Recruitment of Blood-Derived Inflammatory Cells Mediated via Tumor Necrosis Factor-α Receptor 1b Exacerbates Choroidal Neovascularization

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PURPOSE. Tumor necrosis factor (TNF)-α contributes to inflammation-associated angiogenesis, and TNF-α receptor 1b is selectively expressed on immune-competent and endothelial cells. This study investigated the role of TNF-α receptor 1b in the recruitment of circulating inflammatory cells and the development of choroidal neovascularization (CNV).

METHODS. Lethally irradiated Tnfrsf1b-/- mice and their wild-type (WT) controls were transplanted with whole adult bone marrow (BM) cells, competent for both TNF-α receptors 1a and 1b (gfp+ labeled), as well as with BM cells deficient for TNF-α receptor 1b. One month after transplantation CNV was induced by laser damage of Bruch’s membrane. Pathologic angiogenesis was estimated qualitatively and quantitatively by histology on choroidal flatmounts and paraffin cross sections. Macrophage invasion was investigated by immunochrometry.

RESULTS. One month after transplantation the reconstitution rate measured by FACS analysis was >80% in gfp+-chimeric mice. Two weeks after laser injury reduced gfp+-cell invasion to the laser scars and decreased pathologic angiogenesis were observed in Tnfrsf1b-/- versus WT recipients. Approximately 70% of the invaded gfp+ cells were labeled with macrophage marker F4/80. Transplantation of TNF-α receptor 1b–deficient BM cells in WT recipients reduced the CNV lesion compared with WT and Tnfrsf1b-/- recipients that received TNF-α receptor–competent BM cells. Transplantation of receptor 1b–deficient cells to Tnfrsf1b-/- recipients further reduced the degree of CNV formation.

CONCLUSIONS. Signals through TNF-α receptor 1b expressed on BM–derived inflammatory cells mediate an increased inflammatory cell invasion and enhanced angiogenic response after laser-induced rupture of Bruch’s membrane. (Invest Ophthalmol Vis Sci. 2011;52:6101–6108) DOI:10.1167/iovs.10-5996

Inflammatory mechanisms and immune activation have been implicated in the pathogenesis of choroidal neovascularization (CNV). Macrophages can be colocalized with endothelial cells in choroidal neovascular lesions.1,2 It was assumed that leukocytes contribute to CNV formation because their distribution correlates with CNV in humans and in animal models.3 The concept of inflammatory mediated neovascularization is further supported by studies showing that a generalized macrophage depletion reduced the size and leakage of laser-induced CNV.4,5 Migration of monocyte/macrophages from the blood circulation and activation of resident microglia cells in choroid and retina provoke local inflammation, which is commonly thought to be involved in the late stage of AMD.6 Recent studies showed that the CNV cell population has a dual origin (circulating versus resident populations). Circulating hematopoietic stem cells appear to have an important role in the formation of CNV as was shown by recent studies that vascular and inflammatory cells within CNV are derived from circulating precursors.7–11 Therefore, the inhibition of bone marrow (BM) cell mobilization to the eyes could be a new approach for the treatment of CNV. Inflammatory cells, especially blood-derived macrophages, are the most obvious nonocular cell type that might be recruited to CNV. Moreover, Espinosa-Heidmann and co-workers14 and Caicedo and co-workers6 demonstrated that blood-derived macrophages were the predominant form of monocyte at all time points during CNV formation. Infiltrating macrophages seem to play a critical role in the pathogenesis of CNV by secreting different angiogenic and inflammatory growth factors and cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-1β.3,15,16 Macrophage-secreted TNF-α triggers additional production of VEGF by RPE cells17 that was mediated through TNF-α receptor 1b.18 TNF-α further stimulates monocyte adhesion15 and upregulates granulocyte-macrophage colony-stimulating factor.19

