**Differential Role of Tumor Necrosis Factor (TNF)-α Receptors in the Development of Choroidal Neovascularization**

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**Purpose.** Tumor necrosis factor alpha (TNF-α) contributes to inflammation-associated angiogenesis. This study investigates the role of TNF-α receptors 1a and 1b in the development of choroidal neovascularization (CNV).

**Methods.** CNV was induced in Tnfrsf1a<sup>−/−</sup> and Tnfrsf1b<sup>−/−</sup> mice with C57Bl6/J background and their wild-type (WT) (C57Bl/6j) controls by laser damage to the Bruch’s membrane. TNF-α expression in RPE/choroid was determined by Western blot analysis. Pathologic angiogenesis was estimated qualitatively and quantitatively by fluorescein angiography and histology on choroidal flat mounts and paraffin cross-sections. Inflammatory cell invasion was investigated by clodronic acid depletion of circulating macrophages and immunohistochemistry, and the apoptotic activity was investigated by TUNEL assay and by caspase-3 and caspase-8 expression. Receptor 1b-specific Bmx/Etk kinase was detected by immunohistochemistry.

**Results.** TNF-α levels were elevated after laser treatment. Severe CNV lesions and increased macrophage invasion were observed in Tnfrsf1a<sup>−/−</sup> compared with WT and Tnfrsf1b<sup>−/−</sup> mice. Increased immunoreactivity for Bmx/Etk kinase corresponded to the severity of CNV formation. Reduced pathologic angiogenesis and macrophage invasion in Tnfrsf1b<sup>−/−</sup> mice (vs. WT and Tnfrsf1a<sup>−/−</sup>) was accompanied by enhanced endothelial cell apoptosis and by caspase-3 and caspase-8 activation.

**Conclusions.** Receptor 1b promotes the recruitment of inflammatory cells to the site of injury and exacerbates pathologic angiogenesis probably by way of the Bmx/Etk kinase–dependent pathway in the absence of receptor 1a. On the other hand, receptor 1a–dependent apoptosis in the absence of receptor 1b leads to reduced inflammatory response and CNV lesions after laser treatment. This demonstrates the potential for specific targeting of TNF-α receptors for future therapies of inflammation-associated choroidal neovascularization.

Choroidal neovascularization (CNV) in age-related macular degeneration (AMD) is the leading cause of blindness in people older than 50 years of age in the western world. Inflammation is critically involved in the formation of CNV lesions and may contribute to the pathogenesis of AMD. In choroidal neovascular lesions, macrophages have been localized with endothelial cells. Surgically removed CNV membranes from AMD patients contain macrophages in approximately 60% of the cases. In surgically removed CNV membranes, macrophages express TNF-α, an inflammatory cytokine, suggesting that TNF-α contributes to pathologic angiogenesis in AMD. TNF-α is the prototypical member of a family of cytokines, including FasL, CD40L and TRAIL. TNF-α stimulates the expression of proangiogenic VEGF and intercellular adhesion molecule-1 on vascular endothelium, induces the activation of nuclear factor-κB (NF-κB) protein, and stimulates leukocyte adhesion to endothelial cells. TNF-α is highly expressed in fibrovascular CNV membranes and in blood monocytes of AMD patients. Anti–TNF-α treatment with different inhibitors reduces the size and leakage of laser-induced CNV. Recently, Theodossiadis et al. reported the intravitreal administration of the TNF-α antibody infliximab in patients with neovascular AMD.

TNF-α receptors are expressed on many cell types in the retina and choroid, including endothelial cells of fibrovascular tissue. Both a protective role and a destructive role in cell death signaling has been demonstrated for TNF-α, and the opposing actions are thought to be directly related to the TNF-α receptors Tnfrsf1a and Tnfrsf1b. Activation of Tnfrsf1a causes inflammation, inhibition of endothelial cell (EC) migration, and EC apoptosis through the TNF receptor–associated death domain, or TRADD. Tnfrsf1b affiliates with lymphocyte proliferation and promotes endothelial cell activation, migration, and survival through signals associated with TNF receptor 1b-specific kinase Etk/Bmx, which has been implicated in endothelial cell migration and tube formation in vitro. Activation of NF-κB in endothelial cells (ECs) through Tnfrsf1b stimulates the expression of antipapoptotic genes and increases EC survival. Signaling through Tnfrsf1b is required for NF-κB-mediated VEGF gene expression. TNF-α receptors 1a and 1b play probably differential roles in angiogenesis and CNV formation. Tnfrsf1a signaling probably inhibits, whereas Tnfrsf1b signaling promotes, angiogenesis. This study was conducted to investigate the roles of TNF-α receptors 1a and 1b in the development of CNV after laser photocoagulation.
MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and the institutional animal care and use committee, and the protocols were approved by the Regierungspräsidium Northrhine Westphalia. Tnfrsf1a−/− and Tnfrsf1b−/− mice and the respective wild-type (WT) controls were purchased from Jackson Laboratories (Bar Harbor, ME). Uniformity of genetic backgrounds of WT (C57BL/6J) and Tnfrsf1a−/− mice was ensured through backcrossing Tnfrsf1a−/− and Tnfrsf1b−/− mice onto the C57BL/6j background for at least six generations. For all experiments, age-matched animals of the same sex were used.

