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We have previously shown that tumor necrosis factor (TNF) acts via its two receptors TNFR1 and TNFR2 to elicit distinct signaling pathways in vascular endothelial cells (ECs). Here we used a femoral artery ligation model to demonstrate that TNFR1-knockout (KO) mice had enhanced, whereas TNFR2-KO had reduced, capacity in clinical recovery, limb perfusion, and ischemic reserve capacity compared with the wild-type mice. Consistently, ischemia-initiated collateral growth (arteriogenesis) in the upper limb and capillary formation and vessel maturation (angiogenesis) were enhanced in TNFR1-KO but were reduced in TNFR2-KO mice. Furthermore, our results suggest that vascular proliferation, but not infiltration of macrophages and lymphocytes, accounted for the phenotypic differences between the TNFR1-KO and TNFR2-KO mice. In wild-type animals TNF2 protein in vascular endothelium was highly up-regulated in response to ischemia, leading to increased TNFR2-specific signaling as determined by the formation TNFR2-TRAF2 complex and activation of TNFR2-specific kinase Bmx/Etk. In isolated murine ECs, activation of TNF2 induced nuclear factor-κB-dependent reporter gene expression, EC survival, and migration. In contrast, activation of TNF1 caused inhibition of EC migration and EC apoptosis. These data demonstrate that TNF1 and TNF2 play differential roles in ischemia-mediated arteriogenesis and angiogenesis, partly because of their opposite effects on EC survival and migration. (Am J Pathol 2006, 169:1886–1898; DOI: 10.2353/ajpath.2006.060603)

Endothelial cells (ECs) are among the principal physiological targets of the proinflammatory cytokine tumor necrosis factor (TNF)-α. In ECs, as in other cell types, TNF elicits a broad spectrum of biological effects including proliferation, differentiation, and apoptosis. The nature of TNF effects depends on TNF concentration and the type and growth state of the target cells. Differences in TNF-induced responses are attributable, in part, to the presence of two distinct TNF-specific plasma membrane-localized receptors, type I 55-kd TNFR (TNFR1) and type II 75-kd TNFR (TNFR2).6 TNFR1 is expressed ubiquitously, whereas TNFR2 expression is tightly regulated and found predominantly on ECs and hematopoietic cells. Numerous studies in various cell types, particularly in T cells, suggest that TNFR1 primarily mediates TNF-induced inflammation and cell death, whereas TNFR2 serves to enhance TNFR1-induced cell death or to promote cell activation, migration, growth, or proliferation in a cell-type-specific manner.8–10 Studies with TNFR1- and TNFR2-selective agonists R32W-TNF and D143N-A145R-TNF, respectively, have shown that signaling events elicited by TNF in ECs, as in other cell types, are primarily dependent on the interaction of TNF with TNFR1.11,12 The role of TNFR2 in TNF signaling in ECs is not clear. Studies using receptor-specific neutralizing antibodies have shown that TNFR2 contributes to effects of lower concentrations of TNF, possibly serving

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to capture and pass TNF to the less abundant signaling (TNFR1) receptor. An alternative view is that TNFR2 primarily responds to TNF expressed as an integral membrane protein on the surface of activated macrophages, whereas TNFR1 primarily responds to soluble TNF, which is derived from membrane TNF by the action of a metalloproteinase called TNF-1-converting enzyme (TACE).

TNFR1 and TNFR2, among members of TNF receptor family, share a similar architecture with characteristic cysteine-rich motifs. Unlike the extracellular domains, the primary amino acid sequences of the cytoplasmic domains of TNFR1 and TNFR2 are unrelated. It is believed that the two receptors initiate distinct signal transduction pathways by interacting with different signaling proteins. A current model postulates that TNF binding triggers trimerization of TNFR1 and TNFR2, which then recruit adaptor proteins and signaling molecules by their intracellular domains to form a receptor-signaling complex.13 Many proteins have been shown to be recruited by TNFR1 including TRADD,14,15 TRADD functions as a platform adaptor that recruits TRAF2, RIP, and FADD to form a TNFR1-signaling complex and activate several distinct signaling cascades including activation of the MAP kinase, nuclear factor (NF)-κB, and caspase-dependent apoptotic pathways.16–20 In contrast, less is known about the proteins recruited to TNFR2 and downstream signaling.21 Like TNFR1, TNFR2 can also recruit TRAF2 and use the two cellular inhibitors of apoptotic proteins (cIAP1 and cIAP2).16,22,23 However, the role of these factors in TNFR2-specific signaling has not been defined.

