Membrane versus Soluble Isoforms of TNF-α Exert Opposing Effects on Tumor Growth and Survival of Tumor-Associated Myeloid Cells

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Abstract
TNF-α, produced by most malignant cells, orchestrates the interplay between malignant cells and myeloid cells, which have been linked to tumor growth and metastasis. Although TNF-α can exist as one of two isoforms, a 26-kDa membrane tethered form (mTNF-α) or a soluble 17-kDa cytokine (sTNF-α), the vast majority of published studies have only investigated the biologic effects of the soluble form. We show for the first time that membrane and soluble isoforms have diametrically opposing effects on both tumor growth and myeloid content. Mouse lung and melanoma tumor lines expressing mTNF-α generated small tumors devoid of monocytes versus respective control lines or lines expressing sTNF-α. The lack of myeloid cells was due to a direct effect of mTNF-α on myeloid survival via induction of cell necrosis by increasing reactive oxygen species. Human non–small cell lung carcinoma expressed varying levels of both soluble and membrane TNF-α, and gene expression patterns favoring mTNF-α were predictive of improved lung cancer survival. These data suggest that there are significant differences in the role of different TNF-α isoforms in tumor progression and the bioavailability of each isoform may distinctly regulate tumor progression. This insight is critical for effective intervention in cancer therapy with the available TNF-α inhibitors, which can block both TNF-α isoforms. Cancer Res; 73(13); 3938–50. ©2013 AACR.

Introduction
TNF-α is a major inflammatory cytokine expressed within the tumor microenvironment. TNF-α is not normally detected in the serum of healthy individuals, but elevated levels have been detected in patients with prostate, pancreatic, renal cell, hematopoietic, and metastatic breast cancers (1–6). The role of TNF-α in cancer progression is conflicting. Multiple studies have shown a protumorigenic role of TNF-α in vivo, in part by inhibiting necrosis of and by stimulating a proangiogenic myeloid phenotype (7, 8). Despite the growing body of evidence showing that TNF-α can function as a tumor promoter, there remain conflicting findings. Several case reports describe a temporal relationship between development of skin malignancies and lymphoma and the use of TNF-α inhibitors (9, 10). Moreover, the use of infliximab, which prevents binding of TNF-α to its receptors, does not improve clinical outcome in renal cell carcinoma (11). Collectively, these data show the complexity of TNF-α in cancer pathogenesis.

The majority of studies to date focus on the 17-kDa soluble moiety of TNF-α, which is released after proteolytic cleavage of the 26-kDa type II transmembrane isoform by TNF-α-converting enzyme (TACE; ADAM-17; ref. 12). The role of membrane form of TNF-α and its expression pattern in different tissue is poorly understood. Cardiac-restricted expression of membrane versus soluble TNF-α (sTNF-α) isoform has been shown to have adverse effect in cardiac remodeling (13, 14). Expression of sTNF-α in cardiomyocytes can cause dilation of left ventricle in mice, whereas the membrane TNF-α (mTNF-α) results in a concentric hypertrophic cardiac phenotype. Increased mTNF-α expression on T cells is shown to modulate monocytes interleukin (IL)-10 production (15). Despite these findings, the role of mTNF-α in tumor biology is unknown (13). Thus far, it is not known whether tumors can express both isoforms. In addition, there is little understanding of the difference in the mechanism of action of sTNF-α versus mTNF-α in regulating tumor behavior or impact on the tumor-infiltratory stroma.

The goal of the current study was to assess if the conflicting data about the association of TNF-α with tumor progression was due to distinct effects of tumor expression of membrane versus soluble isoforms. We found that although sTNF-α expression promoted tumor growth, mTNF-α–expressing tumors exhibited reduced growth and were largely devoid of myeloid cells. Our study showed distinct TNF-α isoform-
dependent effects on myeloid cell survival. Importantly, we showed for the first time that human non-small cell lung carcinoma (NSCLC) tissues exhibit differential expression of membrane versus sTNF-α. Moreover, patients with lung tumors predicted by the molecular signature to have higher mTNF-α had better survival when compared with patients with tumors with higher soluble form of TNF-α expression. Together, these data show that opposing functions of membrane versus sTNF-α significantly impact tumor progression for several cancer types, including NSCLC, one of the most commonly diagnosed cancer in the United States and one of the most difficult to treat (16). Screening patients for expression of each isoform and tailoring inhibitors to specifically target the soluble isoform may result in a better clinical outcome.

Materials and Methods

Mice and cell lines
Wild-type C57Bl/6 (WT) mice were purchased from The Jackson Laboratory. Homozygous mutants for TNFR1 and R2 knockout (TNFR-DKO) on a C57Bl/6 background were a generous gift from Dr. D. Polk (Vanderbilt University Medical Center). Lewis lung carcinoma (LLC), B16F10 melanoma, and RAW 264.7 cells were purchased from American Type Culture Collection and maintained in Dulbecco’s Modified Eagle Medium supplemented with glucose (4.5 g/L) along with penicillin (10 U/L), streptomycin (10 μg/mL), plasmocin (25 μg/mL), and amphotericin-B (2.5 μg/mL). H520, HCC95, SW900, H157, HCC15, and AS49 were provided by Dr. P.P. Massion (The Thoracic Program at Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, TN) and Department of Veterans Affairs, Tennessee Valley Healthcare System, Nashville Campus, Nashville, Tennessee, NV) and were maintained in RPMI-1640 medium (Gibco), AS49 cells were maintained in Ham’s F-12K medium (Gibco). All cell lines were supplied with 10% (v/v) FBS and incubated at 37°C in 5% CO₂.

