Thrombospondin 1 Promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 Cells

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Abstract
Inhibition of tumor growth by thrombospondin (TSP) 1 is generally attributed to its antiangiogenic activity, but effects on tumor immunity should also be considered. We show that overexpression of TSP1 in melanoma cells increases macrophage recruitment into xenograft tumors grown in nude or beige/nude mice. In vitro, TSP1 acutely induces expression of plasminogen activator inhibitor-1 (PAI-1) by monocytic cells, suggesting that TSP1-induced macrophage recruitment is at least partially mediated by PAI-1. Tumor-associated macrophages (TAM) can either promote or limit tumor progression. The percentage of M1-polarized macrophages expressing inducible nitric oxide synthase is increased in TSP1-expressing tumors. Furthermore, soluble TSP1 stimulates killing of breast carcinoma and melanoma cells by IFN-γ–differentiated U937 cells in vitro via release of reactive oxygen species. TSP1 causes a significant increase in phorbol ester–mediated superoxide generation from differentiated monocytes by interaction with α5β1 integrin through its NH2-terminal region. The NH2-terminal domain of TSP2 also stimulates monocyte superoxide production. Extracellular calcium is required for the TSP1-induced macrophage respiratory burst. Thus, TSP1 may play an important role in antitumor immunity by enhancing recruitment and activation of M1 TAMs, which provides an additional selective pressure for loss of TSP1 and TSP2 expression during tumor progression. [Cancer Res 2008;68(17):7090–9]

Introduction
Thrombospondin 1 (TSP1) is a secreted glycoprotein that is predominantly stored in platelets but also secreted at low levels by many cell types, including monocytes and macrophages. TSP1 is rapidly and transiently released in response to tissue injury and is elevated in several chronic diseases (1, 2). TSP1-null mice display acute and chronic inflammatory pulmonary infiltrates and an elevated number of circulating WBCs (3), suggesting an anti-inflammatory role. In contrast, TSP1 expression in ischemic injuries limits tissue survival and restoration of perfusion by blocking nitric oxide/cyclic guanosine 3′,5′-monophosphate signaling (4). The diverse biological activities of TSP1 are mediated by its multiple functional domains that engage corresponding receptors expressed by a variety of cells. Differential expression or activation of cell surface receptors for TSP1, including integrins, CD36, CD47, low-density lipoprotein (LDL) receptor-related protein, proteoglycans, and sulfatides, may dictate the specific responses of each cell type to TSP1 (5).

TSP1 plays several roles in the physiologic functions of phagocytes. TSP1 mediates phagocytosis of neutrophils undergoing apoptosis (6). Macrophage recognition and phagocytosis of apoptotic fibroblasts requires fibroblast-derived TSP1 and CD36 (7). CD36-deficient patients have impaired oxidized LDL-induced nuclear factor-κB activation and subsequent cytokine expression (8). TSP1 modulates expression of interleukin (IL)-6 and IL-10 by monocytes (9) and activation of latent transforming growth factor β (TGFβ); ref. 3). TSP1 stimulates motility of human neutrophils (10, 11) and promotes chemotaxis and haptotaxis of human peripheral blood monocytes (12). In addition, TSP1 enhances cytokine-induced respiratory burst of human neutrophils (13) and enhances chemoattractant fMLP-mediated superoxide anion (O2•-) generation by human neutrophils through its NH2-terminal domain (14, 15). However, the underlying mechanism for regulation of O2•- generation has not been delineated. Here, we provide evidence that soluble TSP1 causes a significant increase in phorbol 12-myristate 13-acetate (PMA)–mediated O2•- generation from IFN-γ–differentiated human monocytes by interaction with α5β1 integrin through its NH2-terminal region and identify a requirement for extracellular calcium to mediate the macrophage respiratory burst.

Macrophages are an important effector cell of innate immunity against tumors. However, tumor-associated macrophages (TAM) can differentiate into either cytotoxic (M1) or tumor growth–promoting (M2) states. This differentiation depends on the tissue microenvironment (16). Macrophages are classically activated toward the M1 phenotype by IFN-γ alone or in concert with microbial products. Alternative activation by stimulation with IL-4 or IL-13, IL-10, IL-21, TGFβ; immune complexes, and glucocorticoids drives macrophages toward the M2 phenotype (17). M2 macrophages are present in most established tumors and promote tumor progression (18).

TSP1 is often down-regulated during tumor progression and inhibits tumor growth when reexpressed (19). This activity is generally attributed to angiogenesis inhibition, but the above results suggest that effects on tumor immunity should also be considered. The current study shows an important role for TSP1 as a positive modulator of innate antitumor immunity by increasing M1 macrophage recruitment and stimulating reactive oxygen species (ROS)–mediated tumor cytotoxicity.