We and others have dissected distinct TNFR1- and TNFR2-mediated TNF signaling pathways in ECs. Besides TRAF2-RIP-IKK-dependent NF-κB-dependent antiapoptotic pathways, TNF via TNFR1 also induces two distinct apoptotic pathways (TNFR1-TRADD-FADD-caspase-8 and TRAF2-AIP1-ASK1-JNK/p38 kinase cascade).24 On the other hand, we have recently identified Bmx/Etk (bone marrow tyrosine kinase in chromosome X)/Etk (endothelial/epithelial tyrosine kinase) as a TNFR2-responsive tyrosine kinase.25 TNFR2, but not TNFR1, specifically associates with and activates Bmx/Etk, which has been implicated in cell migration and proliferation/survival. We further show that Bmx-dependent transactivation of vascular endothelial growth factor (VEGF) receptor 2 is critical for TNF/TNFR2-induced EC migration and tube formation.26 These in vitro findings have recently been confirmed in both organ culture system and in human kidney specimens11 as well as in mouse ischemic hindlimb angiogenesis models.27 Thus, TNFR1-dependent apoptosis and TNFR2-dependent EC proliferation/migration represent two distinct pathways differentially activated by TNFR1 and TNFR2, respectively (see Figure 1a).

The in vivo function of TNFR2 signaling in angiogenesis has not been investigated. In the present study, we used mice genetically deficient in TNFR1 or TNFR2 to determine the in vivo functions of TNFR1 and TNFR2 in arteriogenesis and angiogenesis. Our data demonstrated that TNFR1-knockout (KO) mice had enhanced, whereas TNFR2-KO had reduced ischemia-initiated angiogenesis and arteriogenesis compared with the wild-type (WT) mice in a femoral artery ligation model, a commonly used in vivo arteriogenesis/angiogenesis model. Mechanistic studies suggested that although TNFR1 signaling induces EC apoptosis, TNFR2 activates Bmx/Etk-dependent EC migratory and TRAF2-dependent survival pathways to mediate ischemia-induced arteriogenesis/angiogenesis.

Materials and Methods

TNFR1-KO and TNFR2-KO Mice

WT C57BL/6, TNFR1-KO mice (strain name B6.129-tnfrs-fla), and TNFR2-KO (strain name B6.129-tnfrsflb) were purchased from Jackson Laboratory (Bar Harbor, ME). TNFR1-KO and TNFR2-KO mice were bred with C57BL/6 mice for at least six generations before experiments. Mice were confirmed by genotyping with specific primers suggested by the vendor. All animal studies were approved by the institutional animal care and use committee of Yale University.

Mouse Hindlimb Ischemic Model

Male C57BL/6, TNFR1-KO, and TNFR2-KO mice, 8 to 12 weeks old, were used for all experiments. Mouse ischemic hindlimb model was performed as described previously.28 In brief, after anesthesia (79.5 mg/kg ketamine and 9.1 mg/kg xylazine), the left femoral artery was exposed under a dissection microscope. The proximal portion of femoral artery and the distal portion of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arterectomy was performed. Sham operation is without femoral artery ligation but skin incision.

Blood Flow Measurement and Clinical Score

Blood flow was measured by PeriFlux system with the Laser Doppler Perfusion Module unit (Perimed, Inc., North Royalton, OH). Deep measurement probe was placed directly on gastrocnemius muscle to ensure a deep muscle flow measurement. Ischemic and nonischemic limb perfusion was measured before and after surgery. The final blood flow values were expressed as the ratio of ischemic to nonischemic hind limb perfusion. To evaluate more precisely the mobility of mice after limb ischemia, we designed a scoring system in which 0 = normal; 1 = pale foot or gait abnormalities; 2 = gangrenous tissue in less than half the foot without lower limb necrosis; 3 = gangrenous tissue in less than half the foot with lower limb necrosis; 4 = gangrenous tissue in greater than half the foot; 5 = loss of half lower limb. Clinical outcome of all mice was observed and recorded at the same time points of blood flow measurement.

Micro-Computed Tomography (Micro-CT) Analysis

Four weeks after femoral ligation, mice were anesthetized and perfused with 20 ml of 37°C phosphate-buffered
saline (PBS) plus 10 U/ml heparin at a flow rate of 10 to 15 ml/minute through the left ventricle. To analyze the formation of new vasculature after onset of ischemia we used a silicone-based contrast formulation (Microfil; Flow Tech Inc., Bloomfield, CT) to optimize visualization of the vascular structures in mice. All animals have been imaged using the micro-CT system (O-FLEX; Gamma Medical Ideas, Northridge, CA) implemented in the cone-beam geometry and equipped with a high-resolution GOS/CMOS detector achieving resolution of less than 100 μm. The micro-CT detector was positioned at the distance of 64 mm from the center of rotation. High-resolution CT scans were performed using 512 projections, a 512 x 512 matrix, and 75 kV/250 μA penetration energy. Scans with these parameters provide more than sufficient delineation of internal organs and the vasculature in proximal and distal hindlimb. The contrast CT projection images were reconstructed using commercial software (Cobra; Exxim Computing Corp., Pleasanton, CA) using a cone-beam optimized algorithm. The reconstructed images were visualized using three-dimensional-rendering with Amira software (Visual Concepts GmbH, Berlin, Germany). For qualitative analysis of vasculature, the bone was manually wiped and vasculature volume was quantified by image analyses using Image J (National Institutes of Health, Bethesda, MD).