Constructs and retroviral transductions
Secretable TNF-α was generated by replacing amino acids –76 to –1 containing the cytoplasmic signal-anchor for type II membrane protein and a short extracellular region with a sequence coding for the IL-2 signal peptide (IL-2sp, amino acids –20 to –1) that directs the transport of TNF-α to the outer cellular space and produces a solely secretable form of 17-kDa TNF-α. This was done by amplifying mouse WT TNF-α cDNA using forward primers flanked by BamHI-IL-2sp. The amplified fragment was isolated and purified by gel electrophoresis. The restriction sites at each end allowed ligation of the IL-2spTNF-α fragment into the BamHI–EcoRI site of LZRS-IRES-Neo retroviral vector, conferring neomycin resistance. The mTNFΔ1-9, K11E sequence encoding a mutant transmembrane TNF-α molecule with a deletion at the cleavage site between presequence and mature mTNF-α (BCCM/LMBP plasmid collection; Ghent University, Gent, Belgium) was also cloned into LZRS. This mutation prevents cleavage of the 26-kDa mTNF-α into secretory TNF-α isoform. An empty LZRS vector was used as a control vector.

Surface expression of TNF-α
Cells were detached from tissue culture plate and incubated with anti-TNF-α antibody (1 μL/2.5 × 10⁶ cells; Southern Biotech) for 30 minutes on ice without permeabilization. Phycoerythrin (PE)-conjugated secondary antibody (0.125 μg/10⁶ cells/100 μL) was added for 30 minutes on ice. Surface expression of TNF-α was measured using flow cytometry. Data are presented as percentage of viable cells.

In vivo murine tumor model
Control, IL-2spTNF-α, and mTNF-α cell lines (10⁶ cells in 100 μL of PBS) were implanted subcutaneously into WT, TNFR-DKO, or TNFR-DKO-BMT mice. Mice were sacrificed 15 days postimplantation, tumors were excised, and the volume was calculated by multiplying tumor length by width by height.

BrdU assay
The bromodeoxyuridine (BrdU) ELISA was conducted according to the manufacturer’s instructions. Briefly, 1,000 cells per well were seeded in triplicate in a 96-well plate. Cells were allowed to attach for 8 hours. BrdU label was added to each well and incubated for an additional 4 hours. Absorbance was analyzed at 450 to 540 nm.

Cell viability assay
Cell viability was measured by seeding 5,000 cells per well in a 96-well plate for 48 hours. Cells were labeled with 100 μL of PBS containing 0.5 mg/mL of MTT (Sigma). After 2 hours of incubation at 37°C, cells were lysed with 0.1 mL dimethyl sulfoxide (DMSO). Photometric measurement was carried out at 540 nm.

Leukocyte quantification in tumors
Tumor tissues were finely minced and incubated in 5 mL dissociation solution [RPMI medium supplemented with 5% FBS, and 1 mg/mL of Collagenase type IV (Worthington)] for 30 minutes at 37°C. To obtain a single-cell suspension, cells were passed through 70-μm nylon cell strainer (Becton Dickinson). Cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS, 2 mmol/L EDTA, 0.5% bovine serum albumin) and incubated for 5 minutes in RBC lysis buffer solution (155 mmol/L NH₄Cl, 12 mmol/L NaHCO₃, 0.1 mmol/L EDTA). Cells were washed twice in FACS buffer and incubated with anti-CD3 (BioLegend), -Ly6G (BD Pharmingen), -F480 (eBioscience), and -CD11b⁺ (BD Pharmingen). After 2 washes, labeled cells were resuspended in vital dye 7-Amino-actinomycin D (7-AAD; BD Pharmingen) and subjected to flow cytometry on LSRFortessa flow cytometer (Becton Dickinson) and analyzed by using FlowJo software (TreeStar).

Myeloid cell–trafficking to tumor
Control vector or mTNF-α-expressing tumor cells were injected subcutaneously into WT mice. After 12 days, freshly isolated myeloid cells were labeled with 5(6)-carboxyfluorescein N-hydroxysuccinimidy ester (CFSE) fluorescent tracking dyes and injected into retro-orbital space (5 × 10⁶ cells).
cells/animal). Eighteen hours later, tumors were harvested and single-cell suspensions were made. CFSE-labeled cells were detected using flow cytometry.

Caspase-3/7 activity
Freshly isolated CD11b+ (105/96-well) were cocultured with fixed control, control+recTNF-α (100 U/mL), and mTNF-α B16F10 cells at CD11b+/tumor cells ratio of 1:10 for 5 hours. Apoptosis was quantified in the form of caspase-3/7 activation using the Apo-One fluorometric assay system from Promega Corporation according to the manufacturer’s protocol.

Measurement of intracellular reactive oxygen species
The oxidant-sensing probe CM-H2DCFDA (Invitrogen) was used to detect intracellular reactive oxygen species (ROS). Freshly isolated CD11b+ cells were loaded with 10 μmol/L CM-H2DCFDA, and cocultured with fixed B16F10 control, control+100 U/mL of recombinant TNF-α, mTNF-α, or mTNF-α+2 mmol/L N-acetylcysteine (NAC) for 8 hours. Fluorescence was determined using a luminescence spectrophotometer (Spectra max; Molecular Devices) with an excitation wavelength of 429 nm and emission wavelength of 517 nm.

