Differential Functions of Tumor Necrosis Factor Receptor 1 and 2 Signaling in Ischemia-Mediated Arteriogenesis and Angiogenesis

Dianhong Luo,* Yan Luo,* Yun He,*
Haifeng Zhang,* Rong Zhang,* Xianghong Li,*
Wawrzyniec L. Dobrucki,[†] Al J. Sinusas,[†]
William C. Sessa,^{*,‡} and Wang Min*

From the Department of Pathology,* Interdepartmental Program in Vascular Biology and Transplantation, Boyer Center for Molecular Medicine, and the Departments of Internal Medicine (Cardiology) and Diagnostic Radiology[†] and Pharmacology,[‡] Yale University School of Medicine, New Haven, Connecticut

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) in the lower limb 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 TNFR2 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 TNFR2 induced nuclear factor- κ B-dependent reporter gene expression, EC survival, and migration. In contrast, activation of TNFR1 caused inhibition of EC migration and EC apoptosis. These data demonstrate that TNFR1 and TNFR2 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 .¹ In ECs, as in other cell types, TNF elicits a broad spectrum of biological effects including proliferation, differentiation, and apoptosis.^{2–5} The nature of TNF effects depends on TNF concentration and the type and growth state of the target of cells.⁶ These 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).⁷ 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 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,

Supported by the American Heart Association (established investigator award 0440172N to W.M.) and the National Institutes of Health (grant R01HL65978-05 to W.M.).

D.L., Y.L., and Y.H. contributed equally to this study.

Accepted for publication August 14, 2006.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Wang Min, Interdepartmental Program in Vascular Biology and Transplantation, Department of Pathology, Yale University School of Medicine, BCMM 454, 295 Congress Ave., New Haven, CT 06510. E-mail: wang.min@yale.edu.

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)- κ B, 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- κ B-dependent anti-apoptotic 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 acti-

vates 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-*tnfrsf1a*), and TNFR2-KO (strain name B6.129-*tnfrsf1b*) 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 committees 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 arteriotomy 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 and 3 days, 2 weeks, and 4 weeks 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 were 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 Medica 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 512×512 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 recon-

structed 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, Burlingame, CA). Pictures from four random areas of

a TNF signaling in EC

b Mouse model of critical limb Ischemia

c

d

e

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 $\pm$ SEM, * $P < 0.05$.

each section and five sections per mouse were taken using a Kodak (Eastman-Kodak, Rochester, NY) digital camera mounted on a light microscope ($\times 40$ 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 $1 \mu\text{g}$ 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'-TTCCGATAACGAACGAGACTCT-3', reverse primer: 5'-TGGCTGAACGCCACTTGTC-3'; TNFR1 forward primer: 5'-GGGCACCTTACGGCTTCC-3', reverse primer: 5'-GGTTCTCCTTACAGCCACACA-3'; TNFR2 forward primer: 5'-CAGGTTGTCTTGACACCCTA-C-3', reverse primer: 5'-GCACAGCACATCTGAGCCT-3'; TRAF2 forward primer: 5'-TTCGGCCTTTCCAGATAACG-C-3', reverse primer: 5'-CCTTCCAAGTGCATCCATCAT-TG-3'; BMX forward primer: 5'-TACCTGGCTGAAACT-ACTGCT-3', reverse primer: 5'-CCACATCATATTGCC-CTTCCA-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,²⁵ followed by immunoselection and immortalization modified from the protocol described by Lim and colleagues.²⁹ For immunoselection, $10\text{-}\mu\text{l}$ 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 $100 \mu\text{l}$ of buffer A. Ten μl ($10 \mu\text{g}$) of anti-mouse ICAM-2 or $10 \mu\text{l}$ ($10 \mu\text{g}$) of PECAM-1 were added and rocked at 4°C for 2 hours. Beads were washed for three times and resuspended in $160 \mu\text{l}$ 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 sus-

pension ($\sim 10 \text{ ml}$) 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 .

a CT image

b

Figure 2. Critical roles of TNFR2 in ischemia-induced arteriogenesis. **a:** Four weeks after femoral ligation, mice were anesthetized and subjected to 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 ($\times 4$). A hemocytometer ($1 \text{ mm}^2/\text{grid}$) was used as a standard. Wound area (mm^2) 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 ($\pm \text{SD}$). 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 $\pm$ 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.^{30,31} 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 flow dropped by 80% and returned to a ratio of 1 throughout 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 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 angiotensin. 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-