**Histology and Immunohistochemistry**

Mice were sacrificed at 4 weeks after surgery and muscles of the lower limbs were harvested, methanol-fixed, and paraffin-embedded. Tissue sections (5 μm thick) were stained using anti-PECAM-1 antibody (Pharmingen, San Diego, CA) and anti-smooth muscle α-actin (SMA) antibody (DAKO, Carpinteria, CA). Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed peroxidase substrate kit; Vector Laboratories, 

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Critical roles of TNFR2 in the recovery of hindlimb perfusion after injury. 

- **a:** A schematic diagram for the distinct signaling pathways mediated by TNFR1 and TNFR2. TNF through TNFR1 activates FADD-caspase-8-dependent (not shown in the figure) and ASK1/JNK-dependent apoptotic pathways. TNF activates Bmx/Etk survival and angiogenic signaling through TNFR2. 
- **b:** Ischemic hindlimb model was performed. Blood flow of ischemic and nonischemic limb were measured on gastrocnemius muscle at 30 minutes, 3 days, 2 weeks, and 4 weeks after surgery by using the PeriFlux system with the laser Doppler perfusion measurement (LDPM) unit. Tissues were harvested on day 28 for immunohistochemistry. 
- **c** and **d:** TNFR2-KO mice showed severe phenotype in clinical score (c) leading to necrosis of limb (d). 
- **e:** TNFR2-KO mice show reduced whereas TNFR1-KO mice show augmented recovery of limb perfusion compared with normal C57BL/6 mice (ratio of perfusion unit from nonischemia (left) to ischemia (right) are shown). Number for each strain is shown in parentheses. Data are mean ± SEM, *P < 0.05.
Burlingame, CA). Pictures from four random areas of each section and five sections per mouse were taken using a Kodak (Eastman-Kodak, Rochester, NY) digital camera mounted on a light microscope (×40 objective). Capillary density and SMA positivity were quantified by measuring the percentage of PECAM-positive area or SMA-positive area (in same area of adjacent sections) out of total area using Matlab software (The Math Works, Inc., Natick, MA).

Gene Expression in Ischemic Muscle

Total RNA of lower limb muscles was isolated by using phenol/chloroform and isolated using the RNeasy kit with DNase I digestion (Qiagen, Valencia, CA). Reverse transcription (RT) was done by standard procedure (Super Script first-strand synthesis system; Qiagen) using 1 μg of total RNA. Quantitative real-time polymerase chain reaction (PCR) was performed by using iQ SYBR Green Supermix on iCycler real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Specific primers for mouse TNFR1, TNFR2, TRAF2, Bmx, and 18S ribosomal RNA as an internal control were used as follows: 18S RNA forward primer: 5'-TTCCGATAACGAAACGACTCT-3', reverse primer: 5'-TGGCTGAAACGCACTTGTTC-3'; TNFR1 forward primer: 5'-GGGCACCTTTACGGCTTCC-3', reverse primer: 5'-GGTTCTCCTTACAGGCTTCA-3'; TNFR2 forward primer: 5'-CAGGTTGTCCTGACACCCTA-3', reverse primer: 5'-GCACAGCAGCTGACGCT-3'; TRAF2 forward primer: 5'-TTCGGCCTTTCCAGATAACG-3', reverse primer: 5'-CCTTCCAAGTGCATCCATCAT-3'; BMX forward primer: 5'-TACCTGGCTGAAAACT-3', reverse primer: 5'-CCACATCATATTGCCCTTTCCA-3'. The relative amount of mRNA in mouse lower limb muscle 3 days and 2 weeks after ischemia was quantified.

Cell Culture and Cytokines

Human umbilical vein ECs were from the Boyer Center for Molecular Medicine Endothelial Cell Facility (Yale University). Bovine aortic ECs were purchased from Clonetics (San Diego, CA) and were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Mouse EC isolation from muscle and lung tissues was performed as we described, followed by immunoselection and immortalization modified from the protocol described by Lim and colleagues. For immunoselection, 10-μl beads (per T-75 of mouse lung cells) were washed with 1 ml of buffer A (PBS + 2% fetal bovine serum) for three times and resuspended in 100 μl of buffer A. Ten μl (10 μg) of anti-mouse ICAM-2 or 10 μl (10 μg) of PECAM-1 were added and rocked at 4°C for 2 hours. Beads were washed for three times and resuspended in 160 μl of buffer A. Confluent mouse lung cells cultured in a T-75 flask were placed at 4°C for 5 minutes and incubated with the beads at 4°C for 1 hour. Cells were then washed with warm PBS and treated with 3 ml of warm trypsin/ethylenediaminetetraacetic acid. When cells were detached, 7 ml of growth media were added. An empty 15-ml tube in was placed on the magnetic holder, and the cell suspension (∼10 ml) was added slowly by placing the pipette on the wall of the tube so that the cells pass through the magnetic field. Cells were incubated for 5 minutes, and the media were carefully aspirated. The 15-ml tube was removed from the magnetic holder, and the beads/cells were resuspended in 10 ml of media. The selected cells were plated on 0.2% gelatin-coated flasks and cultured for 3 to 7 days. When the cells were confluent, another round of immunoselection was repeated. Human recombinant TNF and VEGF were from R&D Systems Inc. (Minneapolis, MN) and used at 10 ng/ml.

