fig. 3*A*). However, these L6 transfectants were unequivocally positive when tested for surface TNFα by FACS analysis using soluble TNFR-p55 hcγ3 fusion protein (9) for detection.[2] Therefore, to assess the functionality of cell surface-expressed tmTNFα, TNFα-sensitive L929 indicator cells were co-cultured with the L6-transfected By155.16 cells. Co-cultures of L929 cells with wt TNFα-transfected By155.16, or vector-only (BCMGS-Neo)-transfected By155.16 cells as positive and negative controls, respectively. As shown in Fig. 3*B,* L6 tmTNFα transfectants lyse L929 cells as efficiently as wt TNFα transfectants in a cell contact-dependent manner, whereas the supernatant of L6 overexpressing By155.16 transfectants, even upon 20-fold concentration by ammonium sulfate precipitation, is unable to lyse L929 cells in contrast to the supernatant of the wt TNFα transfectants that exerts potent cytotoxic activity in the L929 bioassay (Fig. 3*A*). The cell contact-dependent lysis of L929 cells is inhibited by neutralizing anti-TNFα antibodies in a dose-dependent manner (Fig. 3*B*).

tm TNFα Expressing By155.16 T Cells Induce VCAM-1 Expression on Endothelial Cells upon Co-culture—To assess the potential proinflammatory properties of tm TNFα, induction of VCAM-1 expression in bEnd.5 endothelioma cells was examined (32). To this end, L6-transfected By155.16 cells, wt TNFα-transfected By155.16, and BCMGS-Neo-transfected By155.16 cells were co-cultured with bEnd.5 cells for 24 h. wt TNFα (Fig. 4*A*) and L6 (tm TNFα) By155.16 transfectants (Fig. 4*B*) induce a marked VCAM-1 up-regulation on a large fraction of bEnd.5 mouse endothelial cells when compared with bEnd.5 cells co-cultured with BCMGS-Neo-transfected By155.16 T cells as a negative control.

Taken together, these results clearly indicate that the L6 TNFα mutant is functional and cannot be cleaved into secreted, bioactive, or inactive TNFα in transfected By155.16 cells.

tm TNFα tg Mice Are Protected from Endotoxic Shock—To further elucidate the distinct functions exerted *in vivo* by tm TNFα and secreted TNFα and hence to assess the potential use of TACE inhibitors to modulate TNFα-mediated effects *in vivo*, we introduced the three mutations of TNFα mutant L6 also in the coding sequence of a genomic clone of mouse TNFα. The resulting transgene is thus under the control of the mouse TNFα promotor, and the AU-rich elements at the 3′ end are maintained. The resulting tm TNFα construct was microinjected into the male pronucleus of $TNF\alpha^{-/-}$ $LT\alpha^{-/-}$ mice (35). The resulting transgenic mice (tmTNFα tg mice) still show an impaired splenic architecture and lack peripheral lymph nodes, as do their nontransgenic littermates.[3] $TNF\alpha^{-/-}$ $LT\alpha^{-/-}$ ($TNF^{-/-}$) mice challenged with LPS + D-galactosamine are fully protected from the LPS-induced mortality observed in B6 × 129 mice (wt TNF mice) (35). Hence, we determined first whether tmTNFα tg mice are also protected from this LPS + D-galactosamine-induced lethality. As shown in Fig. 5*A,* TNF+/+ mice died within 6 h upon administration of LPS + D-galactosamine, whereas tmTNFα tg and $TNF^{-/-}$ mice alike were protected from death throughout the entire observation period of 72 h. No bioactive TNFα was detected in the serum of tmTNFα tg or wt TNF mice 1.5 h post-LPS + D-galactosamine administration, whereas high concentrations of bioactive TNFα were detected in the serum of wt TNF mice, as shown in a TNF bioassay (Fig. 5*B*). The absence of TNFα in the serum of LPS + D-galactosamine challenged tmTNFα tg mice, however, cannot be attributed to a defective transcription of the transgene because in the spleen of these animals, high frequencies of tmTNFα mRNA expressing cells were found (Fig. 5*C*), whereas splenic tissue sections of untreated wt TNF and tmTNFα tg mice showed no cells, or only a few, expressing TNFα mRNA at low levels.[4] To assess the functionality of the tmTNFα transgene, CD4-positive splenic T cells from tmTNFα tg, wt TNF, and $TNF^{-/-}$ mice were isolated and activated *ex vivo* with phorbol 12-myristate 13-acetate and ionomycin. As shown in Fig. 6, only activated CD4 T cells from wt TNF and tmTNFα tg mice lysed the L929 target cells upon co-culture. The cytotoxic activity, however, is generally slightly lower in activated CD4 T cells from tmTNFα tg than from wt TNF donor mice. No cytotoxic activity is detected in the supernatant of activated tmTNFα tg CD4 T cells, whereas L929 cells are lysed by the supernatant of activated splenic CD4 T cells from wt TNF mice.[4]

