Tumor Necrosis Factor Receptor 2–Mediated Tumor Suppression Is Nitric Oxide Dependent and Involves Angiostasis

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Abstract

Tumor necrosis factor (TNF) binds to two different receptors. Although most of its functions are attributed to TNF receptor 1 (TNFR1), the independent role of TNFR2 is still largely unknown. Using TNFR single or double knock-out mice, we show here that the expression of TNFR2 alone on host cells was sufficient to suppress the growth of TNF-secreting tumors in both immune competent and T/B lymphocyte–deficient severe combined immunodeficiency (SCID) mice. Histologic studies showed that TNF recruited, via TNFR2, large numbers of macrophages and efficiently inhibited angiogenesis in the tumor. *In vitro*, TNF activated TNFR1-deficient macrophages to produce nitric oxide (NO). Treatment of TNFR1 knock-out mice with L-NAME, a specific NO synthase inhibitor, almost completely eliminated TNF-induced angiostasis and tumor suppression. Moreover, L-NAME acted only during the first few days of tumor growth. Our results show for the first time that TNFR2 expressed on host innate immune cells is sufficient to mediate the antitumor effect of TNF, and NO is necessary for this process, possibly by inhibition of angiogenesis in the tumor. [Cancer Res 2007;67(9):4443–50]

Introduction

It is well known that tumor necrosis factor (TNF) locally secreted by gene-modified tumor cells inhibits tumor growth (1–5); however, the underlying mechanism is still not clear. A recent study showed that both bone marrow– and non–bone marrow–derived cells were required to express TNF receptor 1 (TNFR1) for the human TNF-induced tumor suppression in mice (6). Among bone marrow–derived cells, T cells may not be essential because the tumor growth was also inhibited in T cell–deficient nude mice (3, 7). In contrast, Mac-1$^+$ cells comprising mainly macrophages and granulocytes are crucial because the antitumor effect of TNF could be completely eliminated by treating the mice with a specific anti–Mac-1 monoclonal antibody (mAb; ref. 2). As for the non–bone marrow–derived cells, systemic administration of high amounts of TNF was shown to induce tumor necrosis by acting on TNFR1-expressing endothelial cells (8).

TNF exerts its functions via two different receptors. It is generally believed that most of the TNF activities are triggered by TNFR1, whereas TNFR2 plays a minor role, e.g., by collecting and handing over TNF to TNFR1, a process called "ligand passing" (9–11). However, the function of TNFR2 should not be underestimated the more because this receptor shows a much more restricted but inducible expression on cells of the immune system and endothelial cells (12, 13). There is now growing evidence for an independent role of TNFR2 in immunologic and inflammatory reactions, such as activation of T cells and myofibroblasts (14–18). Moreover, using receptor-specific agonistic antibodies or TNF muteins that selectively bind to TNFR1 or TNFR2, TNFR2 has been shown to exert tumor cytolytic activity *in vitro* and to induce tumor necrosis *in vivo* (19–21). It is, however, also reported that TNF and its receptor-specific agonistic antibodies may differ in mediating some cellular responses (22). Consequently, the question whether and how TNFR2 is involved in TNF-induced tumor suppression is still to be clarified.

Nitric oxide (NO) is a multifunctional gaseous molecule synthesized from L-arginine by NO synthase (NOS). In contrast to the short-lived, low and constitutive production of NO in neuronal tissue and endothelium by nNOS and eNOS, the production of NO in macrophages sustains over a long time period, is often high, inducible, and catalyzed by the iNOS (23, 24). Lipopolysaccharide (LPS) or cytokines such as TNF and IFN-γ can efficiently regulate *iNOS* transcription within the nucleus of macrophages and lead to NO production through nuclear factor κB (NF-κB) and activator protein 1 (AP-1) (25, 26). Recently, it has been shown that NO is a major effector molecule for the destruction of tumor cells (23, 27, 28). Disruption of *iNOS* gene correlates with impaired tumor rejection in mice (29, 30), and blockage of NO production by macrophages leads to reduced tumoricidal activity *in vitro* (31). Furthermore, expression of NO through an adenoviral vector carrying *iNOS* gene in the tumor tissue prevents tumor metastasis and induces regression of established tumors (32).

In the present study, the independent role of TNFR2 is investigated in TNFR1 single (TNFR1$^{-/-}$) and TNFR1/R2 double knock-out mice (TNFR1$^{-/-}$/R2$^{-/-}$) using tumor cells that were engineered to secrete TNF, and to analyze the role played by innate immune cells, the TNFR1 deficiency was also crossed onto the severe combined immunodeficiency (SCID) background. We show here that expression of TNFR2 alone is sufficient to mediate TNF-induced antitumor activity, whether T/B lymphocytes exist or not. In particular, we show that blocking of NO synthesis *in vivo* completely eliminated TNF-induced angiostasis and growth retardation of tumors in TNFR1-deficient mice.

Materials and Methods

Animals. BALB/c mice were purchased from Charles River and Vital River. CB17 SCID mice were purchased from Bomholtgard. TNFR1$^{-/-}$ mice with a mixed genetic background of 129/Sv/Ev and C57BL/6 were kindly provided by Dr. Horst Bluethmann (Department of Biology, Pharmaceutical Research New Technologies, Hoffmann-La Roche Ltd., Basel, Switzerland; ref. 33). TNFR2$^{-/-}$ mice were obtained from The Jackson Laboratory. Both strains of mice were backcrossed for 12 generations onto the BALB/c background. To obtain TNFR1$^{-/-}$/R2$^{-/-}$ mice, TNFR1$^{-/-}$ mice were paired with TNFR2$^{-/-}$ mice, and subsequent intercross of their offspring led to the