Experimental CNV Model

Laser photocoagulation was performed as described previously14,15 using a diode-pumped, frequency-doubled, 532-nm laser (Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany). Five lesions were induced using a power of 120 mW, spot size of 50 μm, and duration of 100 ms. Laser burns were made on one eye of each animal, and the other eye was used as the control.

Western Blot Analysis

The RPE/choroid layers were extracted and lysed for 30 minutes on ice with lysis buffer supplemented with a mixture of protease inhibitors (Sigma-Aldrich, St. Louis, MO). Two RPE/choroid layers isolated from two mice were pooled and used for one line on the blot. Three different blots were prepared in the same way using protein extracts from different mice each time. The samples were cleared by microcentrifugation and assessed for protein concentration (Bradford assay; Bio-Rad Laboratories, Munich, Germany). Proteins were electrophoretically transferred to a nitrocellulose membrane, followed by treatment with blocking buffer (Starting Block; Perbio Science, Northumberland, UK). TNF-α expression was determined 1 day and 2 weeks after laser treatment with the use of rabbit anti–mouse TNF-α antibody (ab20397, 1:500 dilution; Abcam, Cambridge, UK). β-Actin expression was used as a loading control (A5441, mouse monoclonal, 1:10,000; Sigma-Aldrich, Taufkirchen, Germany). Expression of the active form of caspase-3 and caspase-8 proteins in RPE/choroid layer was analyzed 7 days after laser photocoagulation. The blots were probed with antibodies against caspase-3 (recognizes the cleaved p17 active fragment of caspase-3; A5441, mouse monoclonal, 1:10,000; Sigma-Aldrich, Taufkirchen, Germany), caspase-8 (detects cleaved 18-kDa form; ALX804447, rat anti–mouse, 1:500; Alexis Biochemicals, Grünberg, Germany). The respective secondary peroxidase-labeled antibody was applied at 1:1000 to 1:5000 dilution for 1 hour at room temperature. A chemiluminescence kit (SuperSignal; West Pico Chemiluminescent Substrate, Perbio Science) was used for detection. Density of the immunoreactive bands was measured with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Caspase-3, caspase-8, and TNF-α protein expression values in transgenic and WT mice were normalized by comparison with the expression of β-actin.

Fluorescein Angiography

Fluorescein angiography (FA) was performed using a digital imaging system (Heidelberg Retina Angiograph II; Heidelberg Engineering, Heidelberg, Germany) 2 weeks after laser photocoagulation, as described by Semkova et al.15 Each analysis includes early-phase (1–3 minutes after injection) and late-phase (6–8 minutes after injection) images. Fluorescein angiograms were evaluated qualitatively and quantitatively by a masked observer. Laser-induced lesions were graded according to the increase in fluorescein leakage between early and late phase into four different groups, as described by Semkova et al.15 and as shown in Table 1.

Clodronic Acid Depletion of Circulating Macrophages

Clodronate (dichloromethylene diphosphonate; CL2MDP) liposomes were received from Nico van Rooijen (Department of Molecular Cell Biology, University of Amsterdam). Briefly, 86 mg phosphatidylcholine (Lipoid EPC, Ludwigshafen, Germany) and 8 mg cholesterol (Sigma-Aldrich, St. Louis, MO) were combined with 10 mL clodronate (0.7 M) solution and sonicated gently. Subsequently, the created liposomes were washed to eliminate the free drug. Empty liposomes were prepared similarly using phosphate-buffered saline instead of the clodronate solution. WT, Tnfrsf1a−/−, and Tnfrsf1b−/− mice each received two intravenous injections of 200 μL CL2MDP-LIP or PBS-LIP 2 days before and immediately after laser photocoagulation. Additionally, at these time points, animals received 10 μL CL2MDP-LIP or PBS-LIP into the subconjunctival space with a Hamilton syringe. Mouse blood analysis was performed at an animal clinical diagnostic laboratory (Laboklin, Bad Kissingen, Germany).

