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Transmembrane TNF-α Promotes Suppressive Activities of Myeloid-Derived Suppressor Cells via TNFR2

Xin Hu,*†1, Baihua Li,*†1, Xiaoyan Li,* Xiaoxian Zhao,* Lin Wan,* Guohong Lin,* Min Yu,* Jing Wang,* Xiaodan Jiang,* Wei Feng,* Zhihai Qin,‡ Bingjiao Yin,* and Zhuoya Li*

It has been reported that TNFR2 is involved in regulatory T cell induction and myeloid-derived suppressor cell (MDSC) accumulation, two kinds of immunosuppressive cells contributing to tumor immune evasion. Because transmembrane TNF-α (tmTNF-α) is the primary ligand for TNFR2, we hypothesized that tmTNF-α is mainly responsible for the activation of MDSCs. Indeed, we found that tmTNF-α, rather than secretory TNF-α (sTNF-α), activated MDSCs with enhanced suppressive activities, including upregulating arginase-1 and inducible NO synthase transcription, promoting secretion of NO, reactive oxygen species, IL-10, and TGF-β, and enhancing inhibition of lymphocyte proliferation. This effect of tmTNF-α was mediated by TNFR2, as TNFR2 deficiency significantly impaired tmTNF-α–induced release of IL-10 and NO and inhibition of T cell proliferation by MDSC supernatant. Furthermore, tmTNF-α caused p38 phosphorylation and NF-κB activation, whereas inhibition of NF-κB or p38 with an inhibitor pyrrolidine dithiocarbamate or SB203580 abrogated tmTNF-α–mediated increased suppression of lymphocyte proliferation by MDSCs. Consistently, our in vivo study showed that ectopic expression of uncleavable tmTNF-α mutant by 4T1 cells significantly promoted tumor progression and angiogenesis, accompanied with more accumulation of MDSCs and regulatory T cells in the tumor site, increased production of NO, IL-10, and TGF-β, as well as poor lymphocyte infiltration. In contrast, enforced expression of sTNF-α mutant by 4T1 cells that only released sTNF-α without expression of surface tmTNF-α markedly reduced MDSC accumulation and induced more lymphocyte infiltration instead, showing obvious tumor regression. Our data suggest that tmTNF-α acts as a potent activator of MDSCs via TNFR2 and reveals another novel immunosuppressive effect of this membrane molecule that promotes tumor immune escape.

Tumor necrosis factor-α exists in two biologically active forms, transmembrane TNF-α (tmTNF-α) and secretory TNF-α (sTNF-α). TNF-α is first synthesized as a 26-kDa transmembrane molecule and can be cleaved by the TNF-α–converting enzyme, releasing a 17-kDa soluble cytokine. TNF-α executes its biological effects through two distinct receptors, TNFR1 (p55/p60) and TNFR2 (p75/p80). Although tmTNF-α and sTNF-α bind to the same TNFRs, their different biological effects indicate that both forms of TNF-α trigger distinct signal pathways (1, 2). TNF-α can be produced by tumor and stromal cells in breast, ovarian, colorectal, esophageal, prostate, bladder, and renal cell carcinomas, melanoma, and hematological malignancies (3). Increasing evidence demonstrates that TNF-α acts as a tumor-promoting factor, linking inflammation and cancer. It has been reported that TNF-α−/− mice were unexpectedly resistant to skin carcinogenesis (4), and TNF-α in the tumor microenvironment promotes tumor survival, proliferation, invasion, metastasis, and angiogenesis (5). Additionally, TNF blockade reduced tumor growth as well as decreased colonic infiltration of macrophages and neutrophils in mice (6), and neutralization of TNF-α with specific Ab suppressed the hepatic metastases and prolonged survival (7, 8).

In contrast to the promoting activities of sTNF-α for the tumor development, the role of tmTNF-α is poorly understood. Our previous results showed that tmTNF-α expressed by tumor cells confers the resistance to apoptosis induced by sTNF-α through its reverse signaling (9, 10). Recently it has been reported that in the presence of IL-2, TNF-α selectively activates regulatory T cells (Tregs), leading to proliferation, upregulation of Foxp3 expression, and enhancement of their suppressive activity in a TNFR2-dependent manner (11). Further study demonstrated that Treg induction involves tmTNF-α but not sTNF-α (12). Because tmTNF-α is the primary ligand for TNFR2 (13), it is possible that proinflammatory activities of sTNF-α through TNFR1 (14, 15) synergize with the immunosuppressive effect of tmTNF-α via TNFR2 to promote tumor development and immune escape.

Myeloid-derived suppressor cells (MDSCs) have been reported to facilitate tumor immune escape. This population of cells is markedly increased in tumor-bearing hosts and suppresses anti-tumor immune response through multiple mechanisms (16–18), including high activity of arginase-1 (ARG1) and/or inducible NO synthase (iNOS), production of NO and reactive oxygen species (ROS), as well as release of IL-10 and TGF-β. In mice, the cell

*Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People’s Republic of China; and †National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People’s Republic of China

†X.H. and B.L. contributed equally to this work.