Figure 2. Critical roles of TNFR2 in ischemia-induced arteriogenesis. a: Four weeks after femoral ligation, mice were anesthetized and subjected to microfil perfusion. Arteriogenesis was determined by micro-CT analyses as described in Materials and Methods. b: Vascular growth was quantitated as ratio of vascular density (left/right). n = 10 for each strain. *P < 0.05.
Indirect Immunofluorescence Confocal Microscopy

Fixation, permeabilization, and staining of cultured ECs were performed as described previously.24 Alexa Fluor 488 (green)- or Alexa Fluor 594 (red)-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope (La Jolla, CA) and acquired images were transferred to Photoshop 6.0 (Adobe Systems, San Jose, CA) to generate the final figures.

EC Migration Assay and Image Analysis

EC migration was modified from the method as described previously.25 In brief, infected ECs were cultured in 0.5% fetal bovine serum for overnight and subjected to wound injury with a yellow tip. Cells were plated with fresh media and were further cultured for 12 to 16 hours. The EC migration in culture was determined by measuring wound areas in cell monolayers. Three different images from each well along the wound were captured by a digital camera under a microscope (×4). A hemocytometer (1 mm²/grid) was used as a standard. Wound area (mm²) was measured and analyzed by NIH Image 1.60. Statistical analyses were performed with StatView 4.0 package (Abacus Concepts, Berkeley, CA). Data are presented as means (±SD). Differences were analyzed by unpaired two-tailed Student's t-test. Values of \( P < 0.05 \) were taken as significant.

Statistical Analysis

All data are expressed as means ± SEM. Statistical differences were measured by the nonparametric Mann-Whitney test. A value of \( P < 0.05 \) was considered as statistically significant.

Results

TNFR1-KO Augments, Whereas TNFR2-KO Blunts, Perfusion Recovery in the Ischemic Hindlimbs

C57BL/6, TNFR1-KO, and TNFR2-KO male mice were subjected to femoral artery ligation and various analyses at different time points as diagramed in Figure 1b. On days 14 to 28 after surgery, TNFR2-KO mice showed various degrees of clinical phenotype compared with C57BL/6 and TNFR1-KO mice (Figure 1c), based on the clinical scoring system that we described recently.26 Four of 10 R2-KO mice had severe necrosis of the feet (Figure 1d). To precisely determine functional defects in TNFR2-KO mice, blood flow was measured and ischemic and nonischemic limb perfusion were measured before and after surgery and 3 days, 2 weeks, and 4 weeks after surgery. Before surgery, the ratio of left leg to right leg gastrocnemius blood flow was 1. After surgery, blood flow dropped by 80% and returned to a ratio of 1 throughout the 4 weeks in C57BL/6 mice. TNFR1-KO mice showed augmented recovery of hindlimb perfusion and flow returned to normal in 2 weeks. In contrast, there was a statistically significant impairment in gastrocnemius blood flow in TNFR2-KO mice, suggesting that the impairment in blood flow in TNFR2-KO is associated with a marked increase in clinical severity (Figure 1e).

Postischemic Arteriogenesis Is Enhanced in TNFR1-KO Mice but Is Impaired in TNFR2-KO Mice

Enhanced clinical recovery and limb perfusion could be attributable to increased arteriogenesis from existing vessels of the upper limb or and increased neovascularization/vessel maturation in the lower limb. To better quantify vascular density in the upper limb (thigh muscle) and lower limb (gastrocnemius) of mice, we performed three-dimensional micro-CT analyses. The vasculature was volume rendered using the three-dimensional semiautomated image analyses described in Materials and Methods. Both vasculature and the bone are displayed on three-dimensional volume (Figure 2a). The bone was manually wiped, and vasculature was quantitated by image analyses (see Materials and Methods). Vascular density was increased in TNFR1-KO but was significantly reduced in TNFR2-KO mice compared with WT mice (Figure 2b). These data suggest that TNFR1 functions as a negative regulator, whereas TNFR2 acts positively, in ischemia-mediated arteriogenesis and vascular growth.

Ischemia-Induced Angiogenesis and Vessel Maturation Are Enhanced in TNFR1-KO Mice but Are Impaired in TNFR2-KO Mice

Stable angiogenesis is believed to occur contemporaneously with pericyte recruitment (vessel maturation) mediated by platelet-derived growth factor and/or angiopoietin. We next characterized the ischemia-induced angiogenesis and vessel maturation in the lower limb by immunostaining with anti-CD31 or anti-smooth muscle α-actin (SMA) antibody. We first characterized the ischemia-responsive regions in the lower limb and found that a triangle area of gastrocnemius muscle was most re-
responsive to ischemia when measured for increased capillary formation, macrophage infiltration, and myocyte proliferation. After 4 weeks of ischemia, both CD31-positive capillaries surrounding the skeletal muscle fibers and SMA-positive capillaries were significantly increased in C57BL/6 mice (Figure 3a). We further quantified isch-