Materials and Methods
Proteins and peptides. Human TSP1 was purified from the supernatant of thrombin-activated platelets obtained from the NIH Blood Bank (20). Recombinant human TSP1 was obtained from EMP
Genetech. Recombinant proteins containing various domains of TSP1 and TSP2 were prepared as previously described and provided by Dr. Deane Mosher (University of Wisconsin, Madison, WI; refs. 21–23). The α5β1 integrin inhibitory peptide (LALERKDHSQG) derived from TSP1 and the control peptide (LALARKDHSG) were prepared as described (24). Xanthine, xanthine oxidase, and superoxide dismutase (SOD) were obtained from Stratagene.

Reagents. Rat anti-mouse CD68 antibody (clone FA-11) was from AbD Serotec. Monoclonal neutralizing antibody (clone 9016) against human TGFβ1 recombinant human and mouse IFN-γ, recombinant human TGFβ1, and recombinant human IL-4 were from R&D Systems. Rabbit polyclonal to plasmogen activator inhibitor-1 (PAI-1) and rabbit polyclonal to inducible nitric oxide synthase (iNOS) were from Abcam, Inc. Anti-actin (Ab-1) mouse monoclonal antibody and EGTA were from Calbiochem. The function-blocking rat anti-human α5 integrin monoclonal antibody (clone GOH3) was from Chemicon International, Inc. FITC-conjugated rat anti-human α5 monoclonal antibody (clone GOH3) and the isotype control were from BD Biosciences. Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and PMA were from Sigma-Aldrich. The inhibitor of iNOS, aminoguanidine, was from Sigma-Aldrich. The α5β1 integrin antagonist (4-[[(2-methylphenyl)amino]carbonyl][aminocarbonyl][aminocarboxy]-acyetid-LDVP (25) was obtained from Bachem. The calcium indicator Fluor-4/AM, rabbit polyclonal anti-fluorescein/Oregon Green, and Pluronic F-127 were from Molecular Probes.

**THBS1-transfected cells.** MDA-MB-435 cells transfected with the *THBS1* expression plasmid (clone TH26, 7.5-fold higher TSP1 expression than control) or the empty pCMVBamNeo vector (Neo) were described previously (26).

**Cell culture and differentiation.** Transfected MDA-MB-435 cells were cultured at 37°C, 5% CO2 in complete RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (FBS; Biosource), 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 750 μg/mL geneticin (Life Technologies). The human monocytic line U937 (27), kindly provided by Dr. Mark Raffeld [National Cancer Institute (NCI), NIH, Bethesda, MD], was cultured at 37°C, 5% CO2, in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% endotoxin-tested FBS (Biosource). For differentiation with IFN-γ, 2.0 × 10^6/mL U937 cells in complete growth medium containing 1 mmol/L sodium pyruvate, 0.1 mmol/L MEM with nonessential amino acids (Cellgro), and 100 units/mL recombinant human IFN-γ were incubated for 3 d at 37°C. For differentiation with IL-4, 2.0 × 10^6/mL U937 cells in AIM-V + Albumax serum-free medium (Life Technologies) containing 10 ng/mL recombinant human IL-4 were incubated for 3 d at 37°C. MDA-MB-231, MDA-MB-435, and MCF-7 cells were cultured in RPMI 1640 containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Murine macrophage cell lines ANA-1 (28) and RAW264.7 were cultured in DMEM (Life Technologies) supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% endotoxin-tested FBS. Human peripheral blood mononuclear cells (PBMC) were prepared by gradient centrifugation. In brief, human freshuffy coat (NIH Blood Bank) was diluted 1:4 with sterile Dulbecco's PBS (Life Technologies). Human PBMCs were isolated by mixing 1.077 g/mL Lymphocyte Separation Medium (Cambiox) and the diluted human blood and centrifuged for 30 min at 900 × g, 18°C to 20°C. Human monocytes were isolated from PBMCs by adherence to plastic.

**Tumorigenesis assay in nude mice.** Groups of 10 female NIH-bg/nu mice, ~8 wk of age, were injected in the mammary fat pads with 8 × 10^6 Neo or TH26 cells. Primary tumor size was determined twice weekly by length × width × height measurement. The primary tumors were removed on week 11.

A total of 15 female NIH-bg/nu mice, 7 wk of age, were s.c. injected in the right hind leg with 5 × 10^6 MDA-MB-435 cells. Five animals were injected with Neo cells and 10 with TH26 cells. Primary tumor size was determined twice weekly, and tumor volume was calculated as (width)^2 × length/2. Primary tumors were removed when the volume was 300 to 400 mm³ or at week 7. For histopathologic analysis, tumor tissues were fixed in buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with H&E. Animal experiments were conducted in an accredited facility according to NIH guidelines under a protocol approved by the NCI Animal Care and Use Committee.