Figure 3. Critical roles of TNFR2 in ischemia-induced neovascularization and pericyte recruitment. **a:** Four weeks after femoral ligation, gastrocnemius muscles were harvested. **a:** Capillary density and pericyte recruitment were immunostained with CD31 (an EC marker) and SMA (a smooth muscle/pericyte marker). Representative sections from nonischemic and ischemic groups of C57BL/6 mice are shown. **b:** Representative staining of CD31 and SMA in ischemic groups of TNFR1-KO and TNFR2-KO mice are shown. **c–f:** Quantification of capillaries (number/ mm^2 muscle area), ratio of CD31/muscle fiber, SMA-positive staining (number/ mm^2 muscle area), and percentage of SMA-positive staining (SMA/capillaries) are shown. Data from different mice are shown in graphics and $n = 4$ for each strain. * $P < 0.05$.

sponsive to ischemia when measured for increased capillary formation, macrophage infiltration, and myocyte proliferation.²⁷ 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-

Figure 5. Ischemia induces TNFR2-specific signaling. **a:** C57BL/6 ($n = 3$) 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.

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 capillary (Figure 3f). TNFR2-KO muscle tissues appeared to be less sensitive to ischemic responses. Importantly, CD31-positive capillaries surrounding the skeletal muscle fibers (neovascularization) 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).

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 macrophage 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

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. $*P < 0.05$.

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- κ B activation/EC survival and EC migration, respectively, are indicated. **b:** Effects of TNFR2 on NF- κ B reporter gene. NF- κ B-dependent luciferase reporter gene (1 μ g) and a constitutive expression vector for Renilla luciferase (0.5 μ g) were cotransfected with a vector control (VC), a TNFR2 mutant, TNFR1, TRAF2, or Bmx/Etk (1 μ g 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 ($\pm$ 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 (**e**), with quantification in **f**. Data presented are means ($\pm$ 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.¹¹ 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 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 ki-

nase 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- κ B activation and cell survival.¹⁷ 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- κ B and NF- κ B-dependent anti-apoptotic signaling.¹⁷ 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>).

amjpathol.org). The effect of TNFR2 on NF- κ B activation was analyzed in a κ B-reporter gene assay. Consistent with previous findings,^{34,35} expression of TNFR1 or TRAF2 activates the NF- κ B reporter gene. Expression of Etk-SK (a constitutively active form of Bmx/Etk) alone did not induce NF- κ B activation. However, expression of TNFR2-WT and TNFR2-16, but not TNFR2-mTR2 or TNFR2-59, induced activation of the NF- κ B reporter gene. (Figure 6b). 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- κ B 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 had reduced capacity in clinical recovery, limb perfusion, and ischemic reserve capacity compared with the WT mice. Imaging and immunohistochemistry analyses indicated that TNFR1-KO mice had increased, 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- κ B 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 pathological 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.³² It was then elegantly demonstrated that TNF mediates macrophage-induced angiogenesis in a cornea angiogenesis assay.³⁶ 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.³⁷ 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.³⁸ 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.³⁹ 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's³⁹ studies.

Second, Dr. Hoefler's group³⁹ 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 arteriectomy 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.^{38,40} In contrast, we have monitored the phenotype up to 10 weeks and found that the blood flow was recovered in the ischemic limb only after 4 weeks when inflammation/ischemia was declined to the baseline. Thus postinflammatory responses such as adaptive arteriogenesis and vascular/skeletal remodeling play critical roles in the tissue repair. In supporting this conclusion, both TNFR1 and TNFR2-KO mice displayed reduced inflammatory responses compared with WT mice, yet these mice showed opposite phenotypes in the ischemia-induced arteriogenesis/angiogenesis and functional flow recovery. Thus, regional microenvironment in the ischemic tissue such as shear stress, EC survival, proliferation, and vessel maturation play essential roles in the adaptive process. Our data suggest that TNF receptor signaling in local vasculature contributes to the vascular remodeling.

The mechanisms by which TNFR1 inhibits, but TNFR2 signaling promotes, ischemia-induced arteriogenesis and angiogenesis are not clear. We showed that activation of TNFR1 signaling caused inhibition of EC migration and EC apoptosis. In contrast, activation of TNFR2 signaling induced EC survival/proliferation and migration, leading to increased arteriogenesis/angiogenesis. These data suggest that TNFR1 and TNFR2-mediated apoptosis versus survival/proliferative pathways in ECs represent critical parallel and counterbalanced pathways in ischemia-mediated vascular remodeling. A recent study showed that EC-specific transgenic mice expressing a membrane-bound TNF (mtTNF, a strong activator of TNFR2 *in vivo*) induced constitutive EC activation and inflammatory angiogenesis.⁴¹ The role of TNFR2 activation in these mice has not been determined. Our results showed that TNFR2 signaling is specifically activated in vascular ECs in ischemic tissue. Mechanistic studies suggest that specific activation of TNFR2 (via overexpression of TNFR2) induces TRAF2-dependent NF- κ B survival and Bmx/Etk-dependent EC migration, two components of angiogenesis pathways. Deletion of both Bmx/Etk-binding site and TRAF2-binding site on TNFR2 had greater impairment than deletion of either site on the