Immunohistochemistry on Choroidal Flatmounts

Immunohistochemistry was performed on RPE/choroidal flatmounts 2 weeks after laser injury using Alexa 488–conjugated isoelectric IB4 (Invitrogen). Eyes were isolated and fixed in 2% paraformaldehyde for 1 hour at room temperature. Flatmounts were prepared and postfixified for another 30 minutes with 2% paraformaldehyde and then were subjected to immunohistochemistry. Eyecups were incubated overnight at 4°C with 0.5% Alexa 488–conjugated isoelectric IB4.

For quantification of the CNV size on choroidal flatmounts, all images of the laser scars were captured with a digital video camera (Hamamatsu Orca, Hamamatsu City, Japan) coupled to a computer system with image analysis software (Openlab; ImproVision Inc., Lexington, MA).

Choroidal flatmounts were double stained with Alexa 488–conjugated isoelectric IB4 (1:200) and rat anti–mouse antibody against macrophage marker F4/80 (MCA 497G, 1:200; Serotec, Oxford, UK). The secondary Cy5-conjugated goat anti–rat antibody (1:400; Dianova, Hamburg, Germany) was used. The area of F4/80-positive cells close to the laser scars was measured by ImageJ software (in pixels). We also measured the orange areas (colocalization endothelial cells and macrophages; Alexa 488–conjugated isoelectric IB4 green; Cy3-F4/80 red) within the laser burns with ImageJ software and normalized these areas to the total size of the laser burns (100%).

Histology and Immunohistochemistry on Paraffin Sections

Paraffin sections were coimmunostained with polyclonal rabbit anti–human von Willebrand factor (vWF; A0082; DakoCytomation, Glostrup, Germany) to visualize the blood vessels and F4/80 antibody (rat anti–mouse; 1:200) to label the inflammatory cells. Goat anti–rabbit FITC-
conjugated antibody (1:300; Chemicon) was used as the detection antibody for vWF, and goat anti-rat Cy3-conjugated antibody (1:300; Dianova) was used for the detection of F4/80. Bmx/Etk kinase was detected in a single staining on parallel sections (because of the common origin of the vWF and Bmx/Etk antibodies) using goat anti-Bmx antibody (C17, sc-8874, 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA). As secondary antibody, donkey anti–goat IgG–FITC-conjugated (1:200; Santa Cruz Biotechnology Inc.) was used. Neighboring sections were stained with vWF to evaluate vascularization.

**Determination of Apoptosis by TUNEL Assay**

Apoptosis was examined by TUNEL assay (In Situ Cell Death Detection Kit; Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions and was analyzed by fluorescence microscopy. Staining was performed 14 days after laser photoagulation, which was within the period of active CNV formation. To test the specificity of the TUNEL assay, slides were stained with label solution without terminal transferase (negative control). As expected, in this case no apoptotic nuclei were observed. In addition, a positive control was prepared by treatment of the slices with DNase (Sigma-Aldrich). Here, TUNEL-positive cells were observed in all retinal nuclear layers and in the choroid and sclera. All sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) after the TUNEL reaction to verify that TUNEL staining was localized in the nucleus. Further, the apoptotic cells were counted within the area of the CNV lesion/section.

**Statistical Analysis**

All results are presented as mean ± SEM. Analysis of variance (ANOVA) and Student’s t-test were used to perform statistics on all experiments in the study. *P < 0.05* was considered statistically significant. Significance was classified by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001). Statistical analysis was conducted with statistical software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL).