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emia-induced angiogenesis and vessel maturation by measuring capillary number/mm² (Figure 3c), ratio of capillary/fiber (Figure 3d), SMA-positive capillaries/mm² with quantification of the number of capillaries/mm² (Figure 3e), and percentage of SMA-positive capillaries (Figure 3f). TNFR2-KO muscle tissues appeared to be less sensitive to ischemic responses. Importantly, CD31-positive capillaries surrounding the skeletal muscle fibers (neo-vascularization) and SMA-positive SMCs (pericyte recruitment) were significantly increased in TNFR1-KO mice but reduced in TNFR2-KO mice compared with C57BL/6 secondary to ischemia (Figure 3b with quantification in Figure 3, c–f).

Figure 5. Ischemia induces TNFR2-specific signaling. a: C57BL/6 (n = 5) mice were subjected to hindlimb ischemia, and hindlimbs were harvested on days 0, 3, 14, and 28 after surgery as indicated. Gene expression of TNFR1, TNFR2, TRAF2, and Bmx/Etk were determined by qRT-PCR. 18S rRNA was used for normalization. Fold induction (left/right) is shown. b and c: Day 3 samples were used for protein analyses. TNFR1, TNFR2, TRAF2, and Bmx/Etk, as well as Bmx/Etk phosphorylation (pY40), were determined by Western blot with respective antibodies. β-Tubulin was used as a control. Relative levels of pY40 and Bmx expression are shown, with ischemic WT as 1.0. Similar results were obtained in an additional five mice from two independent experiments (total n = 6). d: TNFR2-TRAF2 complexes in the lower limb tissues were determined by immunoprecipitation with anti-TRAF2 or normal rabbit serum (NRS) followed by Western blot with anti-TNFR2 antibody. TRAF2 in the immunoprecipitates was determined by Western blot with anti-TRAF2 antibody. Similar results were obtained from two additional experiments. e: TNFR2 was induced in vascular endothelium. TNFR2 protein in the frozen sections of nonischemic and ischemic (day 3) lower limb were detected by immunohistochemistry with anti-TNFR2 antibody. Capillaries (CD31-positive) found to be TNFR2-positive or -negative are indicated by arrows and arrowhead, respectively.

Figure 4. Characterization of infiltrated immune cells, tissue necrosis/apoptosis, and cellular proliferation in TNFR1-KO and TNFR2-KO mice in response to ischemia. C57BL/6, TNFR1-KO, and TNFR2-KO mice were subjected to ischemia ligation, and tissues were harvested at indicated times. Tissue infiltrates, apoptosis, and cellular proliferation in gastrocnemius were determined by immunohistochemistry with respective markers. a–c: Recruitment of macrophages/lymphocytes in response to ischemia as determined by anti-F4/80 and anti-CD3 antibody, respectively. Representative images of nonischemic and ischemic hindlimbs in C57BL/6 on day 3 are shown in a in which macrophages and lymphocytes are indicated by arrows. F4/80- and CD3-positive cells were counted as number of infiltration/mm² muscle area in C57BL/6, TNFR1-KO, and TNFR2-KO mice on day 3 after ischemia (b, c). d–f: Cellular proliferation in nonischemic and ischemic tissues (day 28) was determined by PCNA staining. d: PCNA-positive capillaries (CD31-positive) and muscle fibers are indicated by arrows and arrowhead, respectively. e: Representative images of nonischemic and ischemic hindlimbs in C57BL/6, TNFR1-KO, and TNFR2-KO mice are shown. f: PCNA-positive cells were counted as number/mm² muscle area. Data from different mice groups are shown in graphics and n = 4 for each strain. *p < 0.05. g and h: Characterization of tissue apoptosis in TNFR1-KO and TNFR2-KO mice in response to ischemia. Tissues were harvested at day 0, 3, 7, and 28 after surgery, and apoptosis in gastrocnemius was determined by TUNEL assay (Roche). Apoptosis peaks at day 3, as shown in g. No apoptosis was detected in nonischemic tissues (not shown). f: TUNEL-positive cells were counted as number/mm² muscle area. Data from different mice groups are shown in graphics and n = 4 for each strain. *p < 0.05.