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generation of the double knock-out mice. TNFR1-deficient SCID (R1 − S) and TNFR1−competent SCID mice (R1 + S) were generated by pairing of TNFR1$^{-/-}$ mice with CB17 SCID mice and subsequent intercross of their offspring. Genotyping of the mice was done by flow-cytometric analysis with B cell–specific mAb (anti–B220-PE, RA3-6B2; BD PharMingen) and PCR to distinguish between the normal and mutated TNFR1 alleles. For PCR, genomic DNA from the tail was prepared and amplified with the following primers: 5′-CTC TCT TgT gAT CAg CAC Tg-3′ and 5′-CTg gAA gTg TgT CTC AC-3′ for the *TNFR1* gene, 5′-CTg gAA gTg TgT CTC AC-3′ and 5′-CCA AgC gAA ACA TCg CAT CgA gCg A-3′ for the *neo*^{R} gene. The primers used for genotyping of the TNFR2$^{-/-}$ mice are 5′-CCT CTC ATg CTg TCC Cgg AAT-3′ and 5′-AgC TCC Agg CAC AAg ggC ggg-3′ for the *TNFR2* allele and 5′-Cgg TTC TTT TTg TCA AgA C-3′ and 5′-ATC CTC gCC gTC ggg CAT gC-3′ for the *neo*^{R} gene. Mice were bred in the animal facilities of the Institute of Biophysics (Beijing, China) and Max-Delbrück-Center for Molecular Medicine (Berlin, Germany). In all experiments described here, sex- and age-matched mice were used. Animal studies were conducted after the approval of the corresponding authorities.

Cell lines. The BALB/c-derived plasmacytoma cell line, J558L cells (34) were cultured in RPMI 1640, and the macrophage-sensitive mastocytoma P815 cells were cultured in DMEM supplemented with 10% FCS and antibiotics. The peritoneal macrophages were cultured in RPMI 1640 supplemented with 25 mmol/L HEPES, 10% FCS, and antibiotics. J-mTNF10 cells were established by cotransfection of J558L cells with the plasmid pBA-mTNF (35), in which the mouse *TNF* (mTNF) gene was driven by β-actin promoter and the neomycin resistance gene containing plasmid pWL-neo in a 10:1 molar ratio. Similarly, the human TNF-producing J-hTNF31 cells were established by cotransfection of J558L cells with the plasmid pBA-hTNF and pWL-neo by electroporation using a Gene-Pulser. As mock transfectants, J558L cells were transfected with the plasmid pWL-neo alone (J-neo).

***In vivo* studies.** Exponentially growing tumor cells were harvested, washed, and injected s.c. into the left or right abdomen region of mice in a volume of 0.2 mL in D-PBS. To block the NO synthesis *in vivo*, 50 mg/kg/day of L-NAME (N^G-nitro-L-arginine-methyl ester, N5751, Sigma) was injected in 0.15 mL D-PBS s.c. on the indicated days. Tumor size was measured in millimeters using a caliper and was recorded as published previously (2, 35).

Cytokine detection. To determine the mouse or human TNF secreted by gene-modified tumor cells, J558L transfectants were cultured in RPMI 1640 (1×10^6 cells/mL) at 37°C for 48 h. Cell culture supernatants were then harvested, and TNF was determined using the corresponding ELISA kits according to the manufacturer's instruction (BD Biosciences). Although J-mTNF10 cells secreted 980 ± 40 pg/mL of mTNF, J-hTNF31 cells secreted 3100 ± 150 pg/mL of hTNF. To compare the bioactivity of the mouse and human TNF produced by J-mTNF10 and J-hTNF31 cells, the respective cell culture supernatants were also determined for the cytotoxic activity on L929 cells as described previously (2). One unit was defined as the amount of TNF required for 50% cytolysis of L929 cells. The detection limit was ~0.3 units/mL. Whereas J-mTNF10 cells secreted 10 units/mL, J-hTNF31 cells secreted 31 units/mL of TNF activities.

Tumoricidal activity of peritoneal macrophages. TNFR1$^{-/-}$ and control TNFR1$^{+/-}$ mice were i.p. injected with 0.5 mL of a solution of 0.2% casein in D-PBS. Peritoneal exudate cells (PEC) were then harvested by i.p. lavage, and macrophage monolayer was prepared by seeding 1.25×10^5 PECs per well in 100 μL in a 96-well flat-bottomed plate. After 2 h incubation at 37°C and three washes with medium, adherent cells were incubated for 24 h with or without the addition of cytokine stimulators, TNF (R&D Systems) and/or IFN-γ (BD PharMingen). Subsequently, 5,000 of ^{3}H-thymidine–labeled P815 cells were added in 0.2 mL per well to the macrophage monolayer and incubated for 18 h. The remaining cells were harvested and assayed for radioactivity on a gamma counter (top count; Packard). The percentage of specific killing was calculated as (Spontaneous retention of DNA − Experimental retention)/Spontaneous retention.

Nitrate/nitrite fluorometric assay. Peritoneal macrophages from TNFR1$^{-/-}$ and TNFR1$^{+/-}$ mice were obtained as described above and cultured for 12 h. Approximately 1×10^4 adherent cells per well were distributed in 100 μL of serum-free hybridoma medium (Life Technologies BRL) in a 96-well flat-bottomed plate and cultured for another 2 h. Cells were then exposed to mTNF (100 ng/mL), hTNF (1,000 ng/mL), or IFN-γ (10 units/mL) and LPS (1 μg/mL). After 24 h of stimulation, cell culture supernatants were harvested and tested for NO by a nitrate/nitrite fluorometric assay kit (Cayman Chemical Company).

Immunohistochemical analysis. Isolation of tumor tissues, preparation of cryostat sections, and immunostaining were done as described previously (2). The mAbs used for staining were anti-CD31 (MEC13.3), anti–Gr-1 (RB6-8C5), anti–Mac-1 (M1/70), anti–Mac-3 (M3/84), and isotype-matched control mAbs (all from BD Biosciences). As secondary reagents, the alkaline phosphatase–conjugated goat anti-rat immunoglobulin G (IgG) and biotin-conjugated rabbit anti-rat IgG (H + L) were used (Jackson Immunoresearch). All sections were counterstained with Mayer's hematoxylin (Zhongshan Golden Bridge). Tissue sections of three mice per group were evaluated. The apoptosis of blood vessel endothelial cells were detected by a VasoTACS *in situ* kit (R&D Systems) and anti-CD31 mAb. Rhodamine (TRITC)-conjugated streptavidin and Cy3-labeled goat anti-rat IgG were used as the secondary reagents (Jackson Immunoresearch). The tissue section stained was evaluated with an Olympus *FV*1000 confocal microscope.