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Address correspondence and reprint requests to Zhuoya Li and Bingjiao Yin, Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan 430030, People’s Republic of China. E-mail addresses: zhuoyali@ hust.edu.cn (Z.L.) and bingjiao.yin@ hust.edu.cn (B.Y.)

Abbreviations used in this article: ARG1, arginase-1; DCFDA, 2′,7′-dichlorofluorescein diacetate; iNOS, inducible NO synthase; KO, knockout; MDSC, myeloid-derived suppressor cell; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; sTNF-α, secretory TNF-α; tmTNF-α, transmembrane TNF-α; Treg, regulatory T cell; wtTNF-α, wild-type TNF-α.
surface phenotype of MDSCs is identified as CD11b+ and Gr-1+. The expansion of MDSCs can be induced by the stimulatory factors of myelopoiesis such as GM-CSF, stem cell factor, and vascular endothelial growth factor (19–21), released primarily by tumor cells, and the activation of MDSCs is associated with proinflammatory mediators, including IL-1β, IL-6, PGE2, and S100A8/9 proteins (22–25), produced by tumor cells, tumor stroma, and tumor-infiltrating immune cells. For example, IL-1β has been reported to promote tumor progression by increasing the accumulation of MDSCs (26), whereas IL-1R–deficient mice have a delayed accumulation of MDSCs and reduced tumor progression.

NF-κα is an important proinflammatory cytokine, and a more recent report (26) showed that TNFR deficiency impairs MDSC accumulation by promoting apoptosis, suggesting a requirement of TNF-α–mediated signaling for MDSC survival and accumulation. However, which form of TNF-α exerts effects and whether TNF-α affects MDSC functions are still unknown. We hypothesized that tmTNF-α contributes to activation of MDSCs. Indeed, we found that tmTNF-α, but not sTNF-α, was able to activate MDSCs with enhanced suppressive activities via TNFR2 and promoted tumor progression. Our study reveals a novel immunosuppressive effect of this membrane molecule.

Materials and Methods

Cell lines

The 4T1 mammary carcinoma cell line (BALB/c background) was provided by Prof. Zhihai Qin (National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) and cultured in DMEM (Life Technologies/Invitrogen) supplemented with 10% FCS (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmids and transfer

pTriEx-4C vector is a shuttle plasmid in which the mouse cDNA (NM-013693.2) for wild-type TNF (wtTNF-α) was inserted at EcoRI1 and BamHI cloning sites. Δ1–12TNF cDNA encoding a mutant transmembrane TNF-α molecule, which has 12 aa (residues 1–12) deleted to prevent the cleavage of the transmembrane molecule into the secretory form, was also cloned into pTriEx-4C. Similarly cloned into pTriEx-4C was sTNF-α mutant, which has the region coding for the TNF-α signal peptide (amino acids 76 to 1) replaced with a sequence coding for the human Ig κ-chain secretion signal peptide (5′-ATGGAGACAGACACACTCCTGCTATGG-GTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC-3′) to express a solely secretable form of 17-kDa TNF-α. All of the constructs were sequenced (Invitrogen, Shanghai, China) to confirm the integrity and preciseness of the described mutations. 4T1 cells were transfected with expression plasmids pTriExNeo (as a control), pTriEx/wtTNF, pTriEx/sTNF-α, or pTriEx(tmTNF-α), respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. Cells were subcultured to G418 (Life Technologies, Grand Island, NY) selection and cloned by limiting dilution.

Tumor inoculations

BALB/c mice were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China). TNFR1 or TNFR2 knockout (KO) mice on a BALB/c background were provided by Prof. Zhihai Qin (National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). Mice were bred and maintained under specific pathogen-free conditions and treated according to Tongji Medical College (China) Animal Care Guidelines. The animal study was approved by the Ethical Committee of Tongji Medical College.

Six- and 10-wk-old female BALB/c, TNFR1−/−; or TNFR2−/− mice were inoculated in the mammary fat pad with 5 × 103 4T1 parental cells or with 4T1 cells stably transfected with wtTNF-α, sTNF-α, tmTNF-α, or empty vector. For in vitro experiments, tumor (transfected with TNF-α and its mutants) dimensions were measured with calipers every 3 d. Mean tumor size was calculated by taking width 2 × length × 0.52 (27). All measurements were performed in a coded, blinded fashion.

Isolation, purification, and stimulation of MDSCs

Spleens were isolated from normal and tumor-bearing mice and washed twice with Hank’s solution. After lysis of RBCs, splenocytes were fractionated by Percoll density gradient centrifugation as described by Kusmartsev et al. (28). Cells were harvested from the gradient interfaces between 50 and 65%, and Gr-1+ cells were isolated from this fraction using BD IMag anti-mouse Ly-6G and Ly-6C Particles-DM (BD Biosciences). For the stimulation of MDSCs, 300 ng/ml sTNF-α (PeproTech) or surface TNF-α on 1% parafomaldehyde-fixed 4T1/wtTNF-α cells (at an E:T ratio of 10:1) was added and incubated for 16 h. The supernatants and the cells were then harvested for the corresponding assays in vitro.