**RESULTS**

**Increase of TNF-α Expression in RPE/Choroid after Laser Photocoagulation**

The expression of TNF-α in the RPE/choroid layer of WT, Tnfrsf1a−/−, and Tnfrsf1b−/− animals was determined by Western blot analysis 1 day and 2 weeks (Fig. 1) after laser photoagulation. As controls, nondamaged WT, Tnfrsf1a−/−, and Tnfrsf1b−/− mice were used. The expression of TNF-α in nondamaged mice was set to 1. In accordance with our previous data,6 TNF-α expression was significantly increased in the RPE/choroid layer of WT and TNF-α receptor−deficient animals as early as 1 day after laser treatment compared with nondamaged animals (Fig. 1). An approximately twofold increase in TNF-α expression after laser treatment was observed in all experimental groups compared with nondamaged controls (Fig. 1). Two weeks after laser treatment, TNF-α expression in the RPE/choroid layer of WT and Tnfrsf1a−/− animals remained higher than in nondamaged mice (P < 0.001 for both groups; Fig. 1). However, in Tnfrsf1b−/− animals, 2 weeks after laser photocoagulation, the TNF-α level was not significantly increased compared with the level in nondamaged animals and was significantly reduced compared with levels in WT (P < 0.001) and Tnfrsf1a−/− mice (P < 0.001) (Fig. 1).

**Decreased Fluorescein Leakage in Tnfrsf1b−/− Mice**

Two weeks after laser photoagulation, pathologic leakage representing CNV formation occurred in the mice of the three experimental groups—WT and Tnfrsf-deficient mice, as seen in representative angiograms taken from early (1–3 minutes) and late (6–8 minutes) phases after fluorescein injection (Fig. 2; Table 1). Large and diffuse areas of leakage were observed in Tnfrsf1a−/− and WT mice. Most of the evaluated Tnfrsf1a−/− mice (75%) and WT mice (65%) had + + + + + -grade (severe damage, hyperfluorescence that increases in size and intensity) and + + + + + + -grade (very severe damage, confluent increasing hyperfluorescence, almost complete occupation of the retina by the leakage, confluent laser spots) leakage. In contrast to the WT and Tnfrsf1a−/− mice, only 29% of the Tnfrsf1b−/− mice showed + + + + + + + lesions. The pathologic leakage was reduced in Tnfrsf1b−/− mice, and almost 70% of the animals showed + + -grade and + + + -grade lesions that corresponded to slight and moderate damage (hyperfluorescence without leakage or hyperfluorescence that increased in intensity but not in size) (Fig. 2).
Reduced CNV Lesions in Tnfrsf1b−/− Mice

Isolectin-positive areas representing CNV lesions were observed surrounding the optic nerve 2 weeks after laser injury (Fig. 3A). We identified isolectin-positive tubular structures indicating neovascular growth within the laser scars (Fig. 3A), single laser burns, magnification 20×). These structures were found predominantly at the margin but also in the central areas of the scars. This is in contrast to the homogeneous structure of the nondamaged choriocapillaris stained outside the region of the laser burns. In WT and Tnfrsf1a−/− mice, large areas of five laser scars around the optic nerve were detected (sometimes confluent with each other because of the extensive damage), occupied by contained proliferating flat and vessel-forming, tubule-like endothelial cells (Fig. 3A). In contrast, in Tnfrsf1b−/− mice, laser spots were smaller and had very clear borders. The area of neovascularization (μm²) was significantly smaller in Tnfrsf1b−/− mice (1.21 × 10⁴ ± 0.14 μm² (n = 8) vs. 2.34 × 10⁵ ± 0.97 μm² (n = 7)) than in WT mice (P < 0.001). In Tnfrsf1a−/− mice, the CNV area was larger than in WT control mice (2.91 × 10⁴ ± 0.32 μm²; n = 6) vs. 2.24 × 10⁵ ± 0.32 μm²; n = 6 (P < 0.05) (Fig. 3B).