The discussion of a primary role of inflammation versus a secondary event in the pathogenesis of CNV is still not solved; however, we22 demonstrated that inhibition of TNF-α by either a specific antibody or one of the clinically available inhibitors (etanercept and infliximab) is able to reduce lesion size and pathologic vascular leakage in a mice model of laser-induced CNV. However, little is known about the cellular events involved in TNF-α-macrophage–induced angiogenesis and whether signaling through TNF-α can influence the recruitment of inflammatory cells from the circulation to the site of injury. TNF-α receptors are expressed on many cell types in the retina and choroid, including RPE, Müller cells, and choroidal vascular cells.23 In contrast to the low, constant, and universal expression of TNF-α receptor 1a under normal physiological conditions, the expression of TNF-α receptor 1b is inducible and restricted to hematopoietic cells and endothelial cells.24,25 TNF-α protein expression was strongly enhanced in the RPE/choroid layer at different time points after laser photocoagulation.26 Little is known about the role of receptors 1a and 1b in TNF-α–macrophage–induced angiogenesis and development of CNV.
our recent study we reported that TNF-α receptors 1a and 1b play a differential role in the development of experimental CNV. In Tnfrsf1a−/− mice we observed an increased macrophage activity accompanied by increased angiogenesis and exacerbated CNV formation after laser photocoagulation when compared with WT and Tnfrsf1b−/− mice.24 This suggests that TNF-α through receptor 1b expressed on RPE/endothelial cells and hematopoietic cells might stimulate the vascularization via activation of resident and/or recruitment of inflammatory cells from the blood flow to the site of injury. To investigate this we created WT- and Tnfrsf1b−/− gfp−chimeric mice and followed the behavior of gfp−labeled donor blood cells in a model of laser-induced CNV. Additionally, to investigate the role of TNF-α receptor 1b expressed on the hematopoietic cells, we transplanted TNF-α receptor 1b-deficient donor BM cells to WT and Tnfrsf1b−/− recipients and investigated the development of CNV.

**Material 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 animal care and use committee, and the protocols were approved by the ‘Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.’ 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 Tnfrsf1b−/− mice was ensured through backcrossing Tnfrsf1b−/− mice onto the C57Bl/6J background for at least six generations. Bone marrow was obtained from donor mice C57BL/6-TgACTB-EGFP)1Osb/J transgenic for the chicken ß-actin promoter-GFP and cytomegalovirus enhancer (kindly provided by Bernd K. Fleischmann, Institute of Physiology, University of Bonn, Germany). In this transgenic mouse line with an enhanced GFP, promoter-GFP and cytomelagovirus enhancer (kindly provided by Bernd K. Fleischmann, Institute of Physiology, University of Bonn, Germany) and xylazine (0.1%; Bayer, Leverkusen, Germany) in a concentration of 0.1 mL/10 g mouse body weight. Neosynephrin-POS (5%; Ursapharm) was used to dilate the pupils. Through a coverslip Bruch’s membrane in anesthetized mice. Neosynephrin-POS (5%; Ursapharm) was used as a contact lens, five argon laser spots (100 mW intensity, 100 ms duration, 50 μm diameter) were applied to the retina and choroid. Laser spots were applied in a standardized fashion around the optic nerve. Production of a bubble at the time of laser exposure, which indicates rupture of Bruch’s membrane, is an important factor for inducing CNV, and therefore only mice in which a bubble was observed were included in the study. The development of CNV in these mice was compared with WT and Tnfrsf1b−/− mice that received whole BM isolated from WT donors. At 4 weeks after BM transplantation, the BM of the recipient mice usually regenerates by donor BM cells, laser photocoagulation was performed, and the development of CNV was investigated 14 days later as described below.