MTT cell proliferation assay

4T1 cells (1 × 105) stably transfected with wtTNF-α, tmTNF-α mutant, sTNF-α mutant, or empty plasmid were seeded in 96-well microtiter plates and incubated at 37°C, 5% CO2 for the indicated time. Cells were stained with glucose-PBS containing 0.45 mg/ml MTT (Sigma-Aldrich) for 4 h, followed by lysis with 0.1 ml 100% DMSO. The OD value at 570 nm was measured on a microplate reader (Tecan, Grödig, Austria).

Flow cytometry

Abs used for flow cytometry were fluorescein-conjugated mAbs, including PE-anti-CD3 (145-2C11), FITC-anti-CD11b (ICRF44), PE-anti-Gr-1 (RB6-8C5), PE-anti-TNF-α (MP6-XT22), PE-anti-TNFRI (55R-286), PE-anti-TNFR2 (TR75-89), and isotype controls (mouse IgG1, rat IgG2b, Armenian hamster IgG) from BD Biosciences (San Diego, CA) or BioLegend (San Diego, CA).

Tumor tissue (100 mg) was minced into small pieces (1–3 mm3) and digested for 1 h at 37°C in 5 ml FCS-free RPMI 1640 medium containing type IV collagenase (160 μg/ml), hyaluronidase (250 μg/ml), and DNase (5 μg/ml). After filtration through a 200-mesh sieve, a single-cell suspension was collected and cells were washed three times with PBS. These cells from primary tumor tissue or purified MDSCs from spleen were incubated with the above Abs in PBS containing 2% BSA for 30 min at 4°C and washed twice. Expression of the cell surface molecules was analyzed on an LSR II flow cytometer (Becton Dickinson, San Jose, CA) using BD FACSDiva software.

ELISA for cytokines

Tumor tissue (100 mg) in 1 ml PBS was homogenized at 4°C with a Polytron homogenizer. After centrifugation at 12,000 × g for 30 min at 4°C, the supernatants were collected. Commercial ELISA kits were used to detect IL-10 and TGF-β in supernatants of tumor or of MDSCs from TNFR1 KO, TNFR2 KO, or wild-type tumor–bearing mice after stimulation, as well as sTNF-α in supernatants of transfectants according to the manufacturer’s instructions (BD Biosciences). Plates were read at 450 nm on a microplate reader (Tecan), with 570 nm as the reference wavelength.

NO and ROS analysis

NO in the supernatants of MDSCs was determined by Griess ion sensitive dye 2′,7′-dichlorofluorescin diacetate (DCFDA; Sigma-Aldrich). Purified MDSCs were incubated at 37°C in RPMI 1640 in the presence of 2.0 μM DCFDA for 20 min after stimulation. Analysis was then performed by flow cytometry as described above.

Cytoplasmic, nuclear, or total protein preparation and Western blot analysis

MDSCs (1 × 106) were harvested after stimulation and suspended in precooled buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and incubated on ice for 10 min. The cytoplasmic membrane was then broken down by addition of Nonident P-40 at a final concentration of 0.5% and vortexed for 10 s. The separation of the cytoplasmic protein from the nucleus was performed by centrifugation at 10,000 × g for 1 min at 4°C. The pelleted nuclei were washed three times with ice-cold lysis buffer A, then resuspended in 50 μl ice-cold lysis buffer C (50 mM HEPES [pH 7.8], 0.42 M KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM MgCl2, and 20% glycerin) and incubated...
on ice for 30 min. After centrifugation at 12,000 × g for 20 min at 4°C, the supernatant containing nuclear protein was collected.

For total protein preparation, MDSCs activated with both forms of TNF-α, or 4T1 cells expressing wtTNF-α, sTNF-α, or tmTNF-α were solubilized in precooled lysis buffer (30 mM Tris-Cl, 150 mM NaCl, 1% P-40, 10% glycerol, 0.5 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) on ice for 15 min. The total protein in supernatants was obtained by centrifugation at 13,000 × g for 20 min at 4°C.

The expression of nuclear protein was fractionated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane by electroblotting. The membranes were blocked overnight at 4°C with 5% nonfat dry milk in PBS-Tween 20 (0.05%) and then probed with different primary Abs to TNF-α, ERK, p-ERK, JNK, p-JNK, p38, p-p38, NF-κB p65, IκB, lamin B1, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with corresponding secondary Abs conjugated with HRP. Immunoreactive bands were visualized with a chemiluminescence reagent (Pierce) and then exposed to Kodak Image Station 4000MM (Carestream Health) for imaging.

RT-PCR and quantitative real-time PCR

Total RNA from MDSCs was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. One microgram RNA was reverse-transcribed to cDNA using the RevertAid first-strand cDNA synthesis kit (Fermentas). The sequences of the primers synthesized by Sangon were as follows: TNFR2 (700 bp), 5'-ACCCCTCAACCTCAGAT-CATCTTCTC-3' and 5'-CAGATTGACCTCAGCGCTGAGTTG-3'; TNFR1 (480 bp), 5'-GCCTGTCTGCTACGTCTGCTCTG-3' and 5'-AGTCTC-CTGGGGGTGTTGACATTTGC-3'; TNFR2 (500 bp), 5'-AGCGTGCC-GAATCTCAGGAATA-3' and 5'-AGCTGTAAAGGCTTGGTGT-3'; β-actin (450 bp), 5'-TCAACCCACCTGCCCCCTCATCAGA-3' and 5'-CATCGGAAACCCTCCTGCCAACAG-3'. The reaction was performed in 30 cycles (94°C, 20 s; 58°C, 20 s; and 72°C, 30 s).