tm TNFα tg Mice Produce More IL-12 Than wt TNF Mice upon Activation with LPS in Vivo—Mycobacteria- or LPS-induced production of IL-12 has previously been shown to be TNF-dependent (37). To determine the effect of transmembrane TNFα on the production of IL-12 p70 *in vivo*, $TNF^{-/-}$, wt TNF, and tm TNFα tg mice were challenged with 100 μg of LPS alone. 3 and 6 h later, mice were sacrificed to collect serum for subsequent determination of IL-12 p70 levels. As shown in Fig. 7, 3 h following LPS administration, IL-12 p70 serum levels were significantly increased in wt TNF mice over $TNF^{-/-}$ mice. In tm TNFα tg mice, however, IL-12 p70 serum levels significantly ($p = 0.014$) exceeded even those seen in wt TNF mice. At 6 h after LPS administration IL-12 p70 serum levels are

[3] C. Mueller and M. Bühler, unpublished observations.

[4] N. Corazza and C. Mueller, unpublished observations.

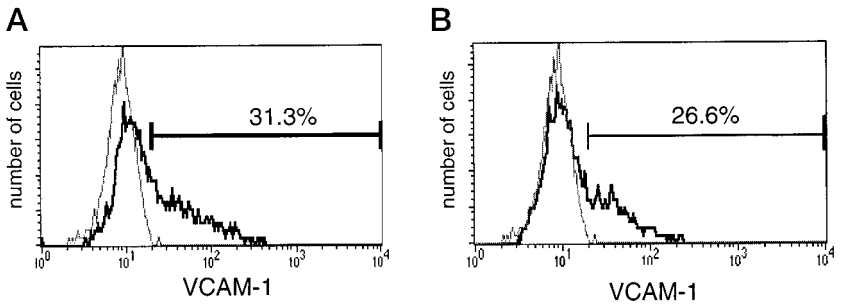

FIG. 4. **VCAM-1 expression on the mouse endothelial cell line bEnd.5 upon co-culture for 24 h with By155.16 wt TNFα (*black line*) (*A*) and By155.16 L6 mutant TNFα (*black line*) (*B*).** VCAM-1 expression of bEnd.5 upon co-culture with By155.16-Neo is indicated as a *thin gray line* in both panels as a negative control.

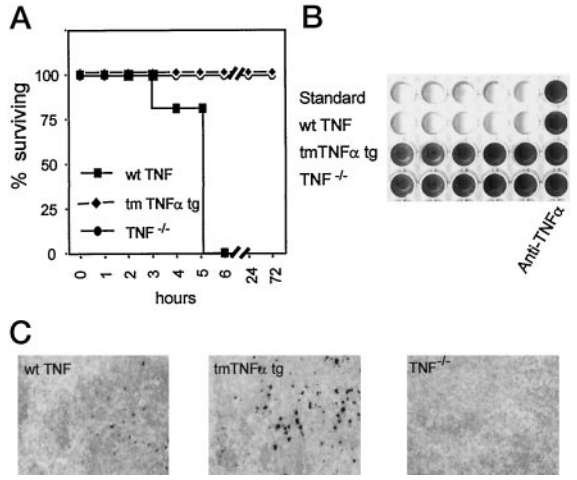

FIG. 5. **LPS + D-galactosamine-induced mortality.** *A,* survival curve of wt TNF mice (*squares*), tm TNFα tg mice (*diamonds*), and $TNF^{-/-}$ mice (*circles*) upon administration of LPS + D-galactosamine. *B,* bioassay for the detection of TNFα in the serum of mice challenged 1.5 h earlier with LPS + D-galactosamine. Shown are sera from $TNF^{-/-}$ mice, tm TNFα tg mice, wt TNF mice, and recombinant TNFα (*Standard*). As a specificity control, 1 μg of a neutralizing polyclonal antiserum against TNFα was added in the last well of each row (*Anti-TNFα*). *C, in situ* hybridization with TNFα RNA antisense probes of splenic tissue sections from B6 × 129 mice (*wt TNF*), tm TNFα tg mice (*tmTNF-α tg*), and TNFα $^{-/-}$ $LT\alpha^{-/-}$ mice ($TNF^{-/-}$) 1.5 h following administration of LPS + D-galactosamine. TNFα mRNA positive cells can be identified by the *dark silver grains* on the tissue section.