Survival analysis
A cohort published by Shedden and colleagues was analyzed for disease-free survival (17). The dataset included gene-expression profiles for 442 lung adenocarcinomas with high-quality gene-expression data, pathologic data, and clinical information. The association between gene expression and the survival was examined using the Cox proportional hazard model. Kaplan–Meier curves were generated to visualize the survival pattern by dichotomizing the gene expression. The subgroup analysis of mTNF-α and TACE was done by dividing the cohort into 4 groups of high TNF-α with high/low TACE or low TNF-α with high/low TACE. Log-rank overall tests were conducted for the 4 groups. All statistical analyses were conducted using R (www.r-project.org).

Statistical analysis
The statistical significance between experimental and control groups was determined by Student t test or ANOVA followed by Tukey posttest using Prism software (GraphPad). A P value of less than 0.05 was considered statistically significant.

Results
mTNF-α isoform reduces tumor growth
In this study, we investigated the effects of different TNF-α isoforms on malignant tumor phenotypes using murine LLC sublines expressing either sTNF-α (LZRS-RES-II-2spTNF-α) or mTNF-α (LZRS-RES-mTNFα1-9) by retroviral transduction (Fig. 1A). Cells transduced with empty vector (LZRS-RES-Neo) were used as control. Untransduced LLC cells exhibited undetectable levels of TNF-α expression as determined by ELISA. The relative expression of sTNF-α and cell surface expression of TNF-α by transduced cells were confirmed by ELISA and flow cytometry, respectively. sTNF-α expression was detected in IL-2spTNF-α-expressing cells at 5 ng/mL. Surface expression of TNF-α was not detected in control and IL-2spTNF-α tumor cells (mean fluorescent intensity of 150 and 202, respectively). In contrast, mTNF-α–expressing LLC cells displayed 7.1- and 5.3-fold increase in surface TNF-α as compared with control and IL-2spTNF-α, respectively (Fig. 1B).

To evaluate whether overexpressing various TNF-α isoforms affected growth or survival of tumor cells, we assessed in vitro proliferation and viability using the BrdU incorporation and MTT assay, respectively. Both IL-2spTNF-α and mTNF-α–expressing cells exhibited similar in vitro growth rates compared with control LLC lines (P > 0.05; Fig. 1C). IL-2spTNF-α and mTNF-α LLC cell lines tested for viability also displayed similar levels of survival rate compared with control (P > 0.05; Fig. 1D). To gain additional evidence that the membrane isoform did not reduce survival or viability and that these observations were not cell specific, we transduced B16F10-melanoma cell lines with retroviral constructs, containing mTNF-α or an empty construct as control cells. Similar to LLC lines, the proliferation rate (P > 0.05) and viability (P > 0.05) were not affected in mTNF-α–expressing B16F10 cells compared with control cells (Supplementary Fig. S1A and S1B). These findings are consistent with earlier studies in which we showed that overexpressing the WT TNF-α in both LLC and B16F10 melanoma did not alter in vitro growth (8).

LLC cell lines expressing soluble (IL-2spTNF-α) or membrane (mTNF-α) isoforms were implanted subcutaneously into the flank of WT C57Bl/6 mice. The same number of cells transduced with empty vector was implanted as a control. After 14 days, LLC tumors expressing IL-2spTNF-α were approximately 7-fold (1,214 ± 122 mm3) larger compared with control tumors (124.4 ± 92 mm3; n = 5; P < 0.0005; Fig. 1E). In contrast, tumors expressing mTNF-α exhibited 65% reduction in tumor volume (105.8 ± 29.3 mm3) compared with control tumor (294.1 ± 35.9 mm3; n = 12; P < 0.0005; Fig. 1F). Similar growth reduction was observed with mTNF-α–expressing B16F10 tumors cell line when compared with matched controls (n = 7; P < 0.05; Fig. 1G). In addition, tumor weight measurement followed similar pattern to tumor volume as presented in Supplementary Fig. S2A–S2C. These data suggested that different TNF-α isoforms have opposite effects on tumor size.

Expression of mTNF-α does not affect tumor proliferation or vascularity in vivo
To evaluated tumor vascular density and tumor cell proliferation rate, histologic sections from control, IL-2spTNF-α, and mTNF-α LLC tumor were immunostained with platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody to evaluate microvessel density, and anti-Ki-67 to assess tumor cell proliferation (Fig. 2A). Histomorphometry of PECAM-1–positive areas showed no difference in vascular density among the tumors expressing different TNF-α isoforms versus control (P > 0.05; Fig. 2B). Furthermore, IL-2spTNF-α and mTNF-α–expressing LLC tumors showed no significant difference in survival.
immunoreactivity with the Ki-67 antibody compared with control tumors ($P > 0.05$; Fig. 2C).