Real-time PCR was performed in duplicate for each sample on MX3000P (Stratagene) using the Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen, Carlsbad, CA). One microliter cDNA was added to a 20 μl reaction mixture containing 10 μl SYBR Green PCR Master Mix and 400 nM primers. The primers included ARG1, 5'-GCAGTGCGTTACACCCTTTTC-3'; TNFR1, 5'-TCACCCACCTGCCCCCTCATCAGA-3' and 5'-GTGAGGCTGCTGATGTC-3'; TNFR2, 5'-GGAAGGCTGAGTTGTTTC-3'. The sequences of the primers synthesized by Sangon were as follows: TNFR2 (700 bp), 5'-ACCCCTCAACCTCAGAT-CATCTTCTC-3' and 5'-CAGATTGACCTCAGCGCTGAGTTG-3'; TNFR1 (480 bp), 5'-GCCTGTCTGCTACGTCTGCTCTG-3' and 5'-AGTCTC-CTGGGGGTGTTGACATTTGC-3'; TNFR2 (500 bp), 5'-AGCGTGCC-GAATCTCAGGAATA-3' and 5'-AGCTGTAAAGGCTTGGTGT-3'; β-actin (450 bp), 5'-TCAACCCACCTGCCCCCTCATCAGA-3' and 5'-CATCGGAAACCCTCCTGCCAACAG-3'. The reaction was performed in 30 cycles (94°C, 20 s; 58°C, 20 s; and 72°C, 30 s).

Statistical analysis

All data were statistically analyzed by the Student t test or one-way ANOVA test. Differences were considered to be statistically significant at p < 0.05.

Results

To observe effects of both forms of TNF-α on MDSC bioactivities, Gr-1+CD11b+ cells were freshly isolated from wild-type tumor-bearing mice. The purity of the Gr-1+CD11b+ MDSC population was >95% as evaluated by flow cytometry (Fig. 1A, upper cyogram). MDSCs were incubated with 300 ng/ml sTNF-α and tmTNF-α on transfectants (at an E:T ratio of 10:1). We found that tmTNF-α activated MDSCs, manifested as a marked increase in mRNA levels for ARGI (Fig. 1A) and iNOS (Fig. 1B), and a significant elevated production of NO (Fig. 1C) and ROS (Fig. 1D). In contrast, sTNF-α inhibited production of NO by MDSCs, as compared with control, but it has no effect on ARGI and iNOS at the mRNA level and on ROS production in MDSCs. Neutralization of tmTNF-α by a specific Ab totally blocked tmTNF-α-induced activation of MDSCs. Furthermore, tmTNF-α has been shown to result in obviously enhanced release of IL-10 (Fig. 1E) and TGF-β (Fig. 1F); however, sTNF-α only lifted TGF-β secretion by ∼2-fold, but it had no significant effect on IL-10 production.

It is well known that MDSCs suppress activation and proliferation of T cells (16). tmTNF-α has been shown above to enhance the release of IL-10 and TGF-β, two inhibitors for T cell proliferation, into the supernatants of MDSCs. To exclude the effect of tmTNF-α on T cells and the competition between MDSCs and T cells for binding to tmTNF-α, we collected supernatants from MDSCs culture to check whether the inhibitory mediators released by tmTNF-α–stimulated MDSCs could inhibit T cell proliferation induced by PMA and ionomycin. As shown in Fig. 1G, PMA and ionomycin induced T cell proliferation by ∼40%. Addition of the supernatant of MDSCs from tumor-bearing mice into the culture partly inhibited T cell proliferation (p < 0.001), whereas the supernatant from tmTNF-α–stimulated MDSCs totally blocked T cell proliferation (p < 0.001). Conversely, T cell proliferation could be recovered by neutralization of tmTNF-α to abolish the activation of MDSCs or blockade of IL-10 in the supernatant from tmTNF-α–stimulated MDSCs using specific Abs.

Activity effect of tmTNF-α on MDSCs is mediated by TNFR2

Although tmTNF-α is the primary ligand of TNFR2 (13), it is able to effectively trigger both of the TNF receptors (31). To know which receptor, TNFR1 or TNFR2, mediated the activating effect of tmTNF-α on MDSCs, we first tested the expression of TNFR1 and TNFR2 by MDSCs at both mRNA and protein levels. RT-PCR results showed that TNFR2 transcripts were more abundant than TNFR1 in MDSCs, whereas the expression of TNFR2 transcripts in MDSCs from both normal and tumor-bearing mice (Fig. 2A). Consistent with the results of RT-PCR, flow cytometry results also showed that the expression level of TNFR2 was much higher than that of TNFR1 in MDSCs, whereas the expression of both TNF receptors on the surface of MDSCs from tumor-bearing mice was slightly increased as compared with those on MDSCs from normal mice, but it had no statistical significance (Fig. 2B).