**Immunohistochemical evaluation.** Slides were deparaffinized in xylene (thrice for 10 min) and rehydrated in graded alcohol (100%, 95%, and 70%). Antigen retrieval was performed in a pressure cooker containing Target Retrieval Solution (pH 6.10; Dako Corp.) for 30 min (CD68 antibody) or 10 min (PAI-1 antibody) or 10 mmol/L citrate buffer (pH 6.0) for 10 min, followed by cooling at room temperature for 20 min (INOS antibody), and then washed with PBS twice for 10 min. Endogenous peroxidase activity was quenched by 0.3% H2O2 in water. After washing the slides with Wash Buffer Solution (Dako), nonspecific binding was reduced using Protein Block Serum-Free (Dako) for 10 min. The slides were incubated with CD68 antibody (1:100, overnight at 4°C). PAI-1 antibody (1:250 dilution, 1 h at room temperature), and INOS antibody (1:50, 1 h at room temperature). Slides were then incubated with streptavidin-biotin (Dako LSBA+ kit, horseradish peroxidase). 3,3’-Diaminobenzidine (Dako) was used as chromogen for 5 min, and hematoxylin was used for counterstaining. Negative control slides omitted the primary antibody. CD68 was located predominantly within the cells. Nuclei were negative. Cytoplasmic and extracellular staining in macrophages was considered positive for PAI-1. Cytoplasmic staining in macrophages was considered positive for iNOS. The intensity of the staining was evaluated using a Nikon Eclipse E1000 microscope equipped with a microcolor camera (RGB-M5-C). The acquisition software was IPLab-Scientific Imaging Processing 3.5.5.

**Measurement of monocyte chemotactic protein-1, PAI-1, and IL-10.** Monocyte chemotactic protein-1 (MCP-1), PAI-1, and IL-10 levels in differentiated U937 cell supernatants were measured with a multiplex ELISA array (Quansys Biosciences). All samples were run in replicate.

**Western blotting.** RAW264.7 cells were serum deprived for 48 h before addition of TGFβ1 or TSP1. After 2 to 4 h of incubation at 37°C, 5% CO2, in AIM-V + Albumax serum-free medium, cells were lysed at 4°C in 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/L HEPES, 1% Triton X-100, 1% NP40, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Cell pellets were vortexed briefly and centrifuged at 14,000 rpm for 15 min. Cell lysates (15 μg protein) were boiled for 5 min in SDS sample buffer, electrophoretically separated on NuPAGE 10% Bis-Tris gels (Invitrogen) for 1.5 h at 150 V, and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) for 2 h at 100 V. Membranes were blocked in 5% bovine serum albumin (BSA)/0.1% Tween 20/ PBS and incubated overnight with rabbit polyclonal to PAI-1 (2.5 μg/mL). Enhanced chemiluminescence (Upstate) was used for detection. Stripped membranes were reprobed with actin antibody to confirm protein loading levels.

**Real-time quantitative reverse transcription-PCR analysis.** Total RNA was extracted from Neo and TH26 tumors using Trizol (Invitrogen) according to the manufacturer’s instructions. Total RNA was treated with recombinant DNase I (DNA-free kit, Applied Biosystems) and quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). cDNA was synthesized from total RNA using iScript cDNA Synthesis kit (BioRad). Real-time PCR for mouse iNOS and arginase-1 expression profiling was performed on a 7500 Real-Time PCR instrument (Applied Biosystems) using Taqman oligonucleotide primers (Mm00440485_m1 and Mm00475988_m1, respectively. Data were normalized against mouse hypoxanthine phosphoribosyltransferase 1 (HPRT1; Mm00446968_1). Relative iNOS and arginase-1 expression was calculated using the 2^-ΔΔCT method.

**U937 and ANA-1 cell-mediated cytolyis.** MDA-MB-231, MDA-MB-435, and MCF-7 target cells were seeded into 16-well plates in 150 μL of growth medium. Cell growth was dynamically monitored using RT-CES system (ACEA Biosciences) for 24 h. Differentiated U937 effector cells at an E/T ratio of 40:1 were added into wells containing target cells. ANA-1 cells were activated for 20 h at 37°C with 10 ng/mL of LPS and 100 units/mL of IFN-γ in complete medium and also used at an E/T ratio of 40:1. After addition of effector cells, measurements were automatically collected by the analyzer every 10 min for up to 48 h.
were seeded into 96-well plates in 100 μL of growth medium containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in the presence or absence of xanthine/xanthine oxidase for 72 h at 37°C. Media were collected to assess lactate dehydrogenase (LDH) release due to cell death. LDH release was quantified using a colorimetric assay (Promega). All samples were run in triplicate.

**Cytotoxicity assay.** MDA-MB-231 cells (2 x 10^4 per well) were seeded into 96-well plates in 200 μL of growth medium in the presence or absence of soluble TSP1 for up to 72 h at 37°C. MDA-MB-231 cells (2,500 per well) were seeded into 96-well plates in 100 μL of RPMI 1640 containing 1.25% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in the presence or absence of xanthine/xanthine oxidase for 72 h at 37°C. Media were collected to assess lactate dehydrogenase (LDH) released due to cell death. LDH release was quantified using a colorimetric assay (Promega). All samples were run in triplicate.

**Superoxide production.** O2⁻ levels in differentiated U937 and activated ANA-1 cell supernatants were quantified using the LumiMax Superoxide Anion Detection kit (Stratagene). Anion Detection kit (Stratagene). After staining, propidium iodide was added and the cells were analyzed on a FACScan flow cytometer (Becton Dickinson). The analysis software was FlowJo (7.2.1).