Vascular density and cell proliferation analyses were also conducted between B16F10 melanoma-derived tumors expressing mTNF-$\alpha$ versus control (Supplementary Fig. S3A). Similar to LLC tumors, no significant difference was observed in microvessel density and in vitro proliferation between the control and mTNF-$\alpha$–expressing melanoma tumors ($P > 0.05$; Supplementary Fig. S3B and S3C).

mTNF-$\alpha$–expressing tumors are devoid of tumor-associated myeloid cells

It is often assumed that TNF-$\alpha$–mediated tumor promotion is secondary to TNF-$\alpha$–mediated inflammation. To determine whether mTNF-$\alpha$–expressing tumors had altered composition of inflammatory cells, we quantified LLC tumor-associated T cells (anti-CD3), B cells (anti-B220), neutrophils (anti-Ly6G), and myeloid-monocytic lineage (anti-ER-HR3, CD11b, F4/80) using immunohistochemistry staining and flow-cytometric analysis of single-cell suspension of tumors. Both immunostaining and flow-cytometric analysis showed no significant difference in T-cell content among control, IL-2spTNF-$\alpha$, and mTNF-$\alpha$–expressing tumor cells (Supplementary Fig. S4A–S4C). Anti-B220 staining revealed only rare, infiltrating B cells for all tumor groups (data not shown). Flow-cytometric analysis of anti-Ly6G showed no significant difference in neutrophils population within control and mTNF-$\alpha$–expressing tumors.

Figure 1. mTNF-$\alpha$–expressing tumor cells show delayed tumor growth. A, schematic representation of TNF-$\alpha$ mutant, which has the region coding for the TNF-$\alpha$ transmembrane domain (TMD) replaced with region coding for IL-2sp to generate sTNF-$\alpha$ and TNF-$\alpha$ lacking TACE cleavage site ($\Delta$1-9, K11E) to generate mTNF-$\alpha$. B, expression of transmembrane TNF-$\alpha$ on the surface of LLC tumor cells transduced with empty vector (control), IL-2spTNF-$\alpha$, or mTNF-$\alpha$ vectors was analyzed by flow cytometry. C and D, proliferation rate and the viability of transduced tumor cells were determined by BrdU and MTT-labeling assays, respectively. All cell lines showed no significant difference in proliferation or viability. Data are representative of 3 independent experiments expressed as the mean ± SEM. E–G, in vivo growth of tumor cells transduced with control vector was compared with LLC lines expressing IL-2spTNF-$\alpha$ (E), or mTNF-$\alpha$ (F), and B16F10 expressing mTNF-$\alpha$ (G) by subcutaneous implantation in WT C57Bl/6 mice for 14 days. Each point represents an individual animal and the horizontal bar is the mean. *, $P < 0.05$; **, $P < 0.0005$; Student $t$ test. Photomicrograph of each tumor is shown with each experimental group.
(P > 0.05; Supplementary Fig. S4D). Interestingly, when we evaluated tumor-associated myeloid cell population a significant difference was observed in mTNF-α-expressing tumors compared with control and IL-2spTNF-α tumors. Histologic staining of mTNF-α-expressing LLC tumors for ER-HR3, a myeloid marker reactive to approximately 70% of circulating monocytes and a subset of mature tissue macrophages (18), had fewer number of infiltrated ER-HR3⁺ cells (0.04% ± 0.02%) versus control (6.4% ± 0.37%) or IL-2spTNF-α tumors (16% ± 1.03%; P < 0.005; Fig. 3A and B). Further evaluation of single-cell suspension of tumors showed significantly lower number of CD11b⁺ myeloid cells (52.17% ± 6.1%; P > 0.005) and F4/80 macrophages (3.75% ± 2.4%; P > 0.005) in mTNF-α-expressing tumors as compared with control (Fig. 3C–F). Anti-F4/80 staining of tumor sections further confirmed the significant reduction of F4/80-positive macrophages in mTNF-α-expressing tumors (Fig. 3G and H).

To test whether the tumor inhibitory effects of mTNF-α required the presence of TNF-α receptors, tumor growth was assessed in TNF-α receptors–deficient mice (TNFR1 and TNFR2 double knockout, TNFR-DKO). Mice were implanted with LLC cell line expressing various TNF-α isoforms. LLC lines expressing mTNF-α isoform did not generate significantly smaller tumors (110.5 ± 17.6 mm³) in comparison with their paired control tumors (161.7 ± 29.6 mm³; n = 5; P > 0.05; Supplementary Fig. S5A). In addition, implantation of mTNF-α-expressing LLC tumor cells in TNFR-DKO mice restored the ER-HR3⁺ myeloid population in mTNF-α-expressing tumors (0.91 ± 0.16% ER-HR⁺ positive area/total area in mTNF-α vs. 0.67% ± 0.16% in control; P > 0.05; Supplementary Fig. S5B). Similar results were observed in B16F10 line (data not shown).