Because MDSCs express both TNFRs, we isolated MDSCs from TNFR1 or TNFR2 gene KO tumor-bearing mice to study which TNFR mediates the effect of tmTNF-α on MDSCs. As shown in

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Fig. 2C, deficiency of TNFR1 had no influence on the suppression of T cell proliferation by MDSCs or tmTNF-α-activated MDSCs. However, deficiency of TNFR2 significantly impaired tmTNF-α increased MDSC inhibition of T cell proliferation by PMA and ionomycin in a contact-independent manner. Similar results were also observed in T cell proliferation induced by anti-CD3/anti-CD28 (Fig. 2D). Because IL-10 in the supernatant released by tmTNF-α-activated MDSCs showed an inhibitory or stimulatory effect on NO production of MDSCs, respectively (Fig. 1C), a substance also responsible for inhibition of T cell proliferation. We found that TNFR1 KO completely removed the suppressive effect of sTNF-α, whereas TNFR2 KO totally cleared up the activatory effect of tmTNF-α on MDSCs to compare with those effects of the both forms of TNF-α on MDSCs from wild-type tumor-bearing mice.

**FIGURE 1.** Activation of MDSCs by tmTNF-α. Purified Gr-1⁺CD11b⁺ MDSCs (1 × 10⁶/ml) from wild-type tumor-bearing mice were incubated for 16 h with 300 ng/ml sTNF-α or with tmTNF-α expressed on the 1% paraformaldehyde-fixed 4T1 cells (1 × 10⁷) transfected with wtTNF-α (at an E:T ratio of 10:1). The specificity of TNF-α activity was confirmed by a 30-min preincubation of the transfectants with an mAb against murine TNF-α. The purity of MDSCs was evaluated by flow cytometry [cytogram at the top of (A)]. (A and B) Total mRNA was isolated from treated MDSCs and transcripts of ARG1 (A) and iNOS (B) were analyzed by real-time PCR. The production of NO (C) and ROS (D) by MDSCs was tested by Griess agent and DCFDA, respectively, and the release of IL-10 (E) and TGF-β (F) from MDSCs was determined by ELISA. (G) The percentage of proliferated CD3⁺ T cells was tested by CFSE dilution assay. Naïve splenocytes (5 × 10⁶) labeled with CFSE were stimulated for 72 h with 10 ng/ml PMA and 0.5 µg/ml ionomycin in the presence of supernatant (SN) from MDSCs activated for 24 h by tmTNF-α. Cells were stained with PE-anti-CD3 Ab, and the proliferation of T cells (gated on CD3⁺ cells) was analyzed by flow cytometry. Nontreated T cells served as a control. Data are presented as means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control (A–F) or PMA plus ionomycin alone (G). \*\*\*p < 0.001 versus SN of MDSCs treated with nothing (G).
FIGURE 2. tmTNF-induced MDSC activation via TNFR2. (A) mRNA levels of TNFR1 and TNFR2 in purified Gr-1⁺CD11b⁺ cells from naive or tumor-bearing mice were tested by RT-PCR and normalized to β-actin gene expression. (B) Expression of TNFR1 and TNFR2 on Gr-1⁺CD11b⁺ cells was analyzed by flow cytometry. Upper and middle panels of (A) and (B) show the representative results from one experiment out of three, and lower histograms show the means ± SD of three independent experiments. (C and D) MDSCs (1 × 10⁶/ml) from wild-type, TNFR1 KO, or TNFR2 KO tumor-bearing mice were incubated for 24 h with medium alone or with 1 × 10⁷ fixed 4T1 cells transfected with tmTNF-α (at an E:T ratio of 10:1). The supernatants (SNs) were added to naive splenocytes labeled with CFSE and incubated for 72 h in the presence of 10 ng/ml PMA and 0.5 μg/ml ionomycin (C) or of 2 μl anti-CD3/anti-CD28 beads plus 20 IU/ml recombinant murine IL-2 (D). The proliferated T cells (gated on CD3⁺ cells) were determined by flow cytometry after staining with a PE-anti-CD3 Ab. Nontreated T cells served as a control. (E and G) MDSCs from wild-type, TNFR1 KO, or TNFR2 KO tumor-bearing mice were incubated for 24 (E) or 16 h (G) with 4T1 cells transfected with tmTNF-α (at an E:T ratio of 10:1) or 300 ng/ml sTNF-α (G). IL-10 was detected by ELISA (E), and NO was determined by Griess agent (G). For neutralization of tmTNF-α, a specific Ab (20 μg/ml) was used to pretreat the transfectant for 30 min. (F) MDSCs (1 × 10⁶/ml) from wild-type tumor-bearing mice were pretreated at 4˚C for 30 min with anti-TNFR1, anti-TNFR2, or IgG (eBioscience) in the concentration of 15 μg/ml and then stimulated with tmTNF-α for 24 h as described above. SNs were collected (Figure legend continues).
mice (Fig. 2G). These data strongly implied that TNFR2 mainly mediated the activatory effect of tmTNF-α on MDSCs.