Statistical analysis. Data were analyzed by the Wilcoxon signed-ranks test, Student's *t* test, or one-way ANOVA. The differences were considered significant when the *P* value was <0.05.

Results

TNFR2 expression on host cells is sufficient to mediate the antitumor effect of TNF. To analyze whether TNFR2 expressed on host cells can independently mediate TNF-induced antitumor effect, mTNF-secreting J-mTNF10 tumor cells were injected s.c. into the naive TNFR1$^{-/-}$ and TNFR1$^{-/-}$/R2$^{-/-}$ mice. As control, the growth kinetics of the mock-transfected J-neo cells in these mice was also monitored. As shown in Fig. 1, both J-mTNF10 cells and J-neo cells grew progressively in TNFR1$^{-/-}$/R2$^{-/-}$ mice, indicating there was no direct effect, or that the direct effect of TNF on tumor cells did not influence their growth *in vivo*. However, J-neo cells grew well in TNFR1$^{-/-}$ mice, and the tumor size reached 1,000 ± 200 mm^3 in 16 days, whereas the growth of J-mTNF10 cells in these mice was drastically inhibited, and the mean tumor size was 50 ± 30 mm^3 at the same time point (Fig. 1). Actually, most of the J-mTNF10 tumors did not grow out during the whole observation period of more than 40 days (data not shown). This shows that expression of TNFR2 alone is sufficient to mediate TNF-induced antitumor effect in TNFR1$^{-/-}$ mice.

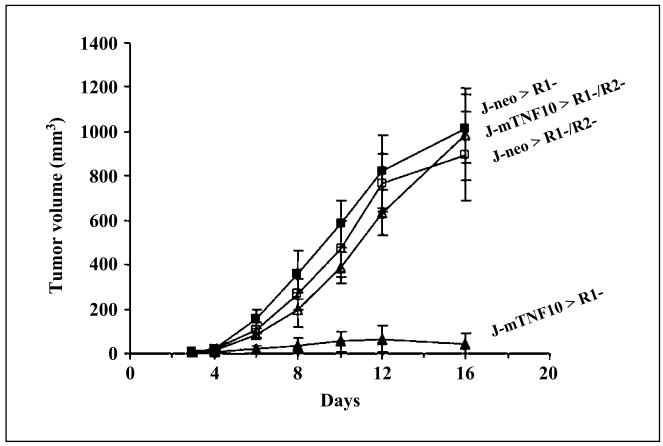

Figure 1. TNFR2 alone is sufficient to mediate TNF-induced antitumor effect. TNFR1$^{-/-}$ mice (*closed*) or TNFR1$^{-/-}$/R2$^{-/-}$ mice (*open*) were s.c. injected with 5×10^6 J-mTNF10 (*triangles*) or as control, J-neo cells (*squares*). Tumor growth was monitored every 2 d. Each group contains five to six mice. Graph is the representative result of two experiments with similar results.

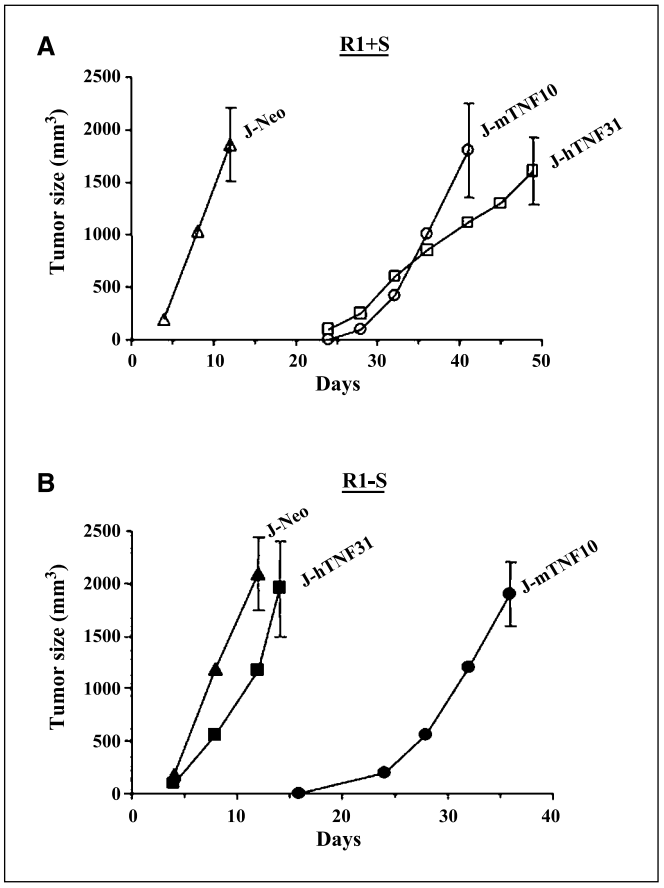

Figure 2. TNFR2-mediated antitumor effect is independent of T/B lymphocytes. Groups of (*A*) R1 + S mice (*open*) or (*B*) R1 − S mice (*closed*) were s.c. injected with 5×10^6 of J-neo (△ or ▲), J-hTNF31 (□ or ■), or J-mTNF10 cells (○ or ●). Tumor growth was monitored. *Points,* mean tumor volumes in cubic millimeters of 8 to 10 mice in each group. Similar results were obtained in another experiment.