Ischemia-Induced Cellular Proliferation Is Enhanced in TNFR1-KO Mice but Is Impaired in TNFR2-KO Mice

To determine the molecular mechanism by which TNFR1 and TNFR2 differentially regulate ischemia-induced arteriogenesis/angiogenesis observed above, we examined the recruitment of inflammatory cells (primarily macrophages and lymphocytes) that have been shown to be critical for inflammatory angiogenesis.32,33 Infiltration of macrophages and lymphocytes to ischemic hindlimb was determined by immunostaining with anti-F4/80 and

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Figure 6. Roles of TNFR2 signaling in EC survival and migration in vitro. **a:** Schematic structure of TNFR2. The numbers refer to amino acid number indicating the boundary of the extracellular and intracellular domains. TRAF2- and Bmx/Etk-binding motifs, which are critical for TNFR2-mediated NF-κB activation/EC survival and EC migration, respectively, are indicated. **b:** Effects of TNFR2 on NF-κB reporter gene. NF-κB-dependent luciferase reporter gene (1 μg) and a constitutive expression vector for Renilla luciferase (0.5 μg) were cotransfected with a vector control (VC), a TNFR2 mutant, TNFR1, TRAF2, or Bmx/Etk (1 μg each) into TNFR2-null MECs as indicated. Reporter gene activities were measured. Data are presented from mean of duplicate samples from four independent experiments. **c** and **d:** Effects of TNFR2 on EC apoptosis. MECs were infected with retrovirus expressing GFP (VC) or a Myc-tagged TNFR2 mutant. EC apoptosis was determined by nuclear condensation by DAPI staining. The representative pictures for each construct are shown. Quantification of EC apoptosis is shown in **d.** Three images from each well were captured, and apoptotic and total nuclei were counted. Data presented are ratio of apoptotic nuclei (±SEM) from four independent experiments. *P < 0.05. **e** and **f:** Effects of TNFR2 on EC migration. MECs were infected with retrovirus expressing GFP (VC) or a TNFR2 mutant. EC migration was performed as described in Materials and Methods. EC migration in culture was determined by measuring wound areas in cell monolayers **(f)**, with quantification in **f.** Data presented are means (±SEM) of the two triplicates from two independent experiments. *P < 0.05.
anti-CD3 antibodies, respectively. No macrophages were detected in nonischemic limb. Ischemia induced a drastic increase in infiltration of macrophages, peaking at day 3 after surgery (~500 macrophages/mm²) (Figure 4a and quantification in Figure 4b) and declining to baseline by day 7 in C57BL/6 mice. Similar kinetics were observed for lymphocyte infiltration (Figure 4a and quantification in Figure 4c). We then determined infiltration of macrophage/lymphocytes in TNFR1-KO and TNFR2-KO mice, and results showed that both TNFR1-KO and TNFR2-KO mice showed significantly reduced macrophage infiltration compared with C57BL/6 mice (Figure 4, b and c, for quantification on day 3 after surgery). These data suggest that infiltration of cells to ischemic hindlimb in TNFR1-KO and TNFR2-KO mice did not account for the phenotypic differences between the two mice.

Previously, we have shown that TNFR1 mediates apoptosis/necrosis signals, whereas TNFR2 mediates cellular proliferation responses in human kidney organ culture models. To determine whether the ischemia-induced cellular proliferative/apoptotic responses contribute to the differences in the tissue repair between TNFR1-KO and TNFR2-KO mice, we measured cellular proliferation by proliferating cell nuclear antigen (PCNA) staining and tissue apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Cellular proliferation started at day 7 and was sustained until 4 weeks. Both capillaries and myocytes showed PCNA-positive staining (Figure 4d, arrowheads and arrows, respectively), consistent with the increased total numbers of capillaries and muscle fibers at 4 weeks of ischemia (Figure 3). Notably, nuclei of the newly generated myocytes in ischemic tissue were localized in the center of cells (Figure 4d, arrows). Importantly, both PCNA-positive ECs and myocytes were significantly increased in TNFR1-KO but were dramatically reduced in TNFR2-KO mice compared with WT mice (Figure 4e with quantification in Figure 4f). Kinetics studies suggested that apoptosis peaked at day 3 after surgery (data not shown), suggesting that ischemia-induced apoptosis is an early event in the adaptive response. More importantly, ischemia-induced tissue apoptosis was dramatically decreased in TNFR1-KO mice but was increased in TNFR2-KO mice compared with WT mice (Figure 4, g and h). These data suggest that TNFR1-mediated apoptosis and TNFR2-dependent proliferation are critical components in ischemia-mediated tissue remodeling.

**TNFR2 Signaling Complexes Are Highly Induced in Vascular Endothelium of Ischemic Hindlimbs**

The above results prompted us to reason that the intrinsic TNFR1 and TNFR2 signaling in ischemic hindlimb could play more important roles in ischemia-induced arteriogenesis/angiogenesis. To test our hypothesis, we examined expression of TNFR1 and TNFR2 signaling molecules in ischemic hindlimb. The TNFR2 gene was significantly induced by ischemia as determined by qRT-PCR. Likewise, TNFR2-specific kinase Bmx/Etk was also strongly induced (Figure 5a). In contrast, expression of TNFR1 and the adaptor protein TRAF2 were not significantly altered (not shown). As controls, TNFR2 mRNA was not detected in TNFR2-KO mice, and TNFR1 mRNA was not detected in TNFR1-KO mice (data not shown). We next examined TNFR2 protein and TNFR2 signaling complexes by Western blot with respective antibodies. TNFR2 was drastically up-regulated on day 3 after ischemia compared with nonischemic muscle in C57BL/6 mice. In contrast, TNFR1 was only slightly increased, whereas TRAF2 was not altered in response to ischemia (Figure 5b). Likewise, expression and activation of Bmx/Etk, as determined by Western blot, were greatly induced in response to ischemia. Furthermore, Bmx/Etk expression and activation (phosphorylation of pY40) were significantly reduced in TNFR2-KO mice (Figure 5c). Interestingly, TNFR2 expression and Bmx/Etk activation were enhanced in TNFR1-KO mice compared with C57BL/6 mice (Figure 5, b and c). We then determined the formation of TNFR2-TRAF2 complex, which is critical for TNFR2-induced NF-κB activation and cell survival. Although TRAF2 protein was not up-regulated, TNFR2-TRAF2 complex was dramatically increased as determined by immunoprecipitation assays (Figure 5d).