**Flow cytometry analysis.** Direct immunofluorescence was performed by incubating 1 x 10⁶ cells with 50 μg/mL of FITC-conjugated rat anti-human αi antibody (clone G0H3) or isotype control for 45 min at 4°C in HBSS containing 0.1% BSA and 0.1% sodium azide (Sigma-Aldrich). After staining, propidium iodide was added and the cells were analyzed on a FACScan flow cytometer (Becton Dickinson). The analysis software was FlowJo (7.2.1).

**Measurement of intracellular free Ca²⁺.** Differentiated U937 cells were incubated with loading solution consisting of HEPES-buffered saline [116 mM HEPES (Cellgro) in HBSS] supplemented with 2 μmol/L Fluo-4, 0.02% Pluronic F-127, and 1% BSA for 30 min and then incubated in loading solution without Fluo-4 for 30 min to allow deesterification of the probe. Loading solution was replaced with HBSS containing anti-fluorescein antibody and TSP1. The cells were then placed in a fluorometer (GENios Plus Tecan), and measurements were collected every 5 min for up to 40 min.

**Statistical analysis.** All data are shown as mean ± SD except where indicated. Significance was determined with one-tailed distribution Student’s t test analysis. The difference was considered significant when P < 0.05 (*) and P < 0.001 (**).

**Results**

**TSP1 overexpression increases tumor macrophage recruitment in vivo.** Expression of TSP1 in the melanoma cell line MDA-MB-435 significantly inhibits tumor growth and metastasis (26). Notably, increased infiltration of monocytes was observed in clone TH26 tumors that highly express TSP1 (26) and in tumor-bearing mice treated with TSP1 peptides (29). Because macrophage recruitment into wounds is TSP1 dependent (1), we examined the effect of tumor cell TSP1 over expression on TAM recruitment. Using the previously described conditions of injection in the mammary fat pads (26), we confirmed that TH26 tumors showed delayed growth in female NIH-bg/nu mice relative to control-transfected clones (data not shown). The primary tumors were removed on week 11, and seven randomly selected mice were analyzed by immunohistochemistry. TAMs were detectable in nonnecrotic areas of tumor stained with H&E (data not shown). Based on CD68 antibody staining, the percentage of TAMs was significantly higher in TH26 tumors than in Neo tumors (P < 0.05; Fig. 1A).

Mammary fat pad injection was used previously as an orthotopic model of breast carcinoma growth, but recent studies have confirmed that the MDA-MB-435 cell line is a derivative of the M14 melanoma cell line (30). To determine whether TSP1 suppresses tumor growth at a site more appropriate for melanoma, 15 female NIH-μu/μu mice were s.c. injected in the right hind limb with TH26 or Neo cells. TSP1 overexpression in TH26 cells also inhibited s.c. tumor growth (P ≤ 0.001; Fig. 1B). The primary tumors were removed when the volume was 300 to 400 mm³ or at week 7 and analyzed by immunohistochemistry. Sections of tumor were stained with H&E and anti-mouse CD68 antibody. Increased TAM infiltration was observed in s.c. TH26 versus Neo tumors (P < 0.001; Fig. 1C and D).

**Role of MCP-1 and PAI-1 in TSP1-dependent TAM recruitment.** MCP-1 is an important regulator of monocyte recruitment (31), and a deficit in MCP-1 was proposed to account for decreased infiltration of macrophages into an excisional wound of TSP1-null mice (1). However, 12 h of stimulation with different doses of soluble TSP1 resulted in no significant change in total MCP-1 release from differentiated U937 human mononcytic cells (Fig. 2A, left).

Macrophage migration in vitro and in vivo also depends on PAI-1 (32, 33), and elevated PAI-1 expression was previously
reported in TH26 versus Neo cells (34). Therefore, increased PAI-1 could also account for increased TAM recruitment into TH26 tumors. Because this result was obtained using a stable transfectant, it was unclear whether TSP1 directly regulates PAI-1 expression rather than the increased PAI-1 being an indirect adaptation of the transfected clones. Incubation of differentiated U937 cells with TSP1 resulted in an acute dose- and time-dependent increase in PAI-1 expression, with a maximal 50-fold