T/B lymphocytes are not necessary for the TNFR2-mediated tumor suppression. Both T and B lymphocytes express TNFR2. To analyze whether these cells are required for the TNFR2-mediated tumor suppression, TNFR1-deficient genotype was crossed onto the SCID background to obtain TNFR1-deficient SCID (R1 − S) and TNFR1-competent SCID mice (R1 + S). It is well known that mTNF binds to TNFR1 and TNFR2, whereas hTNF binds only to the mouse TNFR1. To confirm the above finding that the antitumor effect of TNF can be efficiently mediated by TNFR2 alone, tumor growth of mTNF- and hTNF-secreting J558L cells were compared in R1 + S and R1 − S mice. As shown in Fig. 2*A*, local production of both mTNF and hTNF led to tumor growth inhibition in R1 + S mice. In R1 − S mice (Fig. 2*B*), although hTNF producing cells grew progressively, the growth of J-mTNF10 cells was strongly suppressed. On day 14, all J-hTNF31 tumor-bearing R1 − S mice had to be killed due to large tumors of about 2,000 mm^3, whereas none of the R1 − S mice that had received J-mTNF10 cells had got a palpable tumor. The difference cannot be explained by different TNF activities the tumor cells produced because J-hTNF31 secreted even more TNF bioactivity than J-mTNF10 cells in a standard L929 assay (31 units/mL/48 h versus 10 units/mL/48 h). The fact that all J-mTNF10 tumors grew eventually out after 30 days is consistent with the previous finding that T cells are necessary for the complete tumor rejection (6). Furthermore, the growth of J-mTNF10 cells in R1 − S and R1 + S mice was not significantly different ($P > 0.05$), suggesting that TNFR2 alone can efficiently mediate the TNF-induced antitumor response in the absence of T and B lymphocytes. In other words, TNFR2 expression by innate immune cells and non–bone marrow–derived cells is sufficient to mediate tumor suppression.

TNF can induce macrophage infiltration and angiostasis in the tumor via TNFR2. To identify effector cells for the TNFR2-mediated tumor suppression, the J-mTNF10, J-hTNF31, and J-neo cells were s.c. injected into R1 − S and R1 + S mice. Tumors were isolated 5 days later, and tissue cryosections were stained with mAbs against macrophages and granulocytes. Table 1 summarizes the findings of a series of immunohistologic analysis. Few infiltrating cells could be found in J-neo tumors from either R1 − S or R1 + S mice. In R1 + S mice, both mTNF and hTNF secretion led to a clustered accumulation of Mac-1^+ and Gr-1^+ cells in the tumor. In R1 − S mice, however, only the secretion of mTNF, but not hTNF by tumor cells, led to the infiltration of macrophages and granulocytes (Table 1).

In the presence of T/B lymphocytes, innate immune cells were also the major components of tumor-infiltrating cells. Figure 3 shows tumor sections prepared from $TNFR1^{-/-}$ and $TNFR1^{-/-}/R2^{-/-}$ mice 10 days after challenge with J-mTNF10 cells. Again, clusters of Mac-1^+ and Gr-1^+ cells were found in the middle of the tumor from $TNFR1^{-/-}$ mice, but not from $TNFR1^{-/-}/R2^{-/-}$ mice. Staining with anti-CD31 mAb showed that whereas in $TNFR1^{-/-}/R2^{-/-}$ mice, tumor blood vessels grew well into the tumor mass, in $TNFR1^{-/-}$ mice, much less and truncated blood vessels were observed. This inhibition of angiogenesis could partly be due to the apoptosis of endothelial cells. As shown in Fig. 3*G* and *H*, there were many apoptotic endothelial cells detectable along the tumor vasculature in $TNFR1^{-/-}$ mice (Fig. 3*G*), whereas in sharp contrast, almost no apoptotic endothelial cells were found in $TNFR1^{-/-}/R2^{-/-}$ mice (Fig. 3*H*). Together, the results above clearly show that the high local concentration of TNF can recruit innate immune cells, mainly macrophages, and inhibit tumor-associated angiogenesis by acting via TNFR2.

TNFR2 mediates macrophage activation and NO production *in vitro*. Macrophages are the major tumor-infiltrating cells in

Table 1. mTNF- but not hTNF-secreting tumors are infiltrated by macrophages and granulocytes in R1 − S mice

Tumor cell	SCID mouse	Staining*		
		Mac-1	Mac-3	Gr-1
J-neo	$TNFR1^{-/-}$	<5	0	<5
	$TNFR1^{+/-}$	<5	0	<5
J-hTNF31	$TNFR1^{-/-}$	<10	<5	<10
	$TNFR1^{+/-}$	80 ± 26	20 ± 5	64 ± 18
J-mTNF10	$TNFR1^{-/-}$	45 ± 20	15 ± 5	41 ± 16
	$TNFR1^{+/-}$	75 ± 24	20 ± 10	60 ± 22

NOTE: A series of consecutive cryosections were stained with mAbs against Mac-1, Mac-3, and Gr-1. Then, positive cells were counted under microscopy. Values are mean ± SD of positive cell numbers in one optical field with 200× magnification. At least three random sections were evaluated for each value.