To determine which cell type expressed TNFR2, we determined TNFR2 in ischemic limb tissue by immunohistochemistry with anti-TNFR2 antibody. TNFR2 was highly induced in vascular endothelium as shown by positive staining with anti-CD31 antibody (indicated by arrowheads in Figure 5e). However, TNFR2 was not detectable in nonischemic vasculature (indicated by arrows) or ischemic tissue of TNFR2-KO (not shown). Interestingly, Bmx/Etk was also induced in vascular endothelium in response to ischemia (Supplemental Figure S1, see http://ajp.amjpathol.org). Thus, activation of TNFR2 signaling in ischemic vasculature is consistent with the role of TNFR2 in ischemia-induced responses.

**Both TNFR2-Associated Bmx/Etk and TRAF2 Are Critical for TNFR2-Induced EC Survival and Migratory Signaling**

To understand the mechanisms by which TNFR2 signaling (Etk and TRAF2) mediates arteriogenesis/angiogenesis, we isolated TNFR2-null mouse ECs (MECs) from TNFR2-KO muscle tissue and determined the role of TNFR2 in EC survival and migration, two critical steps for angiogenesis. We first determined the effect of TNFR2 on EC survival. TRAF2 is critical for TNFR2-mediated activation of NF-κB and NF-κB-dependent anti-apoptotic signaling. We generated a retroviral system expressing EGFP- or Myc-tagged TNFR2-WT, TNFR2–16 lacking Bmx/Etk-binding, mTR2 lacking TRAF2-binding, and TNFR2–59 lacking binding for both TRAF2 and Bmx/Etk (Figure 6a). The retroviruses could effectively transduce TNFR2-null MECs and express TNFR2 as visualized by indirect immunofluorescence microscopy with anti-Myc antibody (Supplemental Figure S2, see http://ajp.
The effect of TNFR2 on NF-κB activation was analyzed in a κB-reporter gene assay. Consistent with previous findings, expression of TNFR1 or TRAF2 activates the NF-κB reporter gene. Expression of Etk-SK (a constitutively active form of Bmx/Etk) alone did not induce NF-κB activation. However, expression of TNFR2-WT and TNFR2–16, but not TNFR2-mTR2 or TNFR2–59, induced activation of the NF-κB reporter gene. We next determined the effects of TNFR2 on EC survival/death. MECs were transduced with retrovirus expressing EGFP (VC) or various TNFR2 mutants and EC apoptosis was determined by 4,6-diamidino-2-phenylindole (DAPI) staining for nuclear fragmentation. Expression of TNFR2-mTR2 lacking the TRAF2-binding site induced EC apoptosis. TNFR2–59 lacking both TRAF2- and Bmx/Etk-binding sites showed much stronger activity in inducing EC apoptosis (Figure 6c with quantification in Figure 6d). In contrast, TNFR2-WT and TNFR2–16 did not significantly induce EC death. As controls, TNFR1 (but not TRAF2 or Etk-SK) induced EC apoptosis (Figure 6d). These data suggest that the TRAF2-binding site on TNFR2 is critical for TNFR2-induced NF-κB activation and anti-apoptotic signaling in ECs and that the Bmx/Etk-binding site also contributes to the anti-apoptotic function of TNFR2.

We have previously shown that overexpression of Bmx/Etk is sufficient to induce EC migration in a monolayer injury model (Figure 6f).25,26 We used this model to determine the effects of TNFR2 on EC migration. Expression of TNFR2-WT in MECs strongly induced, whereas expression of TNFR1 inhibited, EC migration compared with the control vector (EGFP) (Figure 6, e and f). TNFR2 lacking Bmx/Etk-binding (TNFR2–16) or TRAF2-binding (TNFR2-mTR2) reduced the ability to induce EC migration. Deletion of both sites (TNFR2–59) diminished TNFR2-induced EC migration (Figure 6e with quantification in Figure 6f). These data suggest that both TRAF2 and Bmx/Etk are critical for TNFR2-induced EC migration in vitro, supporting their roles in TNFR2-mediated arteriogenesis/angiogenesis in vivo.