Figure 2. Effects of TSP1 on MCP-1 and PAI-1 expression in differentiated U937 human monocytic cells and mouse macrophages. A to C, differentiated U937 cells (1 × 10⁶/0.5 mL RPMI 1640 with 0.5% FBS) were incubated in the presence or absence of soluble TSP1, soluble recombinant type 1 repeats of TSP1 (3TSR; B), or soluble recombinant human TSP1 (C, right). After 12 h of incubation (A), or at the indicated times (B and C, right), the supernatants were harvested, and total MCP-1 and PAI-1 were determined using a multiplex ELISA array as described in Materials and Methods. Data are representative of at least four different experiments. C, left, differentiated U937 cells were incubated with neutralizing TGFβ1 antibody (clone 9016) for 15 min before the addition of soluble TSP1 (20 μg/mL). Culture supernatants collected after 12 h were used to measure total PAI-1. Data are representative of two different experiments. D, left, serum-deprived murine RAW264.7 cells were stimulated with TGFβ1 (1 ng/mL), as a positive control, or soluble TSP1 (20 μg/mL) for 2 h. The experiment was repeated thrice, and a representative anti–PAI-1 blot is shown. Actin was used to confirm equal protein loading. Right, paraffin-embedded sections cut from TH26 tumors grown s.c. in NIH-nu/nu mice were stained with rat anti-mouse CD68 antibody (clone FA-11; left) and rabbit anti-mouse PAI-1 antibody (right). Representative photomicrographs of adjacent sections are shown from experiments conducted in tumor samples from 12 NIH-nu/nu mice. Identical patterns were observed in all of the tumors examined. Magnification, ×200.
induction at 8 h \( [P < 0.001; \text{Fig. 2A (right) and B}] \). Because PAI-1 is a TGFβ-inducible gene (35) and bioactive TGFβ is present in TSP1 purified from platelets (36), we asked whether TGFβ contamination or TSP1-mediated activation of latent TGFβ (3) contributed to the PAI-1 response in U937 cells. Interestingly, stimulation with 3 \( \mu \)g/mL of soluble recombinant 3TSR, the type 1 repeat domain of TSP1 responsible for the TSP1-mediated activation of latent TGFβ (37), resulted in no significant change in total PAI-1 release from differentiated U937 cells (Fig. 2B). Therefore, activation of latent TGFβ probably does not account for the effect of TSP1 on PAI-1 expression. The NH2-terminal module of TSP1 increases fMLP-mediated \( \text{O}_2 \) generation and chemotaxis by human neutrophils (10, 14) but also had no effect in PAI-1 expression (data not shown).

TSP1 stimulation of PAI-1 production was then examined in the presence of a neutralizing TGFβ1 antibody. At 5 \( \mu \)g/mL, the neutralizing antibody partially inhibited TSP1-stimulated PAI-1 production (Fig. 2C, left). Furthermore, recombinant human TSP1 at the same concentration, which should lack TGFβ contamination, showed less stimulatory activity than platelet TSP1 (Fig. 2C, right). Therefore, bioactive TGFβ present in platelet TSP1 at least partially mediates the stimulation of PAI-1 production by TSP1, but TSP1 lacking TGFβ is also active.

To address whether TSP1 also induces PAI-1 production in mouse macrophages, we used the RAW264.7 macrophage cell line. Increased PAI-1 expression was detected in whole-cell lysates within 2 h after TGFβ (positive control) or TSP1 treatment (Fig. 2D, left). Furthermore, increased PAI-1 secretion was detected in cell culture supernatants within 4 h after TGFβ or TSP1 treatment (data not shown). We also examined whether PAI-1 is expressed by murine TAMs in vivo. Immunohistochemical analysis showed strong PAI-1 staining in the TH26 TAMs (Fig. 2D, right).

Increased M1 macrophage recruitment into TSP1-overexpressing tumors. Activated murine macrophages metabolize \( \text{L-arginine} \) via two main pathways that are catalyzed by the inducible enzymes iNOS and arginase. Increased iNOS is characteristic of M1 macrophages, and arginase-1 is a marker of M2 macrophages (38). To compare iNOS expression in vivo, total RNA extracted from six randomly selected Neo and TH26 tumors was analyzed using real-time PCR. A 3.8-fold increase in iNOS expression was found in TH26 tumors (Fig. 3A). In contrast, the M2 marker arginase-1 was equally expressed in both tumors (data not shown). Staining of tumor sections using an iNOS antibody showed an increased percentage of iNOS-expressing TAMs in TH26 versus Neo tumors (\( P < 0.001; \text{Fig. 3B and C} \)). Taken together, these data show that M1 cytotoxic macrophages are a minor fraction of the TAMs in MDA-MB-435 tumors, but their recruitment or differentiation is increased when the tumors express TSP1.

To address whether M2 macrophage differentiation or function is sensitive to TSP1 in vitro, U937 cells were differentiated using IL-4 for 72 h in the presence or absence of soluble TSP1. IL-10 levels in the cell culture supernatants, a marker of M2 polarization, showed no significant differences (Fig. 3D, left). Similarly, incubation of IL-4–differentiated U937 cells with soluble TSP1 (20 \( \mu \)g/mL) for 12 h did not alter IL-10 secretion (Fig. 3D, right).