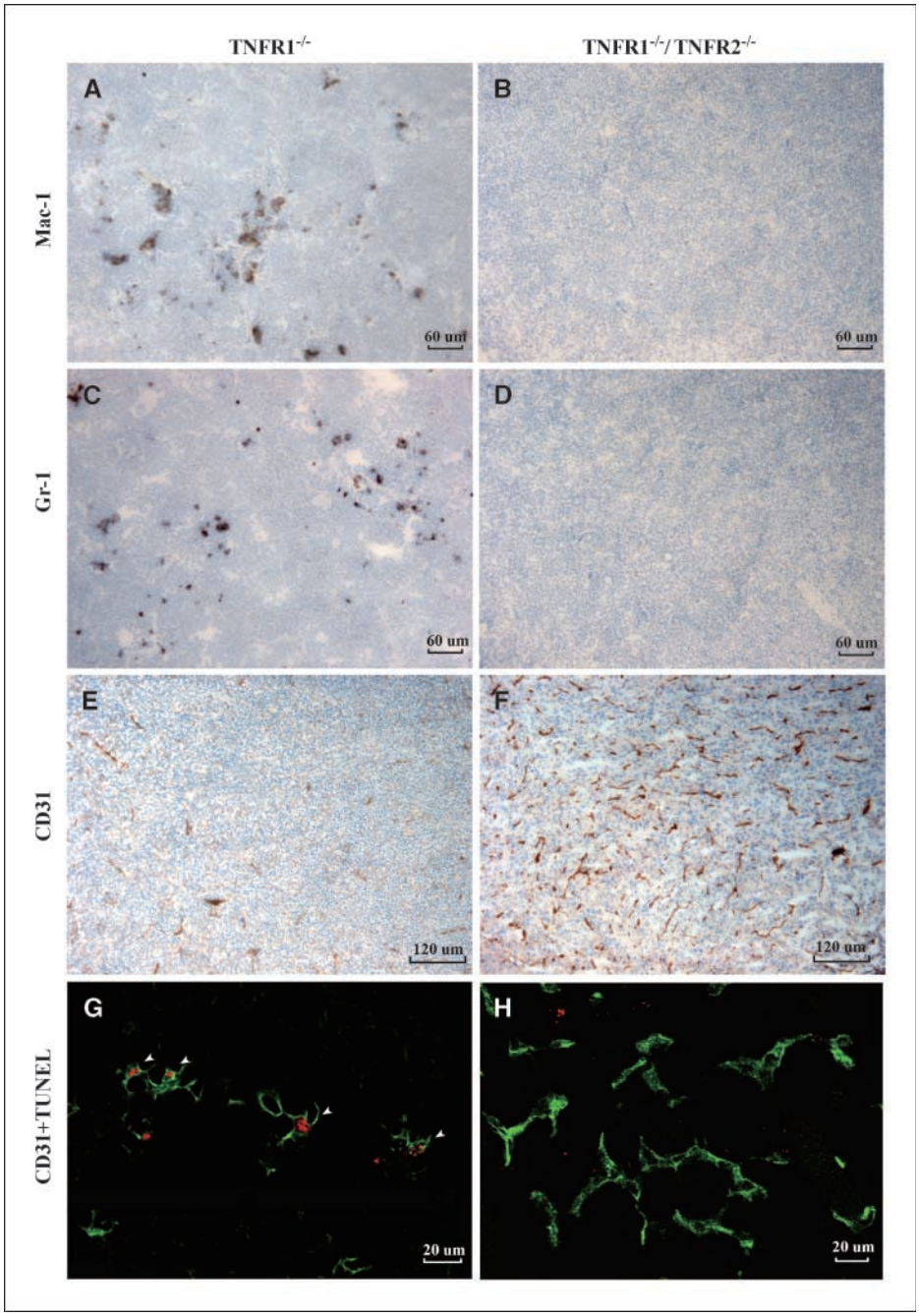

Figure 3. mTNF induces infiltration of innate immune cells and angiostasis in tumors from TNFR1$^{-/-}$ but not TNFR1$^{-/-}$/R2$^{-/-}$ mice. *A* to *F,* TNFR1$^{-/-}$ mice or TNFR1$^{-/-}$/R2$^{-/-}$ mice were injected s.c. with 5×10^6 of J-mTNF10 cells. Ten days after tumor cell challenge, tissue sections of the injection site were stained for Mac-1$^+$ (*A* and *B*), Gr-1$^+$ (*C* and *D*), and CD31$^+$ (*E* and *F*) cells as indicated. *G* and *H,* for confocal microscopy analysis, tissue sections were stained with Cy3-labeled anti-CD31 for blood vessel endothelial cells (*green*) and the VasoTACS *in situ* kit to detect apoptosis (*red*). *Arrows,* apoptotic endothelial cells in the tumor.

regressing tumors from TNFR1$^{-/-}$ mice, and blocking migration of Mac-1$^+$ cells completely abolished the mTNF-induced tumor suppression in normal syngeneic mice (2). To investigate how these cells contribute to the TNF-induced tumor suppression in this model, TNFR1-deficient macrophages were isolated and analyzed at first for the target cell killing upon specific stimulation *in vitro*. Figure 4*A* shows that in the presence of IFN-γ, peritoneal macrophages isolated from TNFR1$^{+/-}$ mice were activated by both hTNF and mTNF in a dose-dependent manner. However, TNFR1-deficient macrophages can be activated only by mTNF to kill the P815 targets.

Nitric oxide has been shown to be an effector molecule for the regulation of angiogenesis (23, 27, 36) and macrophage-mediated killing (37). Its production can be efficiently up-regulated by TNF and IFN-γ. Therefore, we assumed that the TNFR2-mediated antitumor effect might rely on the production of NO by macrophages. Thus, macrophages from casein-treated TNFR1$^{-/-}$ mice were stimulated with mTNF, hTNF, or LPS/IFN-γ as control, and cell culture supernatants were then detected for the NO production. Figure 4*B* shows that casein-activated macrophages spontaneously produced about 10 μmol/L of NO in serum-free medium, and the optimal stimulation of macrophages with LPS/IFN-γ led to a 4-fold increase of NO release. Whereas hTNF had no effect on TNFR1-deficient macrophages, stimulation of the same cells with mTNF led to a NO release of 36 μmol/L, reaching almost the level achieved with the optimal stimulation in this system. Therefore, TNFR2 can transmit signals for the activation of macrophages independently on TNFR1.

Blocking NO synthesis *in vivo* eliminates the antitumor effect of TNF. To analyze whether NO produced by macrophages contributes to the TNFR2-mediated tumor suppression *in vivo*, TNFR1$^{-/-}$ mice were injected with J-mTNF10 cells and treated either with PBS as control, or with L-NAME, a well-known inhibitor of NO synthesis. Figure 5*A* shows that although the growth of J-mTNF10 tumors was strongly suppressed in TNFR1$^{-/-}$ mice, upon application of L-NAME, these cells grew progressively in seven out of seven mice. At day 12 after tumor cell challenge, the mean tumor size was 50 $\pm$ 20 mm^3 for the control group, and the blockage of NO synthesis led to an increase of tumor size to 600 $\pm$ 150 mm^3. To exclude other nonspecific effects of L-NAME on the growth of J-mTNF10 cells, TNFR1$^{-/-}$/R2$^{-/-}$ mice were also injected with the same tumor cells. As shown in Fig. 5*B*, L-NAME hardly influenced the growth of J-mTNF10 tumors in TNFR1$^{-/-}$/R2$^{-/-}$ mice. Thus, production of NO is necessary for TNF-induced, TNFR2-mediated tumor suppression.