tmTNF-α activates MDSCs via the NF-κB pathway
The NF-κB pathway has been reported to be involved in the activation of MDSCs (32, 33). An inhibitor protein, IκBa, is phosphorylated in response to stimulation with a variety of agents, including sTNF-α, resulting in its degradation and the translocation of NF-κB from the cytoplasm into the nucleus where NF-κB binds to cognate DNA binding sites and activates the transcription of target genes (34). To determine whether the NF-κB pathway mediated tmTNF-α-induced activation of MDSCs, we observed the degradation of IκBa and translocation of NF-κB p65 by Western blot. We found (Fig. 3A) that tmTNF-α significantly induced IκBa degradation in the cytoplasm and the translocation of NF-κB p65 into the nucleus of MDSCs from tumor-bearing mice, although sTNF-α also had a slight activatory effect. When MDSCs were pretreated for 30 min with pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, tmTNF-α–induced NF-κBa activation was markedly suppressed (Fig. 3B), and the increased inhibition of T cell proliferation by MDSCs (Fig. 3C) was abolished.

The p38 pathway mediates tmTNF-α–induced MDSC activation
Because sTNF-α has been well known to be an inducer of the phosphorylation of MAPK, including ERK1/2, JNK, and p38 MAPK (35–37), we examined whether the action of tmTNF-α on MDSCs is related to this pathway. As shown in Fig. 4A, the

and the release of IL-10 was detected by ELISA. Results are shown as means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus TNFR1 (A, B) or stimuli alone for T cell proliferation (C, D) or control (E–G). *p < 0.01, ***p < 0.001 versus SN of MDSCs from wild-type, TNFR1 KO, or TNFR2 KO mice (C, D).
phosphorylation level of p38, but not ERK1/2 and JNK, was significantly elevated by tmTNF-α treatment, although sTNF-α also had a weak effect. This action could be completely abrogated by neutralizing tmTNF-α or using SB203580, a p38 MAPK inhibitor (Fig. 4B), and T cell proliferation could be recovered from inhibition of tmTNF-α–activated MDSCs (Fig. 4C).

**Ectopic expression of tmTNF-α on 4T1 mammary carcinoma induces MDSC accumulation and promotes tumor progression**

To confirm the in vivo effect of tmTNF-α on MDSCs, the murine breast cancer cell line 4T1 was stably transduced with wtTNF-α, which encodes both forms of TNF-α, with a tmTNF-α mutant, which encodes only uncleavable tmTNF-α, and with an sTNF-α mutant, which encodes only sTNF-α. Indeed, our results demonstrated in Fig. 5 that 4T1/wtTNF-α expressed the surface molecule detected by FACS (Fig. 5A) and released the soluble molecule determined by ELISA (Fig. 5B). 4T1/tmTNF-α expressed more of the transmembrane form of TNF-α (94.7%) on the surface than did 4T1/wtTNF-α (43%), but it released undetectable levels of the soluble molecule. However, 4T1/sTNF-α did not express the transmembrane molecule, but it secreted higher levels of the soluble molecule than did 4T1/wtTNF-α cells. Additionally, ectopic expression of wtTNF-α, tmTNF-α, or sTNF-α mutant did not affect the growth curve of 4T1 tumor cells in vitro (Fig. 5C).

Next, we inoculated equal numbers (5 × 10^5) of different forms of TNF-transduced 4T1 cells into BALB/c mice. As expected, the tumors that only expressed tmTNF-α grew faster and were larger than in other tumor groups, displaying a marked tumor progression. Conversely, the tumors that only secreted sTNF-α grew much slower and were smaller than in other tumor groups, showing a significant inhibition of tumor development (Fig. 5D). The tumors that produced both forms of TNF-α showed a middle growth rate, exhibiting a delayed tumor progression. Additionally, MDSC accumulation was significantly increased in tmTNF-α–expressing 4T1 tumor cells.
tumor, but was markedly decreased in sTNF-α-secreting tumor (Fig. 5E, 5F), suggesting that tmTNF-α–triggered tumor progression and sTNF-α–induced tumor inhibition are closely associated with accumulation of MDSCs.

**tmTNF-α–expressing tumor produces more inhibitory mediators and promotes Treg accumulation, but it fails to induce lymphocyte infiltration**

Because MDSCs have been shown to accumulate in tmTNF-α–expressing tumors, we further detected whether inhibitory mediators that were released at least partially by MDSCs would be elevated in the tumor tissue. Indeed, the release of NO (Fig. 6A), IL-10 (Fig. 6B), and TGF-β (Fig. 6C) was significantly enhanced in tmTNF-α–expressing tumors, but not in wtTNF-α–producing and sTNF-α–secreting tumors. MDSCs are known as an important regulator of angiogenesis in tumors (38). As expected, CD31+ staining showed an evident increase in the number of small blood vessels in tmTNF-α–expressing tumors, but a relative decrease in angiogenesis of microvessels in sTNF-α–secreting tumors, indicating a pro- or antivasculization effect of tmTNF-α and sTNF-α, respectively (Fig. 6D). This pro- or antivasculization effect seemed to be associated with increased or decreased MDSC accumulation in tmTNF-α or sTNF-α–expressing tumors.