Reduced Macrophage Infiltration in Tnfrsf1b−/− Mice

F4/80-positive cells were concentrated within the laser burns and around the borders of the laser scars 2 weeks after laser injury (Fig. 4A, choroid flatmounts). No F4/80-positive cells were observed in nondamaged areas of the choroid. Increased infiltration with F4/80-positive cells was observed, especially in Tnfrsf1a−/− mice and in a part in WT mice compared with Tnfrsf1b−/− animals (Fig. 4A). Many of the F4/80-positive cells colocalized with the endothelial cells within the laser burns. In WT mice, the area of colocalization (percentage of the whole burn area) was 6.63% ± 1.9% (n = 6) of the laser burn vs. 9.4% ± 1.5% (n = 6) in Tnfrsf1a−/− mice (P < 0.05). The area of colocalization in endothelial cells/macrophages (percentage from the whole burn) was significantly reduced in Tnfrsf1b−/− mice (3.1% ± 0.6%; n = 6; P < 0.001) compared with both WT and Tnfrsf1a−/− mice (Fig. 4B). Additionally, the areas (pixels) with F4/80-positive cells on the borders and outside the laser scars was significantly increased in Tnfrsf1a−/− (26,400 ± 2564 pixels; n = 6) and WT (19,45 ± 1279 pixels; n = 6) mice compared with the Tnfrsf1b−/− (10,532 ± 1198 pixels; n = 6; P < 0.001) (Fig. 4C). Histopathology on paraffin cross-sections (hematoxylin and eosin staining) 2 weeks after laser photocoagulation confirmed the increased the invasion of F4/80 cells in the retina over the laser scars and within the laser scars of WT and Tnfrsf1a−/− animals compared with Tnfrsf1b−/− mice (Fig. 5). Histology showed a discontinuity in Bruch’s membrane at the area of each laser burn in all mice (Fig. 5). Morphologic features of CNV among the groups were similar. However, smaller CNV lesions were observed in Tnfrsf1b−/− than in WT and Tnfrsf1a−/− mice. Furthermore, vWF stained demonstrated subretinal extension of choroidal vessels through Bruch’s membrane into the subretinal space (Fig. 5). Tnfrsf1b−/− mice exhibited sparse vascularized lesions with rare vessel-like structures (Fig. 5) compared with WT and Tnfrsf1a−/− mice. In all experimental groups, F4/80-positive cells were localized predominantly within the CNV lesions, close to the vascular lumina, or were colocalized with the vessels. F4/80-staining was very intensive in the CNV lesions of WT and Tnfrsf1a−/− mice. Most of the F4/80-positive cells had dendritic forms with many processes, but F4/80-positive cells with ameboid appearances were also observed. Many F4/80-positive cells on sections taken from WT and Tnfrsf1a−/− mice were localized in the retina above the CNV lesions. Large areas were observed with colocalized F4/80-positive cells with the blood vessels. In contrast, the intensity of the F4/80 staining was reduced in the CNV lesions of Tnfrsf1b−/− mice (Fig. 5). In the sections taken from Tnfrsf1b−/− mice, almost no dendritic F4/80-positive cells were observed.
Depletion of Circulating Macrophages by Clodronate Liposomes

To determine the role of both TNF receptors in the recruitment of inflammatory cells to the site of injury after laser treatment, we depleted circulating macrophages with clodronate treatment. This treatment is selective for circulating macrophages and is ineffective in depleting tissue-resident macrophages such as microglia. On choroidal flatmounts of PBS-injected WT and Tnfrsf1a−/− animals, large areas with F4/80-immunoreactive cells around and within laser burns can be observed (Fig. 6). In contrast, choroidal flatmounts of clodronic acid-treated WT and Tnfrsf1a−/− mice showed decreased F4/80-immunoreactivity that was accompanied by significant decreases in CNV areas compared with PBS-treated groups (1.9 ± 0.23 vs. 0.765 ± 0.18 μm² in WT, P < 0.001; and 2.4 ± 0.15 vs. 0.86 ± 0.33 μm² in Tnfrsf1a−/−, P < 0.01; n = 8–10 eyes per group). Importantly, in Tnfrsf1b−/− mice, infiltration with F4/80-positive cells and CNV formation were reduced in the PBS-treated control animals compared with WT and Tnfrsf1a−/− animals. Treatment with clodronic acid in these animals did not further reduce CNV size (Fig. 6).

Increased Apoptotic Activity in Tnfrsf1b−/− Mice

One week after laser treatment, expression of the active form of caspase-3 (~17 kDa) was increased in the RPE/choroid from Tnfrsf1b−/− mice (approximately 2.4-fold; P < 0.001) compared with the WT and Tnfrsf1a−/− (Fig. 7 A). Furthermore, we found strong expression of the active cleaved (~18 kDa) form of the caspase-8 protein in Tnfrsf1b−/− mice that was significantly higher than in WT and Tnfrsf1a−/− mice (P < 0.001; Fig. 7B). Densitometric analysis showed an approximately 2.7-fold increase in the activated caspase-8 form in the RPE/choroid complex of Tnfrsf1b−/− mice compared with WT mice (Fig. 7B). We investigated the apoptotic activity by TUNEL assay on paraffin sections (Fig. 8). Without laser treatment, no apoptotic cells were observed in the RPE/choroid area, and a few apoptotic cells were observed in the retina. In contrast, TUNEL-positive nuclei were observed in the laser scars of the WT and the Tnfrsf1b−/− mice 2 weeks after laser injury. Lesions in Tnfrsf1b−/− mice showed a higher number of TUNEL-positive cells (15.5 ± 2.6 [n = 5] vs. 5.3 ± 1.7 in WT; P < 0.01 [n = 4]) than did Tnfrsf1a−/− mice (4.5 ± 2.1; P < 0.01; n = 4) (Fig. 8). The higher apoptotic activity in Tnfrsf1b−/− mice was accompanied by less vascularization than in the WT and Tnfrsf1a−/− mice (Fig. 8).