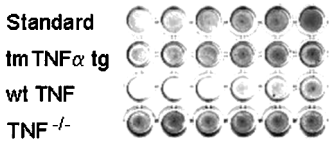

FIG. 6. **TNFα-mediated cytotoxicity of activated splenic CD4 T cells against L929 cells.** L929 target cells were incubated with serial dilutions (1:2) of recombinant TNFα (*Standard*) phorbol 12-myristate 13-acetate-treated and ionomycin-treated splenic CD4 T cells from tm TNFα tg mice (*tm TNFα tg*), B6 × 129 mice (*wt TNF*), and TNFα $^{-/-}$ $LT\alpha^{-/-}$ ($TNF^{-/-}$) mice.

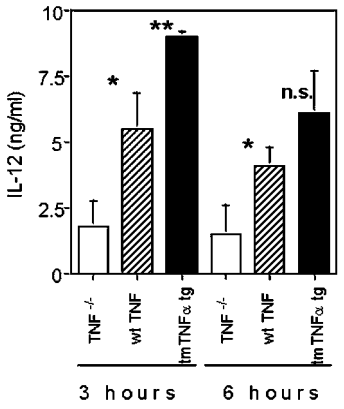

FIG. 7. **IL-12 p70 serum levels after administration of LPS.** IL-12 p70 serum levels in $TNF^{-/-}$ mice, wt TNF mice and tm TNFα tg mice 3 and 6 h after administration of 100 μg of LPS per mouse (mean +S.D.; $n = 3$ mice per group; ** $p < 0.02$; * $p < 0.05$) (*n.s.,* not significant ($p > 0.1$)).

slightly reduced, maximum IL-12 serum levels, however, were still observed in tm TNFα tg mice also at this time point.

DISCUSSION

TNFα, like the majority of its family members, exists as a transmembrane molecule that can be cleaved and secreted as a homotrimer. Although the existence of tm TNFα has been documented soon after characterization of TNFα (38, 39), and although experimental evidence for distinct functions of human transmembrane and secreted TNFα has been provided (19, 20), the functional significance of transmembrane TNFα has been neglected for several years. Recent progress in the understanding of the signaling and the possible TNFR2-mediated functions of tm TNFα (21), together with the characterization and cloning of the metalloprotease(s) responsible for cleaving TNFα (15, 16, 40), rekindled the interest in the biological relevance of tm TNFα. The identification of specific inhibitors of the TNFα processing proteases (24, 41), furthermore, might offer the opportunity to selectively modulate the pleiotropic effects of TNFα through specifically blocking secreted TNFα-mediated effects, such as endotoxic shock induction (24), while maintaining tm TNFα-mediated functions.

Indications for an involvement of transmembrane TNFα in inflammatory reactions *in vivo* have been recently obtained in transgenic mice, overexpressing a mouse Δ1–12 TNFα-human β-globin hybrid transgene (22, 42). These mice developed signs of inflammatory liver diseases upon experimental induction with concanavalin A (23) and were susceptible to the development of arthritis (22). For the generation of this transmembrane TNFα transgene, the 3′ end of the TNFα locus containing the 3′ AU-rich element was replaced by the regulatory 3′ sequence of human β-globin. As has been recently demonstrated by the same group, this modification of the TNFα locus can induce itself immunopathological disorders (26). Furthermore, in these transmembrane TNFα tg Δ1–12 mutant mice, a minor portion of the 26-kDa TNFα is still cleaved to yield a biological inactive, secreted TNFα molecule (22).

Therefore, we decided to make a different construct for the generation of mice transgenic for a nonsecretable homologous tm TNFα by the deletion of all known proteolytic cleavage sites of mouse TNFα. Sequential deletion of the proteolytic cleavage sites in the mouse TNFα cDNA leads to the disappearance of distinct bands in the Western blot analysis of TNFα secreted from transfected NIH-3T3 (Fig. 2*B*) as predicted from the po-

sitions of the mutated sites (Fig. 1*B*). Western blot analysis of the T cell hybridoma By155.16, transfected with the same constructs, however, always revealed secreted TNFα molecules of a uniform size (Fig. 2*C*). This intriguing finding may indicate that either the cellular microenvironment controls the accessibility of the different cleavage sites for the membrane bound metalloproteases or, alternatively, that the 26-kDa TNFα precursor is processed by different proteases expressed in a cell type-specific manner. The distinct amino acid composition forming the three potential cleavage sites may indicate that the 26-kDa TNFα may be processed by different proteases with distinct cleavage specificities.