In contrast to the accumulation of MDSCs, immunohistochemistry (Fig. 7A, 7B) showed a poor infiltration of lymphocytes in the tmTNF-α–expressing tumor as well as in the control. Conversely, in the sTNF-α–secreting tumor, the infiltration of either CD4+ or CD8+ lymphocytes was significantly increased, pointing out a relationship of MDSCs to lymphocyte accumulation. Moreover, MDSCs have been shown to induce Treg differentiation (39), and therefore we detected the accumulation of Foxp3+ T cells in the tumor tissue. Indeed, Foxp3+ T cells were significantly increased in the tmTNF-α–expressing tumors, but slightly decreased in the sTNF-α–secreting tumors (Fig. 7C), indicating a possible association of MDSCs with Tregs. The in vivo data above confirmed again that tmTNF-α, rather than sTNF-α, is responsible for the accumulation and activation of MDSCs, thus promoting tumor progression.

**Discussion**

Previous studies have demonstrated that various proinflammatory mediators, such as IL-1β (22, 40), IL-6 (23), the bioactive lipid PGE2 (24), S100A8/A9 (25, 41), and complement component C5a (42), are involved in the accumulation and activation of MDSCs in tumor-bearing mice and cancer patients. However, whether TNF-α, as a prototype and key proinflammatory cytokine, is also in-
In the present study, we provide firm evidence that tmTNF-α, rather than sTNF-α, is mainly responsible for MDSC activation, manifested as upregulation of iNOS and ARG1, with increased suppressive activities including elevated production and release of NO, ROS, IL-10, and TGF-β, as well as enhanced inhibition of T cell proliferation. Although murine splenic MDSCs exert only Ag-specific T cell inhibition in a contact manner, these cells acquire the ability that tumor-derived counterparts possess to suppress Ag-nonspecific T cell functions when exposed to hypoxia (43). Interestingly, our results show that tmTNF-α also conferred on splenic MDSCs the characteristics of tumor-derived MDSCs, including upregulation of iNOS and ARG1 expression, and thus inhibition of Ag-nonspecific T cell proliferation in a contact-independent manner. Furthermore, it is likely that tmTNF-α–stimulated IL-10 production by MDSCs was required to suppress Ag-nonspecific T cell proliferation, as neutralization of IL-10 completely blocked the inhibitory effect of tmTNF-α.

Because tmTNF-α is the primary ligand of TNFR2, and our results showed that MDSCs expressed mainly TNFR2, we hypothesized that tmTNF-α could activate MDSCs through TNFR2. Indeed, our results support this postulate, as TNFR2 deficiency or neutralization completely abolished the activatory effect of tmTNF-α on MDSCs releasing IL-10 and inhibiting T cell proliferation. Furthermore, TNFR2 KO totally blocked tmTNF-α–induced elevation of NO production by MDSCs, whereas TNFR1 KO entirely prevented sTNF-α–induced inhibition of NO release from MDSCs. It seems that both forms of TNF-α affected MDSCs via different types of TNFRs. Because tmTNF-α has a higher affinity for TNFR2 than sTNF-α (13), this may also account for the difference between two forms of TNF-α in the activation of MDSCs. Furthermore, in contrast to TNFR1, TNFR2 is considered to be associated with a negative regulation of inflammatory pathological process (44, 45). It has been reported that TNFR2-expressing CD4+Foxp3+ Tregs comprise ~40% of peripheral Tregs in normal mice and represent the maximally suppressive subset of Tregs (46). Additionally, TNFR2-mediated signaling has also been demonstrated to be required for MDSC survival and accumulation (26). To our knowledge, in the present study we describe for the first time the association of tmTNF-α/TNFR2 with the functions of MDSCs. It is likely that the interaction of tmTNF-α and TNFR2 preferentially leads to the activation of Tregs and MDSCs, two important negative regulatory cells that are involved in anti-inflammation and tumor immune evasion. Our study demonstrates that the NF-κB pathway and the p38 pathway mediated tmTNF-induced MDSC activation. This is supported by the following evidence: 1) the level of p38 phosphorylation in MDSCs stimulated by tmTNF-α was significantly increased, whereas the phosphorylation of two other MAPK family members, ERK and JNK, was not affected; 2) the NF-κB signaling pathway was also activated in MDSCs by tmTNF-α stimulation, as evidenced by accelerated degradation of IκBα and translocation of NF-κB p65 from the cytoplasm into the nucleus; and 3) upon preincubation of MDSCs with the p38 MAPK inhibitor SB203580 or the NF-κB inhibitor PDTC, suppression of T cell proliferation by tmTNF-α–activated MDSCs was abrogated. It has been reported that the MAPK p38 pathway regulates NF-κB transactivation via direct acetylation of p65 and is required for NF-κB–mediated NF-κB activation (47, 48). Therefore, it is not surprising that inhibition of either pathway was sufficient to block tmTNF-α–induced activation of MDSCs.
TNFR1 and TNFR2. Additionally, TNFR2 is required for TNF-
study was a murine molecule that exerts its effects via both
tansductants. In fact, the tmTNF-
the in vitro results, significant release of NO, IL-10, and TGF-