**FACS Analysis of the Blood**

Peripheral blood samples from the tail vein were used for FACS analysis. Blood drops from the tail vein were diluted in 200 μL PBS + 5 μL Heparin-Na (25,000 U; Roche, Mannheim, Germany). Erythrocytes were removed by lysis with erythrocyte lysis buffer (NH₄Cl, KNO₃, 6% EDTA) for 2 minutes. The reaction was stopped with an equal amount of PBS with 10% FCS. After centrifugation, cells were resuspended in PBS containing 10% FCS for FACS analysis. The samples were analyzed with a FACS Aria analyzer (BD Biosciences, San Jose, CA).

**Crossover Transplantations**

To investigate whether the lack of Tnfrsf1b on BM-derived cells can influence the recruitment of inflammatory cells and the development of CNV, we isolated whole BM cells from Tnfrsf1b−/− donors and transplanted them to lethally irradiated WT and Tnfrsf1b−/− recipient mice as described above. The development of CNV in these mice was compared with WT and Tnfrsf1b−/− mice that received whole BM isolated from WT donors. At 4 weeks after BM transplantation, when the BM of the recipient mice usually regenerates by donor BM cells, laser photocoagulation was performed, and the development of CNV was investigated 14 days later as described below.

**Induction of Experimental CNV via Laser Photocoagulation**

One month after BM transplantation, CNV lesions were induced in recipient mice by argon laser photocoagulation as previously described.28 In short, CNV was created by laser-induced rupture of Bruch’s membrane in anesthetized mice. Neosynephrin-POS (5%; Ursapharm) was used to dilate the pupils. Through a coverslip treated with methyl cellulose gel (2% Methocel; Omnidion, Puchheim, Germany), used as a contact lens, five argon laser spots (100 mW intensity, 100 ms duration, 50 μm size; Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany) were delivered to the retina and choroid. Laser spots were applied in a standardized fashion around the optic nerve. Production of a bubble at the time of laser exposure, which indicates rupture of Bruch’s membrane, is an important factor for inducing CNV, and therefore only mice in which a bubble was observed were included in the study. The development of CNV in the laser lesions was confirmed by fluorescein angiography. Two weeks later, animals were killed, and the eyes were removed for experimental analysis.

**Transcardial Perfusion with Rho-Labeled Concanavalin A**

Mice were perfused with rhodamine-labeled concanavalin A (20 μg/mL, in PBS; Alexis, Grünberg, Germany) that binds to the vasculature

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**Total Body Irradiation**

Recipient mice were irradiated under general anesthesia in an acrylic custom-made case with venting holes. Animals were lined up to posture the neck of each animal on the same line. Irradiation was conducted with a low-voltage radiation unit (U = 120 kV, I = 25 mA). The applied total radiation was 11 Gy. The irradiation source was adjusted to achieve total body irradiation. Irradiated mice in which venopuncture for transplantation was unsuccessful served as control for the lethal irradiation dose.