The effect of NO synthesis blocker on TNFR2-mediated antitumor effect is time dependent. To further investigate how NO acts *in vivo*, J-mTNF10 tumor-bearing mice were treated with

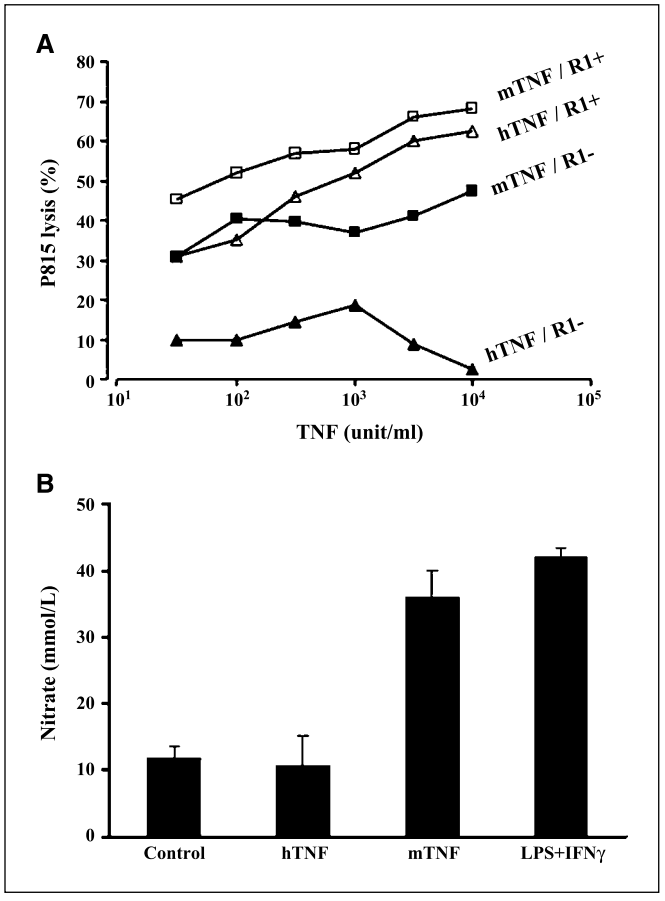

Figure 4. TNF activates TNFR1-deficient macrophages for target cell killing and NO production. *A,* TNFR2-mediated P815 cell lysis. The casein-activated peritoneal macrophages from TNFR1$^{-/-}$ (*closed*) or TNFR1$^{+/-}$ mice (*open*) were stimulated with increasing doses of mTNF (*squares*) or hTNF (*triangles*). Percentages of the specific lysis of P815 cells were shown. Three to five mice per group were analyzed in two experiments with similar results. *B,* TNFR2-mediated NO production. The casein-activated peritoneal macrophages from TNFR1$^{-/-}$ mice were not stimulated as control or stimulated for 24 h as indicated. The nitrate concentrations in millimoles per liter in cell culture supernatants were determined with a nitrate/nitrite fluorometric assay kit.

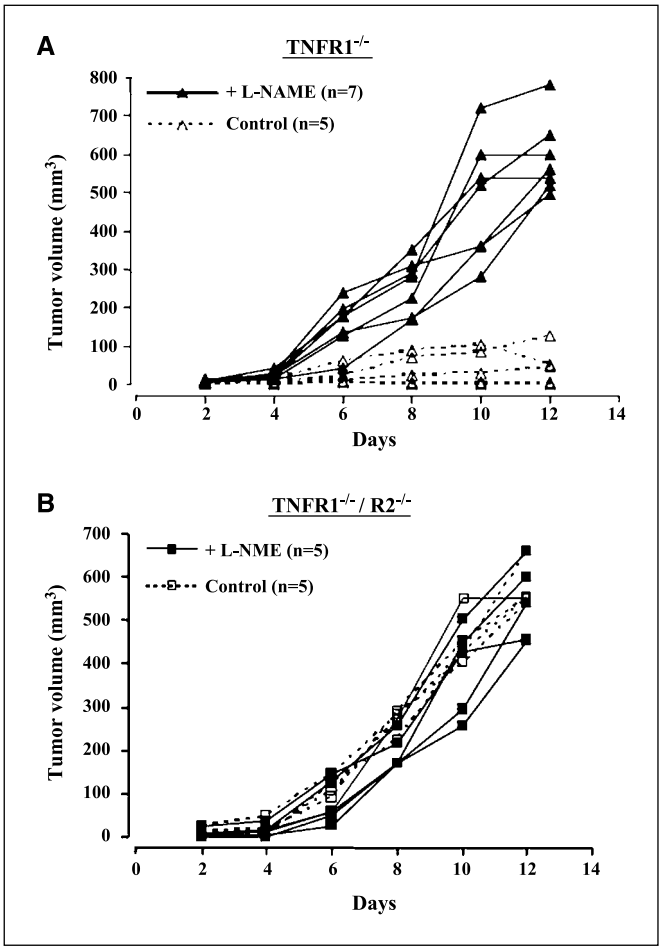

Figure 5. L-NAME eliminates the antitumor effect of mTNF in TNFR1$^{-/-}$ mice. About 5 $\times$ 10^6 J-mTNF10 cells were injected s.c. into (*A*) TNFR1$^{-/-}$ mice or (*B*) TNFR1$^{-/-}$/R2$^{-/-}$ mice. Beginning on day 2, after tumor cell challenge, mice were s.c. injected daily with 50 mg/kg of L-NAME (*closed*) or PBS only daily (*open*). Tumor growth was followed. Each line represents the tumor growth of a single mouse. Numbers of mice analyzed in each group were indicated.

L-NAME starting from different time points: day 0, day 3, and day 9. Mice treated with PBS alone were used as control. As shown in Fig. 6, if the L-NAME treatment started on the same day of or 3 days after tumor cell challenge, there is no significant difference in growth kinetics of J-mTNF10 cells in TNFR1$^{-/-}$ mice and TNFR1$^{-/-}$/R2$^{-/-}$ mice. However, if the mice were treated 9 days later, the effect of L-NAME could not be observed anymore. The fact that L-NAME acted only during the first few days of tumor growth strongly points to the involvement of NO in the inhibition of tumor-induced angiogenesis. However, the possibility that NO directly acts on tumor cells currently cannot be excluded.