FIGURE 7. Poor lymphocyte infiltration, but increased Treg accumulation, in tmTNF-α-expressing tumor. Infiltration of CD4+ (upper panel), CD8+ (middle panel), or Foxp3+ (bottom panel) cells was detected in tumor sections from different forms of TNF-α-producing tumors by immunohistochemistry using specific Abs (original magnification ×200, left). CD4+, CD8+, and Foxp3+ cells were quantitatively analyzed in five fields per section (two sections per mouse and five mice per treated group) by manual cell counting. The quantitative data of immune reactive cells are represented as means ± SD and shown on the right of the corresponding immunohistochemical images. *p < 0.05, **p < 0.01 versus 4T1/Neo.

Our in vivo data further confirmed that tmTNF-α, but not sTNF-
α, promoted activation and accumulation of MDSCs, and thus facilitated tumor progression. In comparison with the control, ectopic expression of uncleavable tmTNF-α promoted 4T1 tumor growth and progression concomitant with significant MDSC accumulation, whereas expression of sTNF-α led to slower tumor growth and significant tumor regression with decreased MDSC recruitment. However, previous studies including ours (2, 49) have indicated that tmTNF-α leads to tumor regression. These contradictory results are likely due to the different types of tumor studied and/or the different species of tmTNF-α expressed by the tansductants. In fact, the tmTNF-α transduced in our previous study was a human molecule that exerts its effects only via TNFR1 in mice (50), whereas the tmTNF-α transduced in this study was a murine molecule that exerts its effects via both TNFR1 and TNFR2. Additionally, TNFR2 is required for TNF-α to activate MDSCs and to induce Tregs (11). In agreement with the in vitro results, significant release of NO, IL-10, and TGF-β was observed in the tumor expressing tmTNF-α, although these inhibitory mediators were only partially produced by MDSCs. These mediators inhibit T cell proliferation, which might account for the poor lymphocyte accumulation in tmTNF-α-expressing tumor. Furthermore, it has been reported that MDSCs down-regulate L-selectin on T cells (51) that is needed for T cell homing to lymph nodes and a tumor microenvironment where they can encounter Ags and be activated. Recently, the ability of MDSCs to promote the de novo development of Foxp3+ Tregs in vivo has been described (39). This is in line with our results showing increased Foxp3+ Tregs in tmTNF-α-expressing tumor with MDSC accumulation. The induction of Tregs by MDSCs was found to require the presence of IL-10 and TGF-β but was independent of NO (39, 52). tmTNF-α-stimulated secretion of IL-10 and TGF-β from MDSCs may favor induction of Tregs, in addition to its possible direct effect on Tregs via TNFR2.

MDSCs have nonimmunosuppressive pro-tumor functions, including augmenting tumor angiogenesis, invasion, and metastasis (38, 53). Yang et al. (38) reported that MDSCs promote tumor angiogenesis by producing MMP9 and differentiating into endothelial cells. Consistent with this observation, our data showed an association of angiogenesis with MDSC accumulation in tmTNF-α- or sTNF-α-expressing tumors. Although tmTNF-α expression has been shown to be upregulated in the tumor endothelium (54) and play an angiogenic role (55), it is possible that tmTNF-α may be a factor in tumor microenvironment that would facilitate MDSC-dependent tumor angiogenesis. Briefly, our in vivo data indicated again that tmTNF-α plays an important role in the activation of MDSCs, and thus promotes tumor immune escape, one of the mechanisms involved in tumor progression.

Interestingly, sTNF-α solely expressed by tumor has opposite effects. It failed to recruit MDSCs as compared with the control, but it attracted more lymphocytes in the tumor, promoting significant tumor inhibition. It appears that sTNF-α is an antitumor factor, instead of being a tumor-promoting factor, in the absence of tmTNF-α. This is consistent with our previous studies (9, 10, 56), showing that when tmTNF-α expression is disturbed, tumor cells become sensitive to the cytotoxic effect of sTNF-α, because tmTNF-α expressed by tumor constitutively activates NF-κB through its reverse signaling, thereby protecting tumor from apoptosis and promoting survival.

In the present study, we demonstrated that tmTNF-α expressed by tumor activates MDSCs through forward signaling via TNFR2 to inhibit antitumor immune responses. However, sTNF-α solely expressed by tumor is beneficial for antitumor immunity. Although the proinflammatory effect of sTNF-α promotes tumor
development, the inflammation conversely favors the development of immune responses. Unfortunately, both forms of TNF-α coexist in the tumor microenvironment. Therefore, selective inhibition of tmTNF-α may prevent tumor-promoting effects of sTNF-α-mediated inflammation while deriving benefit from it for the tumor therapy through weakening immunosuppression and increasing the sensitivity of tumor to apoptosis.

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Disclosures
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References