**Generation of Gfp+-Chimeric Mice**

Reconstitution of lethally irradiated C57BL/6j and Tnfrsf1b−/− mice with total BM cells from gfp+ donors (see above) was performed according to previously described methods by Grant and co-workers,27 Sengahta and co-workers,8 and Espinoza-Heidmann and co-workers.5 Briefly, 10 adult female C57BL/6j and 10 adult Tnfrsf1b−/− mice were used as recipient mice for bone marrow transplantation. After cervical dislocation of gfp− donor mice, the femur and tibia were dissected and placed in DMEM/Ham’s F12 culture medium with t-glutamine (PAA Laboratories, Pasching, Germany) at 4°C. Bone marrow was obtained by slowly flushing medium inside the diaphyseal channel with a syringe through a 27-gauge needle. Bone marrow was homogenized through a 27-gauge needle and filtrated with a nylon filter (70-μm pore size). Bone marrow donor cells were centrifuged and the pellets resuspended in the sterile PBS containing antibiotic mixture. Recipient mice (WT and Tnfrsf1b−/−) were lethally irradiated (see above) 1 day before transplantation. Whole BM mononuclear cells were obtained from gfp+ transgenic mice (heterozygous, >90% gfp+ circulating cells; FACS analysis) and injected into the tail vein of recipient WT and Tnfrsf1b−/− mice (2 × 10⁶ cells/200 μL cell suspension). As a control, whole BM from WT and Tnfrsf1b−/− mice was isolated and transplanted to lethally irradiated WT and Tnfrsf1b−/− mice, respectively, as described above. The survival rate of the mice transplanted with exogenous BM was 100%. Blood components were allowed to flow to the site of injury. To investigate this we created WT- and Tnfrsf1b−/− gfp−chimeric 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 Tnfrsf1b−/− mice was ensured through backcrossing Tnfrsf1b−/− mice onto the C57BL/6j background for at least six generations. Bone marrow was obtained from donor mice C57BL/6-10tgACTB-EGFP)1Osb/J transgenic for the chicken ß-actin promoter-GFP and cytomegalovirus enhancer (kindly provided by Bernd K. Fleischmann, Institute of Physiology, University of Bonn, Germany) and xylazine (0.1%; Bayer, Leverkusen, Germany) in a concentration of 0.1 mL/10 g mouse body weight. Neosynephrin-POS (5%; Ursapharm, Saarbrücken, Germany) was used to dilate the pupils. For all experiments age-matched animals with the same sex were used.
and labels the blood vessels in red. Briefly, 2 weeks after laser photocoagulation, WT and Tnfrsf1b^−/− recipient mice were perfused transcardially with PBS followed by 1% paraformaldehyde and then by rhodamine-labeled concanavalin A lectin. The eyes were enucleated and fixed in 2% paraformaldehyde. As a control, experimental CNV was induced in age-matched WT and Tnfrsf1b^−/− mice, without BM transplantation.

**Evaluation of CNV Areas on Flatmounts**

After transcardial perfusion and fixation of the enucleated eyes (see above) the anterior segment was removed, and the posterior cups of the enucleated eyes were flattened with the neurosensory retina facing up. The eyes were studied using confocal microscopy (Leica, Bensheim, Germany). All images were taken using a ×20 objective and series of Z-stack images taken throughout the entire retina/choroid 2 μm apart starting at the internal limiting membrane. Additionally, the flatmounts were examined by a fluorescence microscope (Zeiss Axioplan; Carl Zeiss Meditec). CNV area was defined as the area of damage due to laser photocoagulation, which is characterized predominantly by newly formed pathologic choroidal (leaky) vessels and migrated inflammatory cells that formed elevated neovascular complexes attached to the choroid. After perfusion with rhodamine-conjugated lectin, used to label the blood vessels, red hyperfluorescent areas (leakage) with tubular structures (vessels) were observed on the flatmounts surrounding the optic nerve. They corresponded to the laser spots (leakage) with tubular structures (vessels) were observed on the flatmounts surrounding the optic nerve. They corresponded to the laser spots.

**Histology and Immunohistochemistry on Cryosections**

For immunostainings 5-μm-thick cryosections were used. After fixation with cold acetone and blocking with normal goat serum the sections were incubated overnight at 4°C with rat anti-mouse CD31 (1:300, Abcam, Cambridge, UK) to visualize the blood vessels and rat anti-mouse F4/80 antibody (1:200; AbD Serotec, Oxford, UK) to label the monocyte/macrophages. As detection antibody rabbit anti-rat Cy3-conjugated antibody (1:300; Dianova, Hamburg, Germany) was used. Immunohistochemistry was evaluated by fluorescence microscopy. GFP- and F4/80-double positive cells were calculated near and within the laser scars.

**Statistical Analysis**

All results are expressed as mean and SD. Student’s t-test was used for populations with normal distribution and equal variance. The in vivo data were analyzed by Mann-Whitney U test with post hoc comparisons tested using Fisher’s protected least significant difference procedure. Differences were considered statistically significant when P values were <0.05. The significance was classified by asterisks: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Statistical analysis was done using commercial software (SPSS v. 15.0 for Windows; SPSS, Chicago, IL).