Increased Angiogenesis and Bmx/Etk Kinase Activity in Tnfrsf1a−/− Mice

TNF-α receptor 1b-responsive tyrosine kinase Bmx/Etk was indicated as critical for receptor 1b-mediated angiogenesis. We stained parallel neighbor sections with antibody against vWF and Bmx/Etk. Bmx/Etk was expressed in the vascular endothe-
lium of WT and Tnfrsf1a<sup>−/−</sup> mice but not in Tnfrsf1b<sup>−/−</sup> mice (Fig. 9). In nondamaged regions far from the laser scars, Bmx/Etk expression was very weak. Increased Bmx/Etk expression corresponded to increased vascular response (vWF staining) in these animals.

**DISCUSSION**

We demonstrate here differences in CNV development between Tnfrsf1b<sup>−/−</sup> and Tnfrsf1a<sup>−/−</sup> mice 2 weeks after laser-induced rupture of Bruch's membrane. The loss of receptor 1a in Tnfrsf1a<sup>−/−</sup> mice led to exacerbation of CNV development compared with WT and Tnfrsf1b<sup>−/−</sup> animals. In contrast, the loss of TNF-α receptor 1b reduced the inflammatory response and pathologic angiogenesis after laser treatment. Our data show that TNF-α receptors 1a and 1b play different roles in the pathogenesis of CNV formation after laser photocoagulation. Although this model does not exactly mimic all the aspects of CNV that occur in association with AMD, it shares two important features. First, the inflammatory invasion and the abnormalities in Bruch's membrane are similar to those in AMD. Second, the new vessels develop from the choroid, grow along the edges of the laser burn, and proliferate into the subretinal space. Macrophage infiltration and increased expression of proinflammatory cytokines such TNF-α contribute to the CNV lesions after laser photocoagulation. We demonstrated previously, and also in this study, an increased expression of TNF-α in RPE/choroid after laser photocoagulation.
also demonstrated that anti–TNF-α treatment (etanercept) reduces the size and the leakage of laser-induced CNV. However, the exact role of TNF-α receptors 1a and 1b in the development of CNV is unknown. Tnfrsf1a is expressed ubiquitously, whereas Tnfrsf1b expression is tightly regulated and found predominantly on endothelial and hematopoietic cells. Under physiological conditions, both TNFR knockout lines did not show any pathologic changes in blood vessel growth within the retina and choroid. However, 2 weeks after laser photocoagulation—the time of peak of CNV extension in rodents severe damage with large areas of fluorescein leakage and advanced histologic changes were observed in WT and Tnfrsf1a−/− animals. We found a large number of cells immunoreactive for the macrophage/microglia marker F4/80 localized within the laser scars or surrounding the scars in Tnfrsf1a−/− and WT mice. Moreover, an increase in the density of F4/80-positive cells was observed in Tnfrsf1a−/− animals, and this correlated with a more severe CNV appearance in these mice compared with WT controls. In contrast, in Tnfrsf1b−/− mice, CNV membranes had smaller lesions and decreased fluorescein leakage compared with WT and Tnfrsf1a−/− mice. Reduced pathologic angiogenesis (2 weeks after laser treatment) was accompanied by decreased macrophage invasion after laser damage in these animals, suggesting that recruitment of inflammatory cells to the site of injury is critical in the development of CNV. It is unclear whether these cells were resident microglia cells or were recruited from peripheral blood monocytes. Depleting circulating macrophages with clodronic acid diminished the density of F4/80-positive cells in Tnfrsf1a−/− and WT mice and reduced CNV formation compared with vehicle treated controls. However, in Tnfrsf1b−/− mice, clodronic acid depletion did not change the macrophage invasion and CNV size, which appeared to be smaller than in other groups. These results suggest that signals through receptor 1b increase macrophage infiltration and recruitment of inflammatory cells to the site of