Discussion

The most important finding of this study is that TNFR1 and TNFR2 play differential roles in ischemia-mediated arteriogenesis and angiogenesis. TNFR1 signaling inhibits, whereas TNFR2 signaling promotes, this adaptive response, likely attributable to their opposite effects on EC survival and migration. This conclusion was based on functional analyses from mice genetically deficient of TNFR1 and TNFR2 in a femoral artery ligation model and mechanistic studies in mouse ECs isolated from these mice. Specifically, TNFR1-KO mice had enhanced, whereas TNFR2-KO mice had reduced ischemia-initiated arteriogenesis, angiogenesis, and associated EC proliferation, neovascularization, and vessel maturation. TNFR2 proteins and signaling (TNFR2-TRAF2 complex formation and Bmx/Etk activation) were highly up-regulated in vascular endothelium in vivo in response to ischemia. Furthermore, mechanistic studies suggested that both Bmx/Etk-dependent EC migratory and TRAF2-dependent NF-κB survival pathways were critical for TNFR2-mediated angiogenesis. In contrast, activation of TNFR1 signaling caused inhibition of EC migration and EC apoptosis. Our study suggests that specific inhibition of TNFR1 or activation of TNFR2 signaling in ECs may be a novel target for the treatment of vascular diseases such as coronary artery and peripheral arterial diseases.

The roles of infiltrated macrophages and their products, such as TNF, in pathologic angiogenesis have been investigated previously. The involvement of monocytes/macrophages, a primary cell type that produces TNF, in the process of adaptive arteriogenesis was first suggested in studies in which a correlation was shown between monocyte adhesion to the intima of coronary collaterals and rapid growth of these vessels.30 It was then elegantly demonstrated that TNF mediates macrophage-induced angiogenesis in a cornea angiogenesis assay.31 However, numerous studies suggest that TNF has either proangiogenic or antiangiogenic activities in various in vitro and in vivo angiogenesis models, even in the similar ischemic hindlimb models. In a rat model of hindlimb ischemia, inhibition of bioactive TNF by intramuscular gene transfer of soluble TNFR1 activates VEGF receptor and accelerated angiogenesis.32 In a rabbit femoral artery occlusion model, monocyte accumulation and TNF production were strongly associated with vessel proliferation, capillary density, and collateral/peripheral conductance. Thus anti-TNF therapy with the murine-human chimeric antibody infliximab or the soluble p75 TNF receptor fusion protein etanercept attenuated adaptive arteriogenesis in the rabbit as measured by collateral conductance with fluorescence microspheres.33 Interestingly, high-resolution angiography showed no significance in number of collateral arteries, but immunohistochemical analyses demonstrated a decrease on mean collateral diameter, proliferation of vascular smooth muscle cells, and reduction of leukocyte accumulation around collateral arteries. Mechanistic studies suggest that anti-TNF therapy induced monocyte apoptosis, suggesting that anti-TNF therapy targets monocytes in this study. The reason for this discrepancy is not clear. It is noted that there are a few differences in these studies including species (rabbit/mouse versus rat) and anti-TNF-reagents (soluble TNFR2 versus soluble TNFR1).

Dr. I.E. Hoefer and colleagues39 have demonstrated that TNF, via its receptor TNFR1, is critical for ischemia-mediated arteriogenesis/angiogenesis using mice lacking TNF or TNFRs.39 In contrast to these results, our data suggest that TNFR1 functions as a negative regulator whereas TNFR2 acts as a positive activator in ischemia-mediated arteriogenesis and angiogenesis. It is noted that there are several differences between our study and that of Dr. Hoefer and colleagues. First, the strain background for WT, TNFR1-KO, and TNFR2-KO is C57BL/6 in our study whereas mixed backgrounds in WT, TNFR1-KO, and TNFR2-KO were used in Dr. Hoefer’s studies.
Second, Dr. Hoefer’s group performed a minor ischemic injury model in which the femoral artery was ligated immediately distal to the inguinal ligament and the femoral was not excited to leave to the vessels intact. In our model, the proximal femoral artery and the distal portion of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arterectomy was performed. Thus, our model may represent a more severe ischemia model. It is possible TNFR2 signaling is activated only in response to severe ischemia; TNFR expression was not determined in their study. Third, in their study only a day-7 time point was examined, when inflammatory responses and ischemia-induced injury remain at the peak. It is conceivable that ischemia/inflammation components, both contributing to the burst in TNF production, play a major role during this period of time. Consistently, anti-TNF treatment in this model primarily inhibits skeletal muscle capillary permeability and perivascular leukocyte infiltration and/or induces monocyte apoptosis, leading to attenuated tissue injury. In contrast, we have monitored the phenotype of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arterectomy was performed. Thus, our model may represent a more severe ischemia model. It is possible TNFR2 signaling is activated only in response to severe ischemia; TNFR expression was not determined in their study. Third, in their study only a day-7 time point was examined, when inflammatory responses and ischemia-induced injury remain at the peak. It is conceivable that ischemia/inflammation components, both contributing to the burst in TNF production, play a major role during this period of time. Consistently, anti-TNF treatment in this model primarily inhibits skeletal muscle capillary permeability and perivascular leukocyte infiltration and/or induces monocyte apoptosis, leading to attenuated tissue injury.

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