ability of TNFR2-induced EC migration, supporting that TRAF2 and Bmx/Etk cooperatively mediate TNFR2-induced angiogenesis. The downstream signaling molecules mediating this synergistic effect are not yet known. Rho family small G proteins (RhoA, Rac1, and Cdc42) have been shown to be involved in cell migration by promoting formation of stress fibers, lamellipodia, and filopodia, respectively.⁴² Previous data suggest that Bmx only activates RhoA but not Rac1 or Cdc42.⁴³ Our data show that Bmx-dependent transactivation of VEGFR2 is critical for TNF/TNFR2-induced EC migration and tube formation.²⁶ Our recent results show that TNFR2 promotes filopodia formation by inducing activation of Cdc42 (W. Min, unpublished data). It is conceivable that TRAF2-dependent Cdc42 activation and Bmx-dependent RhoA as well as VEGFR2 activation cooperatively mediate TNFR2-induced EC migration. Of note, expression of TNFR2-59 (deletion of both TRAF2- and Bmx/Etk-binding sites) spontaneously induced EC apoptosis and inhibition of EC migration, an effect observed for TNFR1 expression. It has been shown in other cell types (primarily T cells) that TNFR2 potentiates TNFR1 killing, likely by facilitating TRAF2 degradation.⁴⁴ It is conceivable that TNFR2-59 may enhance TNFR1-dependent apoptotic signaling in ECs. This possibility is under investigation.

Collectively, 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 disease and peripheral arterial disease.

Acknowledgment

We thank Dr. Jordan S. Pober (Yale University) and Dr. John Bradley (University of Cambridge, Cambridge, UK) for discussion.

References

1. Pober JS, Cotran RS: Cytokines and endothelial cell biology. *Physiol Rev* 1990, 70:427-451
2. Loetscher H, Pan YE, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W: Molecular cloning and expression of the human 55kd tumor necrosis factor receptor. *Cell* 1990, 61:351-359
3. Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GHW, Gatanaga T, Granger GA, Lentz R, Raab H, Kohr WJ, Goeddel DV: Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* 1990, 61:361-370
4. Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG: A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* 1990, 248:1019-1022
5. Madge LA, Pober JS: Tnf signaling in vascular endothelial cells. *Exp Mol Pathol* 2001, 70:317-325
6. Fiers W, Beyaert R, Boone E, Cornelis S, Declercq W, Decoster E, Denecker G, Depuydt B, De Valck D, De Wilde G, Goossens V, Grooten J, Haegeman G, Heynincx K, Penning L, Plaisance S, Vancompernelle K, Van Crieginge W, Vandenabeele P, Vanden Berghe W, Van de Craen M, Vandevoorde V, Vercammen D: TNF-induced intracellular signaling leading to gene induction or to cytotoxicity by necrosis or by apoptosis. *J Inflamm* 1995, 47:67-75
7. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP: Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 1999, 17:331-367

