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 nonsecretatable mutant TNFα efficiently lysed L929 target cells in a cell contact-dependent manner and induced expression of vascular cell adhesion molecule-1 on mouse endothelium 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) 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 promoter, 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-
tected from LPS + t-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) led 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]tgaggagatagctgctggctggccataga; to generate Δ[Leu-2 to Leu-1]gctatgaggtcccgggtggccccc.

To construct the mouse TNFα cDNA mutants, the 1108-base pair EcoRI fragment of the TNFα cDNA (kindly provided by Genentech Inc, South San Francisco, CA) was subcloned into the EcoRI site of M13mp18. DNA sequences encoding amino acids Leu-2 to Leu-1, Leu-2 to Leu-1, and Leu-2 to Leu-2/Ley-12 to Leu-10 were deleted using the U-DNA mutagenesis kit from Roche Molecular Biochemicals, and the resulting EcoRI fragments were isolated and cloned into the EcoRI site of vector Bluescript 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-1]), and L3 TNFα (Δ[Leu-2 to Leu-1/Leu-12 to Leu-19]) were cloned into the XhoI NotI sites of 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 the overlapping extension method (33) employing the following primers (Microsynth, Balgach, Switzerland): μuTNF1 (encompassing NarI at position 4448 of clone Y00467), cactacccggggtggcctccc; muTNF2 (deletion of Leu-2 to Leu-11)), gtggtggccagctggagctcaatctggtgcgg; muTNF3 (deletion of Leu-2 to Leu-11), ggagccatagaactgatgccatttgg-ctagccagctggagctcaatctggtgcgg; and muTNF4 (mutation of Lys-12 to Glu-11), gtggtggccagctggagctcaatctggtgcgg. The resulting muTNFα cDNA genes were verified by the deoxy 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 Dr. Y. Reiss, Max-Planck-Institut, Bad nauheim, Germany) 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 G418 sulfate at 750 µg/ml. Resistant colonies were expanded in medium containing 750 µg/ml G418 sulfate. By155.16 T Cells—Transfection of NIH-3T3 and By155.16 cells was performed using G418 sulfate at 750 µg/ml. Resistant colonies were expanded in medium containing 750 µg/ml G418 sulfate.

Electroporation of By155.16 cells was performed as described above with a few modifications. Typically, log phase NIH-3T3 cells were intensively washed in 1 ml of sucrose buffer, and 5 mM MgCl₂ was added.

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 TNFRp55-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 TNFα from the medium and to reduce more than 90% of bovine serum albumin added to supplement the culture medium.

**Extraction of Membrane-associated TNF**—Membrane-associated TNFα was prepared by lysing TNFα 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), 0.5 mM MgCl₂, and 0.5 mM 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 electroblotting system (Immunetics 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 Endothelial Cells with TNFα-transfected By155.16 Transfectants**—bEnd.5 cells (kindly provided by Dr. Y. Reiss, Max-Planck-Institut, Bad nauheim, Germany) were grown in Iscove’s modified Dulbecco’s medium supplemented with 5% fetal calf serum and 4 mM l-glutamine. By155.16 T hybridoma cells transfected with BCMGS-Neo (mock control), BCMGS-wt TNFα, or BCMGS-L6 (tm TNFα) were co-cultured with bEnd.5 cells at 37°C. As a specificity control, a neutralizing polyclonal rabbit anti-mouse TNFα antibody (Pharmingen) and anti-rat-PE (Southern Biotechnology Associates, Inc.) were used. Stained bEnd.5 cells were incubated with 5 mM EDTA (without Mg²⁺ and Ca²⁺) at 4°C and were subsequently analyzed on a FACSscan using Cellquest software (Becton Dickinson) for data acquisition and analysis.
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, TNFα−/− B6 × 129 mice, and tm TNFαtg TNFα−/− 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-CD8 and anti-B220 monoclonal antibody per 10⁶ 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 × 10⁶ cells/ml in RPMI medium + 10% fetal calf serum in the presence of 3 × 10⁵ 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 × 10⁶ 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 NIH3T3 fibroblasts or the T cell hybridoma By155.16 (28). As shown in Fig. 2A, 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. 2B, 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. 2B, lane 3). On the other hand, deleting the cleavage site at −10 results in the disappearance of the slowest mobility band only (Fig. 2B, lane 4). However, when cleavage sites at +1 and −10 were deleted, only one band was visible on Western blots (Fig. 2B, 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 Lys11 → 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. 2C, lane 4) and in a L929 bioassay (Fig. 2C, lane 5).

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2 M. Imboden and S. Trachsel, unpublished observations.
bioassay (Fig. 3A). However, these L6 transfectants were unequivocally positive when tested for surface TNFα by FACS analysis using soluble TNFR-p55 hcy3 fusion protein (9) for detection. 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. 3B, L6 tmTNFα transfectants lysed L929 cells as efficiently as wt TNFα transfectants in a cell contact-dependent manner, whereas the supernatant of L6 overexpressing By155.16 transfectants, even up to 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. 3A). The cell contact-dependent lysis of L929 cells is inhibited by neutralizing anti-TNFα antibodies in a dose-dependent manner (Fig. 3B).

\( \text{tm TNF} \alpha \) Expressing By155.16 T Cells Induce VCAM-1 Expression on Endothelial Cells upon Co-culture—To assess the potential proinflammatory properties of tmTNFα, induction of VCAM-1 expression in bEnd.5 endothelial 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. 4A) and L6 (tm TNFα) By155.16 transfectants (Fig. 4B) 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.

\( \text{tm TNF} \alpha \) 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 promoter, and the AU-rich elements at the 3′ end are maintained. The resulting tm TNFα construct was microinjected into the male pronucleus of TNFα−/− LTα−/− 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.\(^3\) TNFα−/− LTα−/− (TNFα−/−) mice challenged with LPS + d-galactosamine are fully protected from the LPS-induced mortality observed in B6 × 129 mice (wt TNFα mice) (35). Hence, we determined first whether tmTNFα tg mice are also protected from this LPS + d-galactosamine-induced lethality. As shown in Fig. 5A, TNFα+/+ mice died within 6 h upon administration of LPS + d-galactosamine, whereas tmTNFα tg and TNFα−/− 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 TNFα mice 1.5 h post-LPS + d-galactosamine administration, whereas high concentrations of bioactive TNFα were detected in the serum of wt TNFα mice, as shown in a TNF bioassay (Fig. 5B). 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. 5C), whereas splenic tissue sections of untreated wt TNFα and tmTNFαtg 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 TNFα, and TNFα−/− 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 TNFα and tmTNFαtg 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 TNFα 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 TNFα mice.\(^4\)

\( \text{tm TNF} \alpha \) tg Mice Produce More IL-12 Than wt TNFα 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, TNFα−/−, wt TNFα, and tmTNFαtg 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 TNFα mice over TNFα−/− mice. In tmTNFαtg mice, however, IL-12 p70 serum levels significantly (\( p = 0.014 \)) exceeded even those seen in wt TNFα 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.
Functions Exerted by Transmembrane TNFα in Vivo and in Vitro

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 TNFα mice (squares), tm TNFα tg mice (diamonds), and TNFα−/− 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 TNFα−/− mice, tm TNFα tg mice, wt TNFα 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 TNFα), tm TNFα tg mice (tmTNFα tg), and TNFα−/− LTA−/− mice (TNFα−/−) 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 TNFα), and TNFα−/− LTA−/− (TNFα−/−) mice.

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 understand-

ing 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. 2B) as predicted from the po-
sitions of the mutated sites (Fig. 1B). 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. 2C). 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 may turn out to be indispensable 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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