**RESULTS**

**Bone Marrow Chimera Studies**

One month after transplantation the BM was stably reconstituted and >80% of the blood cells were GFP^+ positive (FACS analysis; Fig. 1). Figure 1 shows a representative dotplot and
The area of neovascularization per choroid (Tnfrsf1b−/−) recipients was significantly smaller in Tnfrsf1b−/− recipients compared with WT recipients (1.95 × 10⁴ ± 0.23 μm² [n = 8] versus 3.6 × 10⁴ ± 0.67 μm² [n = 7]; P < 0.01) (Fig. 3A). The recruitment of gfp+ cells to the site of injury was also reduced in Tnfrsf1b−/− recipients compared with WT recipients. The areas (pixels) with gfp+ cells within the laser scars and on/near their borders was significantly increased in WT mice (67,230 ± 9870 pixels; n = 7) in comparison to the Tnfrsf1b−/− recipients (38,950 ± 7860 pixels; n = 8; P < 0.05) (Fig. 3B).

Cross-transplantations and Quantitative Evaluation of the Laser Burns

In further experiments, we performed laser photocoagulation in WT and Tnfrsf1b−/− recipients after transplantation of TNF-α receptors competent and TNF-α receptor 1b-deficient BM-derived cells. At the first set of experiments, gfp+ BM-derived cells from gfp−-transgenic donors were transplanted to WT and Tnfrsf1b−/− recipients (gfp−/WT and gfp−/Tnfrsf1b−/−) (see also Figs. 2 and 3). Gfp+ cells migrated to CNV lesions and localized within the laser burn or near the borders. Some of the green cells colocalized (orange) with the blood vessels (red) within the scars and at their borders. Outside the laser burns, the typical structure of nondamaged choriocapillaris was visible, and no gfp+ cells were identified. Additionally, we transplanted nonlabeled BM-derived cells (also TNF-α receptors competent) from WT donors that have the same genetic background as gfp−-transgenic mice (see Material and Methods) to WT and Tnfrsf1b−/− deficient recipients (WT/WT and WT/Tnfrsf1b−/−). The degree of CNV lesions histogram from flow cytometric analysis of blood obtained from gfp−-chimeric mice 1 month after transplantation.

Reduced CNV Formation and gfp+ Cell Density in Tnfrsf1b−/− Recipients

CNV lesions and the distribution of gfp+ cells in the recipient Tnfrsf1b−/− and WT mice are shown in Figure 2. The laser burns were visible on the flatmounts as red hyperfluorescent areas with vessel morphology, surrounded by the nondamaged choriocapillaris. We observed massive recruitment of gfp+ cells to the sites of choroidal damage after laser photocoagulation but not to the nondamaged choroid of both experimental groups (Figs. 2 A, 2B). Figures 2C and 2D show single CNV lesions visible under the layer of the retinal vessels. Gfp+ cells infiltrated the overlying neurosensory retina over the CNV lesions (Figs. 2E, 2F). The number of the extravasal BM-derived dendritic cells in the retina was markedly reduced in Tnfrsf1b−/− recipients compared with WT gfp+ chimeras. The area of neovascularization per choroid (μm²) was significantly smaller in Tnfrsf1b−/− recipients compared with WT recipients (107.2 ± 23.4; n = 4 versus 245.7 ± 46.8; n = 5; P < 0.01) (Figs. 2C–F).