Restoration of myeloid cell population in mTNF-α-expressing LLC tumors in TNFR-DKO host prompted us to further evaluate the requirement of TNF signaling in inflammatory cells (i.e., bone marrow–derived cells). Therefore, tumor growth was assessed in WT mice receiving bone marrow transplants from TNFR-DKO mice (referred to as BMT-TNFR-DKO mice). Similar to experiments in TNFR-DKO host, LLC line expressing mTNF-α isoform, implanted into BMT-TNFR-DKO, did not generate smaller tumors in mice engrafted with TNFR-deficient bone marrow (497.2 ± 137.6 mm³) as compared with control tumors (387.9 ± 95.94 mm³; P > 0.05; Fig. 3I). Furthermore, we quantified tumor-associated myeloid cell populations in these tumors isolated from BMT-TNFR-DKO. The overall percentage of myeloid populations in mTNF-α-expressing LLC tumors (7.8% ± 0.4%) was similar to control tumors (7.5% ± 1.4%; P > 0.05; Fig. 3J and K). These data suggested that tumor-derived mTNF-α significantly reduced myeloid population within the tumor microenvironment. Because this effect was abrogated in WT mice transplanted with TNF-α receptor deficient bone marrow, it was concluded that intact TNF-α signaling through its receptor in bone marrow–derived cells was required for this effect, and that this effect was not mediated by secondary factors from the tumor cells.
mTNF-α-expressing tumors are devoid of tumor-associated myeloid cells. A, ER-HR3 staining of LLC tumor cells, expressing various TNF-α isoforms. Control (left), IL-2spTNF-α (middle), and mTNF-α (right) LLC tumor sections from WT bl/6 were stained with ER-HR3 myeloid markers (green). B, percentage of ER-HR3-positive cells in LLC tumors transduced with control or different of TNF-α isoforms was quantitated. There was a significant decrease in the number of ER-HR3-positive cells in LLC tumors expressing mTNF-α isoform compared with control tumors. C and E, representative flow-cytometric analysis of CD11b- and F4/80-positive population in LLC tumor cell suspension. Dot plots show CD11b/7-AAD (C) and F4/80/7-AAD (E) from one representative animal for each group. D and F, percentage of CD11b- and F4/80-positive cells were quantitated in control and mTNF-α tumor cell suspensions. G, representative sections of control and mTNF-α–transfected LLC tumors were analyzed by immunohistochemistry for F4/80+ macrophages. H, number of F4/80-positive cells in control and mTNF-α in LLC tumors was quantitated. I, control and mTNF-α–expressing LLC tumor cells were implanted subcutaneously in WT bl/6 mice that received bone marrow transplant from TNF-α receptors1/2 knockout donor (BMT-TNFR-DKO mice) for 14 days. The mean is shown for each group (n = 6 animals). J, representative ER-HR3 immunofluorescence staining from control and mTNF-α–transduced tumors. K, percentage of ER-HR3-positive cells in control and mTNF-α-expressing LLC tumors from WT mice with BMT from TNFR-DKO donor was quantitated. There was no significant difference between the cohorts for ER-HR3-positive cells (n = 3). Data are presented as mean ± SEM; **, P < 0.005; ***, P < 0.0005; Student t test. DAPI, 4',6-diamidino-2-phenylindole.
mTNF-α-derived soluble factors do not affect CD11b+ myeloid cell migration/recruitment

One possible explanation for the reduction of myeloid cells observed in mTNF-α–expressing tumors was that such tumors exhibited reduced expression of necessary signals for myeloid recruitment. Using a modified Boyden chamber assay, we evaluated the ability of conditioned media (CM) from LLC and B16F10 melanoma cell lines expressing various forms of TNF-α to promote migration (i.e., recruitment) of primary murine CD11b+ myeloid cells. Conditioned media from mTNF-α did not inhibit migration of CD11b+ as compared with control-conditioned media in both LLC and B16F10 melanoma line (Fig. 4A and Supplementary Fig. S6A). An increase in CD11b+ myeloid cells migration was observed in conditioned media derived from both IL-2spTNF-α–expressing LLC (~1.5-fold; Fig. 4B) and B16F10 line (~4-fold; Supplementary Fig. S6B). This may be attributed to the presence of TNF-α itself, which is known to induce chemotactic response (19, 20). These results suggested that the relative paucity of myeloid cells in mTNF-α–expressing tumors was likely not due to reduced expression of key cytokines, necessary for myeloid extravasation and migration into the tumor.

We further evaluated the ability of control and mTNF-α–expressing LLC tumors to effectively recruit myeloid cell in vivo by adoptive transfer of CFSE-labeled CD11b+ into tumor-bearing mice. After 18 hours postinjection, CFSE-positive myeloid cells were quantified in each tumor type by flow analysis of single-cell suspension of tumor digest. The overall number of CFSE-positive cells in mTNF-α–expressing LLC tumors (58 ± 21) was similar to control tumors (68 ± 12.57; P > 0.05; Fig. 4C and D). These data show that reduced myeloid cells in mTNF-α–expressing tumors was not due to impaired recruitment of circulating cells.

mTNF-α induces cell death through apoptosis-independent pathway

To investigate if the distinct TNF-α isoforms exerted cytotoxic effects on myeloid cells, freshly isolated CD11b+ cells (target) were mixed with 1% paraformaldehyde-fixed B16F10 melanoma cells (effector) expressing empty vector with or without 100 U/ml recombinant murine TNF-α (FxB16cont or FxB16cont+rTNF-α) or fixed mTNF-α–expressing B16F10 cells (FxB16mTNF) at an effector:target ratio of 10:1. As measured by the MTT assay (Fig. 5A), FxB16mTNF resulted in more than 60% ± 29% cytotoxicity of CD11b+ myeloid cells after 48 hours of incubation, as compared with CD11b+ cells incubated with FxB16cont (P < 0.005). CD11b+ in the presence of FxB16cont+rTNF-α showed less than 1% cytotoxicity as compared with control. We assessed the activation of apoptotic pathway as the mechanism of mTNF-α–induced myeloid cell death by determining the caspase-3/7 enzymatic activity in CD11b+ cells. Compared with control, CD11b+ myeloid cells cocultured with FxB16cont+rTNF-α or FxB16mTNF did not show any significant increase in the level of caspase-3/7 activity (P > 0.05; Fig. 5B).