injury. Because macrophages in CNV lesions are themselves a source of TNF-α, it is possible that through an autocrine loop the inhibition of macrophage infiltration itself, because of the loss of receptor 1b in Tnfrsf1b−/− animals, resulted in a lower level of TNF-α within the CNV lesions and thus further favored smaller lesion sizes. TNF-α regulates inflammatory cell activation and recruitment. This also favors the hypothesis that macrophage activation, TNF-α expression, and inflammatory cell recruitment may exert an autoregulatory and autocrine loop similar to the expression of VEGF by inflammatory cells. Furthermore, the apoptosis and subsequent clearance (efferocytosis) of inflammatory cells by macrophages are key mechanisms orchestrating successful resolution of inflammation. However, recently Michlewski et al. demonstrated that TNF-α potentially inhibits efferocytosis of neutrophils by monocyte-derived macrophages and therefore exacerbates the process of inflammation. We found increased apoptotic activity in Tnfrsf1b−/− mice compared with that in Tnfrsf1a−/− and WT mice after laser photocoagulation. Lesions in Tnfrsf1b−/− mice showed a higher number of TUNEL-positive cells localized in subretinal space. The higher number of apoptotic cells corresponded with reduced CNV lesions in these animals. This increased apoptotic activity in Tnfrsf1b−/− mice after laser photocoagulation was in accordance with the findings of Goukassian et al., who demonstrated that ischemia-induced endothelial cell apoptosis was greater in the limbs of Tnfrsf1b−/− mice than in those of WT and Tnfrsf1a−/− mice after oxygen challenge. Furthermore, Luo et al. demonstrated that Tnfrsf1a−/− mice experienced enhanced, whereas Tnfrsf1b−/− had reduced, ischemia-initiated angiogenesis and arteriogenesis compared with WT mice in a femoral artery ligation model, a commonly used in vivo arteriogenesis/angiogenesis model. The inhibited angiogenesis in Tnfrsf1b−/− mice was associated with increased endothelial cell death and decreased endothelial cell migration mediated through Tnfrsf1a in these mice.
In contrast to the low, constant, and universal expression of Tnfrsf1a under normal physiological conditions, the expression of Tnfrsf1b is inducible and restricted to hematopoietic cells and endothelial cells. Pan et al. identified Bmx/Etk (bone marrow tyrosine kinase in chromosome X)/Etk (endothelial/epithelial tyrosine kinase) as a Tnfrsf1b-responsive tyrosine kinase. Tnfrsf1b, but not Tnfrsf1a, specifically associates with and activates Bmx/Etk, which has been implicated in cell migration and proliferation/survival. We demonstrate here that Bmx/Etk is expressed in vascular endothelium of Tnfrsf1a−/− and WT mice, but not in Tnfrsf1b−/− mice. Bmx/Etk expression was increased within the laser burns; only weak Bmx expression was detected in the healthy retina/choroid. Increased Bmx/Etk expression correlates with the increased neovascularization in WT and Tnfrsf1a−/− mice. Our data from the CNV model further support the finding that Bmx-dependent transactivation of vascular endothelial growth factor receptor 2 is critical for TNF/Tnfrsf1b-induced endothelial cell migration and tube formation.

In conclusion, we demonstrate here that Tnfrsf1a and Tnfrsf1b play different roles in the formation of CNV after laser photocoagulation in mice. Our data demonstrate that the lack of circulating macrophages diminishes the CNV area in WT and Tnfrsf1a−/− mice 2 weeks after laser treatment. The choroidal flatmounts taken from PBS-treated (black bars) WT and Tnfrsf1a−/− mice showed large CNV areas with F4/80-positive infiltrating cells. Clodronic acid treatment (gray bars) strongly reduced both macrophage recruitment and CNV formation in these animals. F4/80-immunoreactivity and CNV areas were significantly decreased in both PBS-treated and clodronate-liposome-treated Tnfrsf1b−/− mice. Scale bar, 150 μm. *P < 0.05; n.s., not significant.