TSP1 stimulates macrophage cytotoxicity toward breast carcinoma and melanoma cells. To determine whether TSP1 can regulate tumor cell killing by macrophages, we performed dynamic monitoring of macrophage-mediated cytosis. IFN-γ–differentiated U937 cells were incubated with MDA-MB-231 breast carcinoma cells (at an E:T ratio of 40:1) in the RT-CES system. The cell index readout assesses changes in viable adherent cells

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**Figure 3.** Increased iNOS-expressing M1 TAMs in TSP1-overexpressing tumors. A, real-time quantitative reverse transcription-PCR analysis of mRNA expression in TH26 and Neo tumors from six NIH-nu/nu mice. Fold difference was adjusted to HPRT1 internal control values. Relative quantification of iNOS was calculated by the \( 2^{-\Delta\Delta CT} \) method. B, immunohistochemical analysis of TH26 and Neo tumors in NIH-nu/nu mice. Adjacent sections were stained using rat anti-mouse CD68 antibody (clone FA-11; top) and rabbit polyclonal antibody to iNOS to detect M1-differentiated TAMs (bottom). The results are representative of tumor samples from 12 NIH-nu/nu mice, which showed identical patterns. Magnification, \( \times 400 \). C, quantitative analysis of CD68-positive cells expressing iNOS in the tumor specimens performed by evaluating the percentage of iNOS-positive macrophages in multiple \( 100 \times \) fields. The results are presented as the percentage of iNOS-positive TAMs in Neo control (white column) and TH26 tumors (red column). D, left, U937 cells (2 x 10⁷/0.5 mL AIM-V) were incubated with IL-4 to induce M2 differentiation in the presence or absence of soluble TSP1. At the indicated times, the supernatants were harvested, and IL-10 was determined using a multiplex ELISA array as described in Materials and Methods. Right, U937 cells were differentiated with IL-4 for 72 h, and then the differentiated cells (1 x 10⁷/1 mL AIM-V) were incubated with soluble TSP1. After 12 h of incubation, the supernatants were harvested, and IL-10 was determined using a multiplex ELISA array.
by electrical impedance. Differentiated U937 cells expressed constitutive cytotoxic activity against MDA-MB-231 cells (Fig. 4A). Moreover, tumoricidal activity was increased 5-fold after 18 h of incubation with soluble TSP1 (5 μg/mL; Fig. 4A, left). This concentration of TSP1 did not show any direct cytotoxic activity against MDA-MB-231 cells (Fig. 4A, right). TSP1 similarly enhanced cytotoxicity against MDA-MB-435 melanoma and MCF-7 breast carcinoma cell targets (Fig. 4B, left and right, respectively). Because our in vivo model used a human tumor xenograft in mice, we also examined the mouse macrophage cell line ANA-1 as effector against human MDA-MB-231 target cells. Activated ANA-1 cells expressed constitutive cytotoxic activity against MDA-MB-231 cells in the presence of an iNOS inhibitor (0.5 mmol/L aminoguanidine) to permit O₂⁻/C⁰₂⁻ accumulation (39). A

Figure 4. TSP1 increases U937 human monocytic cell–mediated tumoricidal activity. A, left, and B, MDA-MB-231, MDA-MB-435, and MCF-7 target cells (2 × 10⁶/150 μL RPMI 1640 with 10% FBS) were seeded into 16-well sensor plates and incubated for up to 24 h. After this incubation, differentiated U937 effector cells (8 × 10⁶/200 μL RPMI 1640 with 10% FBS) were directly added into wells containing target cells in the presence or absence of soluble TSP1. The measurements were automatically collected by the analyzer RT-CES system for up to 48 h. Data are representative of three different experiments. A, right, MDA-MB-231 cells (2 × 10⁶/200 μL RPMI 1640 with 10% FBS) were seeded into 96-well plates and incubated for up to 72 h in the presence or absence of soluble TSP1 (5–20 μg/mL). At the indicated times, medium samples were collected and LDH released was measured as described in Materials and Methods. The absorbance was recorded at 490 nm. Data are representative of two different experiments. C, MDA-MB-231 target cells (2 × 10⁶/150 μL RPMI 1640 with 10% FBS) were seeded into 16-well sensor plates and incubated for up to 24 h. After this incubation, activated ANA-1 effector cells (8 × 10⁶/50 μL RPMI 1640 with 10% FBS and 0.5 mmol/L aminoguanidine) were directly added into wells containing target cells in the presence or absence of soluble TSP1. The measurements were automatically collected by the RT-CES system for up to 18 h. Data are representative of four different experiments. D, MDA-MB-231 target cells (2,500/100 μL RPMI 1640 with 1.25% FBS) were seeded into 96-well plates and incubated for 72 h in the presence or absence of different doses of xanthine (X) and xanthine oxidase (XO). The medium samples were collected, and LDH released was measured. The absorbance was recorded at 490 nm. The percentage of cytotoxicity is shown above each column and is calculated as (experimental − spontaneous)/maximum − spontaneous) × 100. Maximum LDH release was determined by complete lysis of target cells.
10-fold increase in tumoricidal activity was recorded after 12 h of incubation with soluble TSP1 (5 μg/mL; Fig. 4C).