We further assessed the activation of apoptotic pathway in RAW 264.7 cells by determining the level of Bax/Bcl-2 and cleavage/activation of caspase-3 proteins in RAW 264.7. The data suggested that the Bax/Bcl-2 ratio (Fig. 5C) and active-
caspase-3 (Fig. 5D) proteins in RAW 264.7 cells treated with both sTNF-α and mTNF-α had no significant changes when compared with control. Together these findings are indicative of another death pathway independent of apoptosis.

NF-κB is a critical factor in the determination of cell death versus survival and proliferation (21, 22). In cases of failed NF-κB activation, TNF-α can induce either programmed cell death or necrosis through complex signal transduction cascades (23). To evaluate whether soluble versus mTNF-α isoforms induced distinct cellular responses via regulation of NF-κB, we testedNF-κB activity in RAW 264.7 after incubation with cancer cells transduced with various TNF-α constructs. FxB16cont, FxB16cont + TNF-α, or FxB16mTNF was added for indicated incubation period. RAW 264.7 cells were harvested and total cellular protein was analyzed for Bax/Bcl-2 ratio (C), activated caspase-3 (D), and total and phosphorylated NF-κB p65 (E). Immunoblot analysis showed no differences in Bax/Bcl-2 ratio or caspase-3 and NF-κB pathway activation with different TNF-α isoforms compared with control. **, P < 0.05; one-way ANOVA with Tukey posttest.

Figure 5. mTNF-α induces cell death through apoptosis-independent pathway. A, cytotoxic effect of sTNF-α and mTNF-α on CD11b+ cells measured by MTT assay. B, caspase-3/7 activity (relative fluorescence units, RFU) in CD11b+ cocultured with paraformaldehyde-fixed control (FxB16cont), control + rTNF-α (FxB16cont + TNF-α), or mTNF-α (FxB16mTNF). C–E, kinetics of Bax/Bcl-2, caspase-3, and NF-κB activation in RAW 264.7 after incubation with cancer cells transduced with various TNF-α constructs. FxB16cont, FxB16cont + TNF-α, or FxB16mTNF was added for indicated incubation period. RAW 264.7 cells were harvested and total cellular protein was analyzed for Bax/Bcl-2 ratio (C), activated caspase-3 (D), and total and phosphorylated NF-κB p65 (E). Immunoblot analysis showed no differences in Bax/Bcl-2 ratio or caspase-3 and NF-κB pathway activation with different TNF-α isoforms compared with control.

mTNF-α–induced cell death occurs through induction of ROS

TNF-α can induce cell death by induction of intracellular ROS (24, 25). To test the possibility that sTNF-α versus mTNF-α isoforms induced distinct cellular responses via regulation of intracellular ROS, we evaluated ROS levels in CD11b+ myeloid cells incubated with different TNF isoform by measuring CM-H2DCFDA fluorescence. In CD11b+ cell incubated with FxB16cont + rTNF-α, the CM-H2DCFDA fluorescence did not differ from control, whereas in cells with both FxB16cont + rTNF-α and FxB16mTNF was similar to FxB16cont, suggesting that mTNF-α isoform did not affect the activity of NF-κB p65 compared with sTNF-α.
A 1.6-fold increase was observed after 8 hours of incubation ($P < 0.05$; Fig. 6A). Addition of NAC into cells cultured with FxB16mTNF significantly decreased the intensity of CM-H$_2$DCFDA fluorescence, indicating decrease in the level of ROS ($P > 0.05$). NAC treatment of FxB16mTNF treated CD11b$^+$ reduced mTNF-α induced cytotoxicity (FxB16mTNF, 61.57% ± 29.12% cytotoxicity; FxB16mTNF + NAC, 10.64% ± 29.17% cytotoxicity; $P < 0.05$; Fig. 6B).

In addition, we evaluated ROS generation in individual RAW 264.7 by CM-H$_2$DCFDA fluorescent staining assay (26). The fluorescent intensity in RAW 264.7 cocultured with FxB16cont + rTNF-α was similar to the basal level (4.6% ± 3%). However in the presence of FxB16mTNF, we observed an induction of ROS intensity (82.5% ± 15%) as detected by the presence of green fluorescence staining (Fig. 6C and D). Treatment of RAW 264.7 cocultured with FxB16mTNF cells supplied with the ROS scavenger NAC (FxB16mTNF+NAC) diminished mTNF-α–induced accumulation of intracellular ROS in RAW 264.7 (0.5% ± 0.4%; Fig. 6C and D) and led to abolition of mTNF-α–induced cytotoxicity.

**Figure 6.** mTNF-α–induced cell death occurs through induction of ROS. A, effects of various TNF-α isoforms on intracellular ROS generation in CD11b$^+$ cells. Cells were labeled with ROS detection reagent, CM-H$_2$DCFDA, and incubated with FxB16cont, FxB16cont + rTNF-α, or FxB16mTNF, FxB16mTNF + NAC (2 mmol/L) and then ROS level was quantitatively analyzed. B, cytotoxic effect of mTNF-α on CD11b$^+$ cells decreased in the presence of ROS scavenger NAC. C, fluorescent micrographs of RAW 264.7 [blue: 4,6-diamidino-2-phenylindole (DAPI) nuclear staining] incubated for 8 hours with FxB16cont, FxB16cont + rTNF-α, or FxB16mTNF, FxB16mTNF + NAC (2 mmol/L) and subsequently treated with ROS detection reagent, CM-H$_2$DCFDA (green). D, intensity of CM-H$_2$DCFDA was quantitated. Increase in number of ROS-generating cell was detected in RAW 264.7 cocultured with mTNF-α–expressing B16F10 cells, which was reversed by addition of NAC. E, cytotoxic effect of mTNF-α on RAW 264.7. *P < 0.05; **, P < 0.005; and ***, P < 0.0005; one-way ANOVA with Tukey posttest. DAPI, 4,6-diamidino-2-phenylindole.
(FxB16mTNF, 34% ± 9% cytotoxicity; FxB16mTNF + NAC, 0.1% ± 7% proliferation; P < 0.05; Fig. 6E).