In contrast to the low, constant, and universal expression of Tnfrsf1a under normal physiological conditions, the expression of Tnfrsf1b is inducible and restricted to hematopoietic cells and endothelial cells.25,26 TNF-α receptors 1a and 1b might mediate different effects on endothelial cells. Pan et al.11 identified Bmx/Etk (bone marrow tyrosine kinase in chromosome X)/Etk (endothelial/epithelial tyrosine kinase) as a Tnfrsf1b-responsive tyrosine kinase. Tnfrsf1b, but not Tnfrsf1a, specifically associates with and activates Bmx/Etk, which has been implicated in cell migration and proliferation/survival. We demonstrate here that Bmx/Etk is expressed in vascular endothelium of Tnfrsf1a−/− and WT mice, but not in Tnfrsf1b−/− mice. Bmx/Etk expression was increased within the laser burns; only weak Bmx expression was detected in the healthy retina/choroid. Increased Bmx/Etk expression correlates with the increased neovascularization in WT and Tnfrsf1a−/− mice. Our data from the CNV model further support the finding that Bmx-dependent transactivation of vascular endothelial growth factor receptor 2 is critical for TNF/Tnfrsf1b-induced endothelial cell migration and tube formation.27 This is in accordance with investigations demonstrating an upregulation of Tnfrsf1b proteins and signaling (Tnfrsf1b-TNFR2-complex formation and Bmx/Etk activation) in vascular endothelium in response to ischemia.28 Pan et al.11 have identified Bmx/Etk (Etk (endothelial/epithelial tyrosine kinase) as a Tnfrsf1b-responsive tyrosine kinase.

In conclusion, we demonstrate here that Tnfrsf1a and Tnfrsf1b play different roles in the formation of CNV after laser photocoagulation in mice. Our data demonstrate that the lack of circulating macrophages diminishes the CNV area in WT and Tnfrsf1a−/− mice 2 weeks after laser treatment. The choroidal flatmounts taken from PBS-treated (black bars) WT and Tnfrsf1a−/− mice showed large CNV areas with F4/80-positive infiltrating cells. Clodronic acid treatment (gray bars) strongly reduced both macrophage recruitment and CNV formation in these animals. F4/80-immunoreactivity and CNV areas were significantly decreased in both PBS-treated and clodronate-liposome-treated Tnfrsf1b−/− mice. Scale bar, 150 μm. *P < 0.05; n.s., not significant.

**Figure 6.** Depletion of circulating macrophages diminished the CNV area in WT and Tnfrsf1a−/− mice 2 weeks after laser treatment. The choroidal flatmounts taken from PBS-treated (black bars) WT and Tnfrsf1a−/− mice showed large CNV areas with F4/80-positive infiltrating cells. Clodronic acid treatment (gray bars) strongly reduced both macrophage recruitment and CNV formation in these animals. F4/80-immunoreactivity and CNV areas were significantly decreased in both PBS-treated and clodronate-liposome-treated Tnfrsf1b−/− mice. Scale bar, 150 μm. *P < 0.05; n.s., not significant.

**Figure 7.** Western blot analysis of caspase-3 (A) and caspase-8 (B) protein expression in RPE/choroid 1 week after laser injury in Tnfrsf1a−/− and WT mice. Increased expression of the active form of caspase-3 was determined in Tnfrsf1b−/− compared with WT and Tnfrsf1a−/− mice. Densitometric analysis representing caspase-3 expression determined in three different blots (±SEM). Relative protein expression values are normalized to the corresponding β-actin expression (expression in WT mice set to 1). (B) Western blot analysis of caspase-8 protein expression in RPE/choroid of Tnfrsf1b−/− and WT mice. One week after laser photocoagulation, RPE/choroid complexes were isolated, and caspase-8 levels were determined by immunoblotting. Increased expression of the active form of caspase-8 in Tnfrsf1b−/− compared with Tnfrsf1a−/− and WT mice. Densitometric analysis shows the caspase-8 expression averaged from three blots (±SEM). Relative protein expression values are normalized to the corresponding β-actin expression (expression in WT mice set to 1). ***P < 0.001; n.s., not significant.
of receptor 1a is associated with increased laser-induced CNV size, increased vascular leakage from these neovascular lesions, and increased inflammatory cell recruitment to the site of injury. Tnfrsf1a-dependent apoptosis and Tnfrsf1b-dependent endothelial cell proliferation/migration represent two distinct pathways differentially activated by TNF-α. Together this evidence demonstrates the potential of specific targeting of TNF-α receptors for future therapies of inflammation-induced choroidal neovascularization.

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References