TSP1 has been shown to enhance chemotactic fMLP-mediated superoxide generation in human neutrophils (14), suggesting that increased ROS mediates the tumoricidal activity of TSP1-stimulated cells. To confirm sensitivity to O\textsubscript{2} \textsuperscript{-}, MDA-MB-231 cells were treated with xanthine and xanthine oxidase to generate O\textsubscript{2} \textsuperscript{-}. Addition of O\textsubscript{2} \textsuperscript{-} dose dependently increased LDH release from the cells (Fig. 4D).

**TSP1 increases extracellular release of O\textsubscript{2}**. To determine whether the known activity of TSP1 to enhance O\textsubscript{2} \textsuperscript{-} release in human neutrophils extends to M1-differentiated U937 cells, IFN-γ-differentiated U937 cells were stimulated with PMA (100 ng/mL), and O\textsubscript{2} \textsuperscript{-} generation was assessed using luminol chemiluminescence. Incubation of differentiated U937 cells with soluble TSP1 (20 μg/mL) significantly increased (P ≤ 0.001) PMA-mediated O\textsubscript{2} \textsuperscript{-} production (Fig. 5A, left), and 25 units of SOD completely abolished this signal (data not shown), confirming that O\textsubscript{2} \textsuperscript{-} was responsible for the chemiluminescent signal. This also indicates that TSP1 stimulates extracellular O\textsubscript{2} \textsuperscript{-} production because SOD does not degrade intracellular O\textsubscript{2} \textsuperscript{-}. Similar enhancement of O\textsubscript{2} \textsuperscript{-} generation by TSP1 was observed in monocytes isolated from human PBMCs and in a murine macrophage cell line ANA-1 (Fig. 5A, middle and right). Trimeric recombinant constructs (residues 1–356) containing the N-modules of TSP1 (NoC1) and TSP2 (residues 1–359, NoC2) but not other recombinant regions of TSP1 enhanced O\textsubscript{2} \textsuperscript{-} production (Fig. 5B).

The N-module of TSP1 interacts with several receptors, including α\textsubscript{β}1, α\textsubscript{β}2, and α\textsubscript{β}3 integrins (40). Because α\textsubscript{β}1 integrin recognizes the N-module of TSP1 but not TSP2 (41), we examined the role of α\textsubscript{β}1 and α\textsubscript{β}2 integrins. Differentiated U937 cells are known to express α\textsubscript{β}1 (42), and we found that they also express α\textsubscript{β}2 (Fig. 5C). The α\textsubscript{β}2-binding peptide LAKERDHS (24) and a function-blocking α\textsubscript{β}2 antibody partially inhibited the activity of TSP1 on O\textsubscript{2} \textsuperscript{-} generation. In contrast, the α\textsubscript{β}1 antagonist phLDVP had no effect. These results identify a specific requirement for α\textsubscript{β}1 integrin to mediate TSP1 binding to human monocytes and the subsequent activation of intracellular signaling pathways required for O\textsubscript{2} \textsuperscript{-} production (Fig. 5D).

**TSP1-stimulated O\textsubscript{2} \textsuperscript{-} production in macrophages requires intracellular Ca\textsuperscript{2+}**. Ligation of some integrins triggers a transient elevation in intracellular free Ca\textsuperscript{2+} (43, 44). Ca\textsuperscript{2+} is a second messenger of TSP1 binding to human monocytes and the subsequent activation (Fig. 5B). Ligation of some integrins triggers a transient elevation in intracellular free Ca\textsuperscript{2+} (43, 44). Ca\textsuperscript{2+} is a second messenger of TSP1 binding to human monocytes and the subsequent activation.
mechanism for activation of NADPH oxidase in human monocytes (45). This result suggested that increased levels of Ca^{2+} might account for the enhancement by TSP1 of O_2^- production in differentiated U937 cells. Cells were loaded with Fluo-4 for 30 min and then treated with soluble TSP1 (20 μg/mL) for 25 to 50 min. Addition of TSP1 caused a significant rise in intracellular free Ca^{2+} (Fig. 6A). This increase was eliminated completely following chelation of extracellular Ca^{2+} by the addition of 1 mmol/L EGTA (data not shown). To further confirm the role of Ca^{2+} in respiratory burst activity, differentiated U937 cells were treated with EGTA before the addition of PMA and TSP1. As shown in Fig. 6B, chelation of extracellular Ca^{2+} significantly decreased the stimulatory effect of TSP1 on PMA-mediated O_2^- generation in differentiated U937 cells, suggesting that a Ca^{2+}-dependent mechanism is involved in TSP1 modulation of the macrophage respiratory burst.