**Relative expression pattern of TNF-α/TACE correlates with survival probability in lung cancer patients**

In most studies, the role of TNF-α in cancer has only been investigated in murine models or in modified cell lines and to our knowledge the expression level of mTNF-α has not been evaluated in human tumors. To extend the relevance of our findings from the murine model to human cancer, we examined 62 tissue cores available in a single human NSCLC tissue array containing squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. Using coimmunofluorescent staining with anti-TNF-α and carcinoma-enriched membrane antigen (EMA), we evaluated the distribution of both degree (high- or low-expressors) and localization (membrane, cytoplasmic, or both) of TNF-α staining. Forty of 62 individual tumors were positive for TNF-α expression. Among 40 tumors, expressing either high or low TNF-α, we detected tumors presenting high levels of membrane-localized TNF-α, tumors presenting high cytoplasmic TNF-α (i.e., tumors with higher expression of sTNF-α) or tumors with cytoplasmic and membrane-localized TNF-α. Eighteen of 40 tumors (45%) were high expresser with 27.8% cytoplasmic localization, 16.7% localized on the membrane and 55.5% were positive for both membrane and cytoplasmic TNF-α. Twenty-two (65%) showed low expression of TNF-α with 18.3% cytoplasmic, 31.8% membrane, and 50% with both membrane and cytoplasmic (Fig. 7A). These data are the first evidence for the existence of mTNF-α in human tumors and that its level varies significantly from patient to patient. Moreover, its level and localization varies significantly from patient to patient, which is likely an important consideration in predicted therapeutic response to anti-TNF agents based on our preclinical observations.

To provide further evidence that human tumors exhibit varying expression of TNF-α isoforms, the expression of sTNF-α was determined by ELISA of conditioned media and the expression of mTNF-α was evaluated by immunoblot analysis of cell membrane fraction. The analysis showed significant variation in the relative expression of membrane versus sTNF-α among different human lung cancer–derived cell lines (Fig. 7B).

Next, we investigated the possible association of sTNF-α versus mTNF-α ratio with patient outcome (17). It has been shown that there is a positive correlation between TACE surface expression and TNF-α cleavage. Upregulation of TACE protein has also been shown to be associated with a decline in mTNF-α level and increased soluble level and vice versa (27). Using publicly accessible NSCLC microarray database (n = 442 patients), we divided the gene expression data into 4 groups. The first 2 groups featured low TNF-α gene expression and low or high TACE. The third and fourth groups showed high TNF-α with either low or high TACE. Overall higher TACE level was significantly correlated to lower survival probability. Expression of high TNF-α/low TACE—representing tumors with high mTNF-α/sTNF-α relative expression—was associated with longer survival than expression of high TNF-α/high

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**Figure 7.** Relative expression pattern of TNF-α/TACE correlates with survival probability in patients with lung cancer. A, analysis of TNF-α expression from 40 human NSCLC tissue array. Graph displays the distribution of both degree and localization of TNF-α staining 40 NSCLC patient samples. B, expression pattern of sTNF-α and mTNF-α in human NSCLC cell lines measured by ELISA and immunoblotting methods, respectively. A significant variation was observed in the ratio of mTNF-α to sTNF-α expressed by each cell line. C, association between TNF-α and TACE coexpression pattern and survival probability in patients with NSCLC. Analysis of the publicly available data from the Shedden cohort (17) was used to correlate TNF-α/TACE expression pattern with survival probability (n = 442; log-rank; P = 0.035).
TACE—representing tumors with low mTNF-α/sTNF-α relative expression (log-rank; \( P = 0.035; \) Fig. 7C).

Discussion

Data from both experimental and human cancers have identified TNF-α as key cytokine-modulating tumor progression, yet its effects are incompletely understood. In murine models, deletion or inhibition of TNF-α reduces the incidence of cancer formation and even increases resistance to chemically induced carcinogenesis of the skin (28, 29). Consistent with this, there was a positive correlation between level of TNF-α expression and tumor grade in ovarian tumors (30). On the other hand, antitumorigenic properties of TNF-α are also well documented. In a study by Boldrini and colleagues, assessment of TNF-α expression in 61 NSCLC samples showed expression of TNF-α in 45.9% of cases and directly correlated with a better clinical outcome (31).

Many soluble proteins such as TNF-α are originally expressed as a membrane-bound form and then processed to a secretory form through proteolytic cleavage. Some of these proteins such as Fas ligand, a member of TNF-α super family, have been described to have distinct biologic effects on disease process as a membrane isoform when compared with the soluble isoform (32). In this study, we sought to better understand the role of different TNF-α isoforms in the modulation of tumor progression and to determine if some of the reported opposing effects can be attributed to distinct effects of these isoforms.