TNFR2-mediated angiostasis is abolished by blocking NO synthesis. To further address the question whether TNF-induced NO influences tumor blood supply, TNFR1$^{-/-}$ mice were s.c. injected with J-mTNF10 cells, left untreated, or treated with L-NAME. Ten days later, tumors were isolated and analyzed by a series of immunohistology. As shown in Fig. 7*A*, blocking the production of NO with L-NAME apparently diminished the TNF-induced angiostasis in TNFR1$^{-/-}$ mice. Although control tumors were flat and pale in color, tumors from the L-NAME–treated mice were round and full of blood vessels. Staining with anti-CD31 mAb showed that there were small, truncated blood vessels or

single endothelial cells dispersed irregularly in the control tumor (Fig. 7*B*). However, treatment of mice with L-NAME led to a clear recovery of blood vessels in the center of the tumor (Fig. 7*C*). The confocal microscopic analysis revealed that L-NAME treatment significantly reduced the apoptosis of endothelial cells (data not shown). Together, our results show that TNFR2 independently mediates TNF-induced angiostasis in the tumor, possibly via the induction of endothelial cell apoptosis in the tumor.

Discussion

In the present study, we showed that expression of TNFR2 alone on host non-T/B cells was sufficient to arrest the growth of mTNF-secreting tumors. We showed for the first time that NO was indispensable for this process, possibly by induction of endothelial cell apoptosis and inhibition of tumor-induced angiogenesis.

There are at least two reasons for an extensive study on the role of TNFR2 during tumor regression. First, the expression pattern of TNFR2 is quite different from that of TNFR1. In contrast to the low, constant, and universal expression of TNFR1 under normal physiologic conditions, the expression of TNFR2 is inducible and restricted to hematopoietic cells and endothelial cells (12, 13). This makes it possible for the activation of TNFR2 alone to be sufficient for the effective tumor suppression, but without significant side effects, because TNFR1 is considered as the major responder for the TNF-induced toxicity (33). Second, although the binding affinity of TNFR2 to TNF is much higher than that of TNFR1, the significance of TNFR2 may be hidden in the presence of TNFR1 due, for example, to the process of ligand passing. Therefore, whether TNFR2 can work independently is an important question to explore. In our model, we took advantage of TNFR single or double knock-out mice to exclude the influence of TNFR1. Indeed, we found that TNFR2 has an independent role for mediating the TNF-induced antitumor effect. This is in accordance with the finding that TNFR2-specific TNF mutant expressed by an adenovirus vector induced an antitumor response with reduced toxicity in mice bearing established PyMidT tumors (21).

The cells on which TNFR2 has to be expressed for tumor growth inhibition may not include T and B lymphocytes because mTNF could also suppress tumor growth in TNFR1-deficient SCID mice (Fig. 2). The fact that blocking NO synthesis with L-NAME could eliminate the effect of mTNF completely suggested that TNF acts indirectly through NO on endothelial cells (Fig. 7). In contrast, macrophages should be the crucial effector cells, which respond to TNF via TNFR2 expressed on their cell surface. Histologic analysis showed that large amounts of macrophages infiltrated in regressing tumors in TNFR1-deficient mice (Fig. 3 and Table 1) and inhibition of $Mac1^+$ cell migration abolished the tumor-suppressive effect of TNF (2). Furthermore, both TNFR1-competent and TNFR1-deficient macrophages could be efficiently activated and secrete high levels of NO upon mTNF stimulation *in vitro* (Fig. 4).

We showed here that NO was crucial for the TNF-induced, TNFR2-mediated antitumor effect. Treatment of $TNFR1^{-/-}$ mice with NO blocker eliminated the antitumor ability of TNF (Fig. 5*A*). TNF has already been shown to induce NO production by the up-regulation of *iNOS* gene through NF-κB (23–26). Cooperative signaling through TNFR1 and TNFR2 in NO induction was supposed by experiments using specific antibodies for each receptor (38). However, the fact that TNFR2 alone could activate NF-κB (39, 40) indicates that it might also induce NO production independently. Our results supported this idea by showing that expression of TNFR2 alone on macrophages was sufficient for the TNF-induced NO production.

Although the direct effect of NO on tumor cells has to be excluded (41), our results strongly suggest that NO acts on tumor blood vessels. The NO-mediated apoptosis of endothelial cells is possibly the mechanism underlying the TNF-induced TNFR2-mediated tumor regression. The effect of NO blocker is time