Discussion

Several studies have suggested that TSP1 plays an important role in the recruitment of monocytes and macrophages to sites of tissue injury or inflammation (1, 12). Here, we provide evidence that overexpression of TSP1 in tumors increases macrophage recruitment in vivo. Tumor cells produce a range of chemotactic factors for macrophages. MCP-1 stimulates recruitment of macrophages into tumors in vivo (46), and decreased expression of these chemokines was reported to limit infiltration of macrophages into an excisional wound in TSP1-null mice (1). However, soluble TSP1 did not increase MCP-1 release from differentiated U937 human monocytic cells. PAI-1 is also required for cell migration in vitro (33), and evidence is emerging for a critical role of PAI-1 in macrophage migration in vitro and in vivo (32). TSP1 up-regulates PAI-1 in pancreatic cancer cells (47), and PAI-1 expression is up-regulated in TSP1-expressing MDA-MB-435 cells (34). We found that TSP1 acutely increased PAI-1 production by differentiated human and mouse macrophages, and strong TAM expression of PAI-1 was observed in TSP1-overexpressing tumors in vivo. This acute change in PAI-1 expression in response to TSP1 is at least partially TGFβ mediated. Activation of latent TGFβ is mediated by the type 1 repeats of TSP1, but we found no induction of PAI-1 by this domain. Therefore, TSP1 probably induces PAI-1 via bound active TGFβ rather than activation of latent TGFβ produced by U937 cells. Although additional cytokines may be involved, autocrine induction of PAI-1 via tumor cell produced TSP1 as well as paracrine induction of PAI-1 expression in TAMs could increase macrophage recruitment into the tumor.

Whether increased macrophage recruitment inhibits or enhances tumor growth depends on their differentiation state. The tumor environment can educate TAMs toward a tumor-promoting phenotype (M2; ref. 18), which may prevent further macrophage migration within the tumor and ensure constant production of growth and angiogenic factors. In addition to contributing to macrophage infiltration by stimulating PAI-1 signaling in TAMs, we found that TSP1 expression in the tumor selectively increases M1 macrophage infiltration. This may provide a selective pressure distinct from its antiangiogenic activity to account for the frequently observed down-regulation of TSP1 during tumor progression and its ability to inhibit tumor growth when reexpressed.

In general, M1 macrophages are efficient producers of reactive oxygen and nitrogen intermediates that mediate resistance against tumors (17). Here, we provide evidence that tumor expression of TSP1 increases M1 polarization of TAMs assessed by iNOS expression. TSP1 was previously shown to enhance cytokine-induced and chemoattractant fMLP-induced respiratory burst in human neutrophils (13, 14). We now show that TSP1 enhances PMA-mediated respiratory burst in U937 cells differentiated along an M1 pathway using IFN-γ. TSP1 stimulates the cytotoxic activity of differentiated human U937 cells and murine ANA-1 cells against several human breast carcinoma and melanoma cell lines. This contrasts with the reported activity of U937 cells to support tumor growth in an M2 manner when coinjected with prostate carcinoma cells (48). We found no effect of TSP1 on M2 differentiation of these cells in vitro. Therefore, U937 monocytes have the capacity to differentiate along both pathways, but TSP1 selectively enhances the cytotoxic effector function of M1 macrophages.

The NH2-terminal domains of TSP1 and TSP2 are sufficient for this priming activity but not for PAI-1 induction. The NH2-terminal domain of TSP1 mediates interactions with several integrin and nonintegrin receptors (40). Differentiated U937 cells express α_{vβ1} (42) and α_{vβ1} integrins, and inhibition of α_{vβ1} using a TSP1 peptide or a function-blocking α_{vβ1} antibody provides evidence that this integrin mediates intracellular signaling pathways leading to increased O_2^- production.

Interactions between integrins and their ligands can trigger transient elevation in intracellular free Ca^{2+} (43, 44), and Ca^{2+} is a well-known intracellular second messenger for signaling the generation of O_2^- in human monocytes (45). We found that addition of TSP1 to differentiated U937 cells caused a significant increase in intracellular free Ca^{2+} and that chelation of extracellular Ca^{2+} inhibits the stimulatory effect of TSP1 on mobilization of intracellular Ca^{2+} and PMA-mediated O_2^- generation in differentiated U937 cells. Taken together, these data suggest that a Ca^{2+}-dependent mechanism is involved in TSP1 modulation of the
macrophage respiratory burst. However, we cannot exclude the possibility that additional receptors recognized by the N-domains of TSP1 might contribute to O$_2^-$ production by macrophages. It is interesting that the NH$_2$-terminal region of TSP2 shares this activity to stimulate O$_2^-$ production by macrophages. Loss of TSP2 has also been noted with progression in some cancers, and overexpression of TSP2 limits tumor growth in murine models (19). Our data suggest that this may involve both suppression of angiogenesis and inhibition of tumor growth via its antiangiogenic activity, but our results suggest that TSP1 plays an additional role in tumor immunity by increasing M1 macrophage recruitment and cytotoxicity. Avoiding this innate immune surveillance could provide a second selective pressure to reduce TSP1 expression during tumor progression.

**Tumor cell killing in vitro** via production of reactive oxygen intermediates. In vivo, TSP1 promotes M1 macrophage recruitment into tumors while decreasing tumor growth. Clearly, TSP1 can also inhibit tumor growth via its antiangiogenic activity, but our results suggest that TSP1 plays an additional role in tumor immunity by increasing M1 macrophage recruitment and cytotoxicity. Avoiding this innate immune surveillance could provide a second selective pressure to reduce TSP1 expression during tumor progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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