We generated tumor cell lines expressing either uncleavable mTNF-α or sTNF-α. Subcutaneous implantation of mTNF-α–expressing LLC and B16F10 cancer cell lines resulted in significantly smaller tumors, which was not the result of impaired angiogenesis or reduced tumor cell proliferation but was driven by components of the host-derived cells. This idea was further strengthened by significant reduction of tumor-associated myeloid cell content in mTNF-α–expressing tumors, which were restored in tumor cells transplanted in TNFR-DKO mice. Numerous studies have shown critical roles for tumor-associated stromal cells, specifically, bone marrow–derived myeloid cells, in tumor growth (33, 34). Upon activation by cancer cells, tumor-associated macrophages can release growth factors, cytokines, and inflammatory mediators that may facilitate cancer cell invasion, migration, angiogenesis, tumor progression, or metastasis (35–37). Furthermore, systemic depletion (38) or inhibition (39) of tumor-associated myeloid cells migration into the tumor has shown to significantly reduce tumor growth. In light of our findings, it would be of great interest to determine the precise role of myeloid cells in mTNF-α–mediated tumor growth.

Our study revealed that tumor cell expression of the membrane isoform of TNF-α resulted in tumor-associated myeloid cell death through increased ROS production. It has been shown that TNF-α has the ability to induce necrotic cell death by using death domain-containing adaptor proteins such as RIP1, TRADD, and FADD upon TNFR activation. Once recruited to the TNFR death domain further downstream events lead to ROS generation and cell death (40–42). Multiple pathways have been shown to lead to ROS generation upon TNFR activation (24, 43). Necrotic cell death induced by TNFR has been associated with generation of ROS derived from either mitochondrial or nonmitochondrial sources (43, 44). Mitochondrial complex I–mediated generation of ROS has been linked to direct activation by TNFR and ceramide-mediated activation (40, 41). In a study by Kim and colleagues, TNFR was reported as an activator of Nox1 NADPH oxidase complex in a TRADD- and RIP1-dependent recruitment (45). Our findings suggest that the membrane form of TNF-α is very efficient at stimulating ROS generation and initiating necrotic cell death. This could be due to the ability of mTNF to recruit death domain-containing adaptor proteins more efficiently or mTNF-α activates a pathway that is more efficient in ROS generation. The mechanistic pathway(s) that leads to mTNF-α–induced ROS generation requires further investigation.

To our knowledge, there has not been any study evaluating the relative expression of sTNF-α and mTNF-α during tumor progression in human cancer, including analyses designed to determine if there is any correlation between the level of sTNF-α versus mTNF-α and the cancer outcome. Here, in this study, we observed a positive correlation with the ratio of soluble to mTNF-α in various different subcellular localizations and in vitro assessment of soluble and mTNF-α expression in human NSCLC cell lines showed that the ratio of soluble to mTNF-α varies among different tumor cell types. Furthermore, we verified this by immunofluorescence staining of tumor sections for TNF-α. The fact that mTNF-α is present in tumor and at different levels and subcellular localization may provide important clues to divergent outcomes seen in TNF-α–positive tumor phenotype seen in different patients.

As it previously described, to generate sTNF-α, the membrane-associated TNF-α is cleaved through proteolytic activity of TACE. Although it has been suggested that other proteinases are capable of TNF-α cleavage, it has been shown that TACE has the highest affinity for TNF-α ectodomain shedding among the other known substrates (27). Level of TACE present on the surface of the membrane has been inversely correlated with the level of membrane-associated TNF-α and inhibition of TACE by matrix metalloproteinase inhibitors has shown a transient increase in mTNF-α surface expression (27, 46). These studies suggest that the regulation of TACE activity and subsequent alteration of the sTNF-α to mTNF-α ratio could have a great impact on tumor growth. The association between higher TACE and higher TNF-α gene expression in NSCLC and decreased survival further confirms the importance of different TNF-α isoforms availability on tumor regulation.

Recently, several phase I/II clinical trials have been undertaken with TNF-α antagonists in patients with cancer (47–49). In these clinical trials, TNF-α antagonist treatment resulted in a period of disease stabilization in only 20% of the patients with advanced cancer. It is suggested that to take this forward, we need to identify those patients who are most likely to benefit from TNF-α antagonist treatment. Perhaps determining the predominant form of TNF-α expressed by tumor in these patients would be beneficial for a more effective treatment with TNF-α inhibitors, which can block both soluble and membrane isoforms.
In summary, we show that TNF-α membrane versus soluble isoforms have opposing effects on cancer growth. Expression of both forms of TNF-α in NSCLCs indicates that this finding is relevant to human malignancies and that isoform analysis should be applied to identify candidates for which anti-TNF-α agents are likely to be beneficial versus detrimental.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Ardestani, B. Li, P.P. Young
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ardestani, B. Li, D.L. Deskins, P.P. Massion, P.P. Young
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): S. Ardestani, B. Li, H. Wu, P.P. Massion, P.P. Young

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Correction: Membrane versus Soluble Isoforms of TNF-α Exert Opposing Effects on Tumor Growth and Survival of Tumor-Associated Myeloid Cells

In this article (Cancer Res 2013;73:3938–50), which was published in the July 1, 2013, issue of Cancer Research (1), there is an error in Fig. 6. In all panels, the label "FxB16_cont + NAC" should be "FxB16_mTNF + NAC." The authors regret this error.

Reference


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