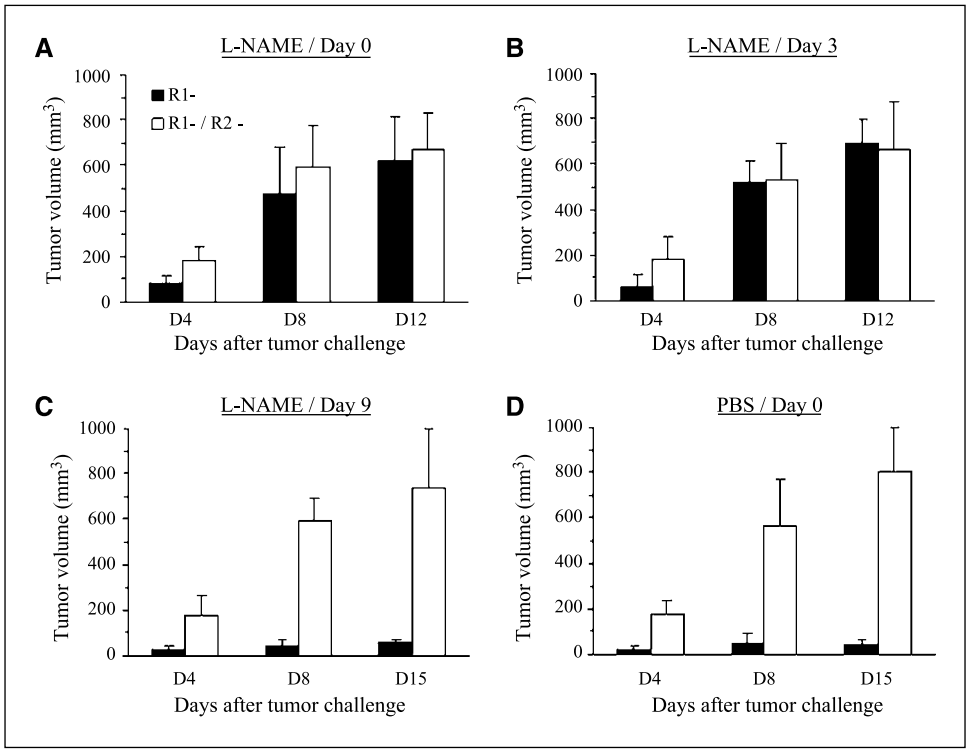

Figure 6. The effect of L-NAME is time dependent. Groups of $TNFR1^{-/-}$ mice (*black columns*) and $TNFR1^{-/-}/R2^{-/-}$ mice (*white columns*) were s.c. injected with 5×10^6 J-mTNF10 cells. Before (*A*), 3 d (*B*), and 9 d (*C*) after tumor cell challenge, mice were s.c. injected with 50 mg/kg of L-NAME or PBS only daily as control (*D*). Tumor growth was followed. *Columns,* mean tumor volumes at days 4, 8, and 12 or 15; *bars,* SD.

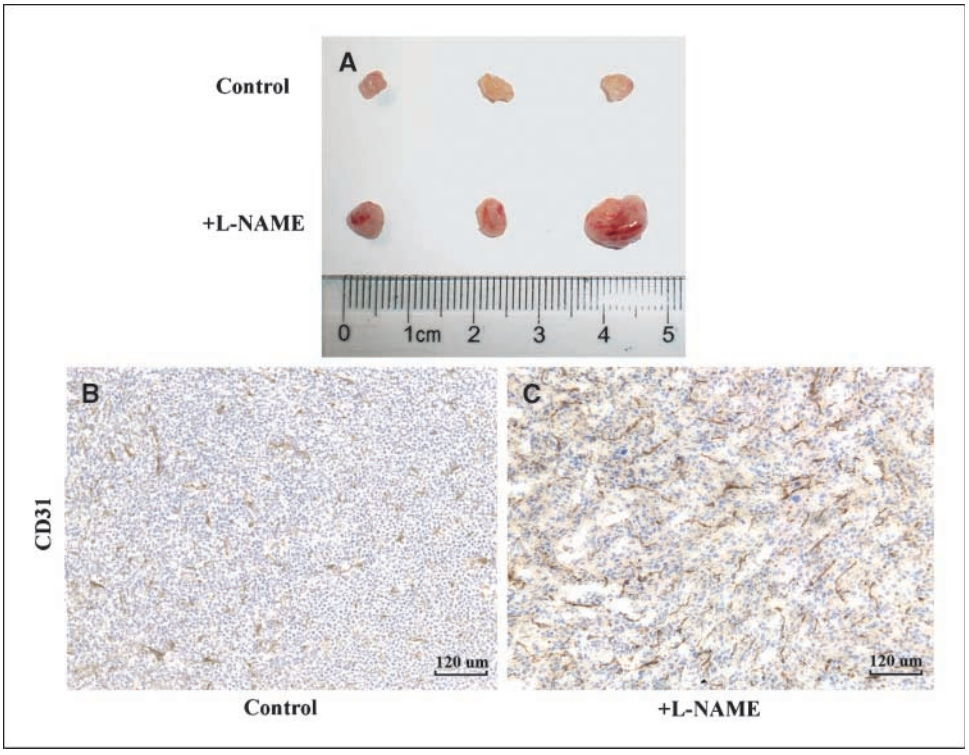

Figure 7. TNF-induced, TNFR2-mediated angiostasis is reverted by blocking NO synthesis. *A,* TNFR1$^{-/-}$ mice were s.c. injected with 5×10^6 J-mTNF10 cells and treated with L-NAME or PBS from day 2 as described above. Ten days after challenge, three pairs of representative tumors were isolated and photographed with a digital camera. A ruler below is used as a scale. Tumors from L-NAME–treated TNFR1$^{-/-}$ mice were bigger and better vascularized. *B* and *C,* tumors isolated as described above were stained with anti-CD31 to detect tumor blood vessels. Three to five mice per group were analyzed, and the representative staining result is shown.

dependent, which worked only if the L-NAME treatment started on days 0 and 3, but not on day 9 upon tumor transplantation (Fig. 6). This suggests that NO may inhibit the process of tumor-induced angiogenesis. In addition, inhibition of angiogenesis has been shown to be a critical effector arm of the antitumor responses mediated by immune cells and cytokines (42, 43). Indeed, apoptosis of endothelial cells was observed by confocal microscopy when high concentrations of TNF were present at the tumor site in TNFR1$^{-/-}$ mice (Fig. 3*E*). Moreover, the blockage of NO synthesis restored the process of angiogenesis (Fig. 7) and led to tumor outgrowth (Fig. 5). This is in correlation with the previously reported antiangiogenic effect of NO (27, 36, 44). Recent studies indicate that high concentrations of NO inhibited phosphorylation of protein kinase C, extracellular signal-regulated kinase, and JUN and the binding activity of AP-1, leading to the NO-induced antiangiogenesis (45, 46). On the other hand, it has also been shown that NO can promote tumor-associated angiogenesis (23, 27, 28, 36, 44). This discrepancy between the pro- and antiangiogenic effects of NO might be explained by differences in experimental models and in local concentration and duration of NO exposure in the tumor. It seems that low concentrations of NO may increase, high concentrations of NO may inhibit the process of angiogenesis (23, 27, 28).

In summary, TNFR2 expressed on innate immune cells, such as macrophages, is sufficient to mediate the antitumor effect of TNF, possibly by the activation of macrophages and induction of NO, which, in turn, inhibit the tumor-induced angiogenesis. Our findings may help to understand the mechanism underlying the TNF-induced antitumor responses and the biological functions of TNFR2. This will shed new light on the clinical application of TNF or TNFR analogues for treatment of related human diseases.

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