The successful use of TNF neutralizing antibodies in the treatment of patients with rheumatoid arthritis (7) or with fistulizing Crohn's disease (8) demonstrates that modulating the activity of TNFα represents a promising strategy to treat inflammatory disorders. The initial observation that due to the absence of secreted TNFα following the administration of TACE inhibitors, mice are protected from endotoxic shock (24), together with the increasing evidence that transmembrane and secreted TNFα may exert quantitatively and/or qualitatively distinct functions, prompted us to generate a tm TNFα tg mouse as a tool to determine distinct effects mediated by transmembrane and secreted TNFα *in vivo*. Such an animal model may turn out to be indispensable in assessing the potential of TACE inhibitors as a more selective treatment of inflammatory disorders. To ensure appropriate transcriptional and posttranscriptional regulation, the transgene was placed under the control of a mouse TNFα promotor, and the regulatory 3′ AU-rich element of the TNF locus were maintained. The functionality of the tm TNFα tg is clearly demonstrated by the cell contact-mediated cytotoxicity of CD4 T cells from tm TNFα tg mice, but not from $TNF^{-/-}$ mice against L929 target cells (Fig. 6). The observed protection of tm TNFα tg mice from endotoxic shock, together with the induced expression of TNFα mRNA in the spleen after LPS treatment in tm TNFα tg mice and wt TNF mice alike (Fig. 5*C*), indicates that the tm TNFα transgene is inducible, although it does not yield biologically active TNFα in the circulation. The histopathological analysis of the liver at the end of the observation period generally revealed more severe damage of the hepatocytes in tm TNFα tg mice than in $TNF^{-/-}$ mice. These alterations, however, were always much less severe than in LPS + D-galactosamine-treated wt TNF mice.[4] Similar observations have previously been made in experimentally induced hepatitis in TACE inhibitor GM-6001-treated mice (43). The observed enhanced production of IL-12 upon LPS stimulation in tm TNFα tg mice when compared with wt TNF mice is intriguing, and the molecular and cellular mechanisms involved have to be defined yet. It is tempting to speculate, however, that secreted TNFα is able to down-regulate production of IL-12, either directly through signaling via TNFRs, presumably through TNFR1, on the IL-12 producing cells (mainly phagocytic cells), or indirectly, through attenuating the activity of T cells. In support of the latter notion are observations by Cope *et al.* (44), who showed that T cells from TNFα-deficient mice and from mice treated with neutralizing anti-TNF antibodies exert enhanced proliferative and functional activity (44). Hence, secreted TNFα may exert immunomodulatory properties and may indeed attenuate T cell activity. Because tm TNFα tg mice lack secreted TNFα (but in contrast to $TNF^{-/-}$ mice), TNFR activation is still possible through tm TNFα, T cells of tm TNFα tg mice may represent potent inducers of IL-12 production in phagocytic cells, *e.g.* through the production of elevated amounts of interferon-γ and enhanced expression of CD154. Indications for quantitatively and qualitatively distinct responses mediated by tm TNFα and secreted TNFα have previously been reported using *in vitro* systems: increased production of prothrombotic tissue factor is observed in co-cultures of human umbilical vein endothelial cells with tm TNFα-transfected Chinese hamster ovary cells when compared with the addition of recombinant soluble TNFα (21), and tm TNFα is able to lyse target cells that are resistant to cytolysis mediated by secreted TNFα (21, 39)

In conclusion, the tm TNFα tg mice described in this report represent a useful tool to further dissect the pleiotropic functions exerted by secreted and transmembrane TNFα. This has been clearly demonstrated by the striking early IL-12 production in tm TNFα tg mice upon LPS administration that exceeds the response seen wt TNF and $TNF^{-/-}$ mice. Due to the central role of IL-12 in determining the functional differentiation of naïve T cells (45), this tm TNFα-mediated effect may have far reaching consequences on the outcome of a cellular immune responses *in vivo*. The definition of the distinct functions exerted by the two molecular forms of TNFα will be instrumental in assessing the potential of TACE inhibitors as a therapeutic agent. Furthermore, the tm TNFα tg mice should allow us to address more specifically the hypothesis that secreted TNFα exerts some of the anti-inflammatory properties described for TNFα *in vivo* and *in vitro* (2, 46, 47).

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