8. Smith CA, Farrah T, Goodwin RG: The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994, 76:959–962
9. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino Jr MA, Goeddel DV: The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* 1991, 88:9292–9296
10. Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 1993, 268:18542–18548
11. Al-Lamki RS, Wang J, Vandenabeele P, Bradley JA, Thiru S, Luo D, Min W, Pober JS, Bradley JR: TNFR1- and TNFR2-mediated signaling pathways in human kidney are cell type-specific and differentially contribute to renal injury. *FASEB J* 2005, 19:1637–1645
12. Chainy GB, Singh S, Raju U, Aggarwal BB: Differential activation of the nuclear factor-kappa B by TNF mutants specific for the p60 and p80 TNF receptors. *J Immunol* 1996, 157:2410–2417
13. Baud V, Karin M: Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 2001, 11:372–377
14. Hsu H, Shu HB, Pan MG, Goeddel DV: TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 1996, 84:299–308
15. Jiang Y, Woronicz JD, Liu W, Goeddel DV: Prevention of constitutive TNF receptor 1 signaling by silencer of death domains [published erratum appears in *Science* 1999 Mar 19;283(5409):1852]. *Science* 1999, 283:543–546
16. Rothe M, Wong SC, Henzel WJ, Goeddel DV: A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 1994, 78:681–692
17. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV: The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 1995, 83:1243–1252
18. Stanger BZ, Leder P, Lee TH, Kim E, Seed B: RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 1995, 81:513–523
19. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995, 81:505–512
20. Baud V, Liu ZG, Bennett B, Suzuki N, Xia Y, Karin M: Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* 1999, 13:1297–1308
21. Carpentier I, Coornaert B, Beyaert R: Function and regulation of tumor necrosis factor type 2. *Curr Med Chem* 2004, 11:2205–2212
22. Rothe M, Sarma V, Dixit VM, Goeddel DV: TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 1995, 269:1424–1427
23. Rothe M, Xiong J, Shu HB, Williamson K, Goddard A, Goeddel DV: I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. *Proc Natl Acad Sci USA* 1996, 93:8241–8246
24. Zhang R, Al-Lamki R, Bai L, Streb JW, Miano JM, Bradley J, Min W: Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. *Circ Res* 2004, 94:1483–1491
25. Pan S, An P, Zhang R, He X, Yin G, Min W: Etk/Bmx as a tumor necrosis factor receptor type 2-specific kinase: role in endothelial cell migration and angiogenesis. *Mol Cell Biol* 2002, 22:7512–7523
26. Zhang R, Xu Y, Ekman N, Wu Z, Wu J, Alitalo K, Min W: Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. *J Biol Chem* 2003, 278:51267–51276
27. He Y, Luo Y, Tang S, Rajantie I, Salven P, Heil M, Zhang R, Luo D, Li X, Chi H, Yu J, Carmeliet P, Schaper W, Sinusas AJ, Sessa WC, Alitalo K, Min W: Critical function of Bmx/Etk in ischemia-mediated arteriogenesis and angiogenesis. *J Clin Invest* 2006, 116:2344–2355
28. Couffignal T, Silver M, Zheng LP, Kearney M, Witzensbichler B, Isner JM: Mouse model of angiogenesis. *Am J Pathol* 1998, 152:1667–1679
29. Lim YC, Garcia-Cardena G, Allport JR, Zervoglos M, Connolly AJ, Gimbrone Jr MA, Luscinskas FW: Heterogeneity of endothelial cells from different organ sites in T-cell subset recruitment. *Am J Pathol* 2003, 162:1591–1601
30. Bauer PM, Yu J, Chen Y, Hickey R, Bernatchez PN, Looft-Wilson R, Huang Y, Giordano F, Stan RV, Sessa WC: Endothelial-specific expression of caveolin-1 impairs microvascular permeability and angiogenesis. *Proc Natl Acad Sci USA* 2005, 102:204–209
31. Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, Rubanyi GM, Qian HS, Murata T, Escalante B, Sessa WC: Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci USA* 2005, 102:10999–11004
32. Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W: Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 1998, 101:40–50
33. Heil M, Schaper W: Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res* 2004, 95:449–458
34. Min W, Pober JS: TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-kappa B and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 pathways. *J Immunol* 1997, 159:3508–3518
35. Zhang H, Zhang R, Luo Y, D'Alessio A, Pober JS, Min W: AIP1/DAB2IP, a novel member of the Ras-GAP family, transduces TRAF2-induced ASK1-JNK activation. *J Biol Chem* 2004, 279:44955–44965
36. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N: Macrophage-induced angiogenesis is mediated by tumor necrosis factor-alpha. *Nature* 1987, 329:630–632
37. Sugano M, Tsuchida K, Hata T, Makino N: In vivo transfer of soluble TNF-alpha receptor 1 gene improves cardiac function and reduces infarct size after myocardial infarction in rats. *FASEB J* 2004, 18:911–913
38. Grundmann S, Hoefer I, Ulusans S, van Royen N, Schirmer SH, Ozaki CK, Bode C, Piek JJ, Buschmann IR: Anti-tumor necrosis factor-(alpha) therapies attenuate adaptive arteriogenesis in the rabbit. *Am J Physiol* 2005, 289:H1497–H1505
39. Hoefer IE, van Royen N, Rectenwald JE, Bray EJ, Abouhamze Z, Moldawer LL, Voskuil M, Piek JJ, Buschmann IR, Ozaki CK: Direct evidence for tumor necrosis factor-alpha signaling in arteriogenesis. *Circulation* 2002, 105:1639–1641
40. Gaines GC, Welborn III MB, Moldawer LL, Huber TS, Harward TR, Seeger JM: Attenuation of skeletal muscle ischemia/reperfusion injury by inhibition of tumor necrosis factor. *J Vasc Surg* 1999, 29:370–376
41. Rajashekhar G, Willuweit A, Patterson CE, Sun P, Hilbig A, Breier G, Helisch A, Clauss M: Continuous endothelial cell activation increases angiogenesis: evidence for the direct role of endothelium linking angiogenesis and inflammation. *J Vasc Res* 2006, 43:193–204
42. Etienne-Manneville S, Hall A: Rho GTPases in cell biology. *Nature* 2002, 420:629–635
43. Kim O, Yang J, Qiu Y: Selective activation of small GTPase RhoA by tyrosine kinase Etk through its pleckstrin homology domain. *J Biol Chem* 2002, 277:30066–30071
44. Li X, Yang Y, Ashwell JD: TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* 2002, 416:345–347