Furthermore, CNV formation was significantly reduced in Tnfrsf1b−/− gfp+ chimeras in comparison with WT gfp+ chimeras. The area of neovascularization per choroid (μm²) was significantly smaller in Tnfrsf1b−/− recipients compared with WT recipients (1.95 × 10⁴ ± 0.23 μm² [n = 8] versus 3.6 × 10⁴ ± 0.67 μm² [n = 7]; P < 0.01) (Fig. 3A). The recruitment of gfp+ cells to the site of injury was also reduced in Tnfrsf1b−/− recipients compared with WT recipients. The areas (pixels) with gfp+ cells within the laser scars and on/near their borders was significantly increased in WT mice (67,230 ± 9870 pixels; n = 7) in comparison to the Tnfrsf1b−/− recipients (38,950 ± 7860 pixels; n = 8; P < 0.05) (Fig. 3B).
BM-derived cells from gfp−/− donors resulted in similar reductions in lesion size compared with WT recipients (Tnfrsf1b−/−/WT recipients). This decrease in lesion size was seen after transplantation of TNF-α receptor 1b−/− competent and TNF-α receptor 1b−/− deficient BM-derived cells to WT recipients (Tnfrsf1b−/−/WT) compared with WT/WT recipient mice when compared with WT recipients. Further, many gfp+ cells with elongated shape were localized close to the blood vessels within the laser scars of Gfp+/WT chimeras, and some of them appear to be well integrated into the neovascular tissue. Other gfp+ cells with dendritic shape were localized in the retina over the laser burns (Fig. 6).

Macrophages stained for F4/80 were present near or within the laser burns of WT and Tnfrsf1b−/− recipients (Fig. 7). The number of green cells expressing macrophage morphology and labeled with the macrophage marker F4/80 (orange) was higher in WT/gfp−/− mice compared with the WT recipients (WT/WT) compared with WT/WT and also WT/Tnfrsf1b−/− recipients. Further decrease in lesion size was seen after transplantation of TNF-α receptor 1b−/−/WT and Tnfrsf1b−/− recipients (Tnfrsf1b−/−/WT and Tnfrsf1b−/−/Tnfrsf1b−/− mice). Significant reduction in CNV size was also measured in WT recipients transplanted with BM-derived cells that lack receptor 1b (Tnfrsf1b−/−/WT) compared with WT recipients (1.45 × 10^4 ± 0.24 μm; n = 5 versus 3.97 ± 10^4 ± 0.3 μm; n = 6; P < 0.001) and WT/Tnfrsf1b−/− mice (1.45 × 10^4 ± 0.24 μm; n = 5 versus 2.12 × 10^4 ± 0.13 μm; n = 7, P < 0.05) suggesting that TNF-α receptor 1b expressed on BM-derived cells plays an important role in the mobilization and recruitment of these cells to the site of injury. Transplantation of TNF-α receptor 1b−/−/WT and Tnfrsf1b−/− recipients (Tnfrsf1b−/−/WT and Tnfrsf1b−/−/Tnfrsf1b−/− mice) further reduced the CNV lesion size compared with Tnfrsf1b−/−/WT recipients (0.87 ± 0.21 × 10^4 μm; n = 5 in Tnfrsf1b−/−/WT recipients versus 1.45 ± 0.2 × 10^4 μm; n = 5 in Tnfrsf1b−/−/WT recipients; P < 0.05) (Figs. 4 and 5). Immunohistochemistry on Cryosections

Cryosections were taken from WT and Tnfrsf1b−/−/gfp+ chimeras. 2 weeks after laser photocoagulation and were stained with antibodies against CD31 to label the blood vessels (Fig. 6) and F4/80 to label the macrophages (Fig. 7). Gfp+ labeled cells were visible throughout the CNV lesions. Many green cells with dendritic shape were observed in the neurosensory retina overlying the laser scars. To localize the migrated BM gfp+ cells in relation to the blood vessels and CNV lesions, we performed staining with CD31 antibody. CNV formation was reduced in Tnfrsf1b−/− recipients when compared with WT recipients. Furthermore, many gfp+ cells with elongated shape were localized close to the blood vessels within the laser scars of Gfp+/WT chimeras, and some of them appear to be well integrated into the neovascular tissue. Other gfp+ cells with dendritic shape were localized in the retina over the laser burns (Fig. 6).

Macrophages stained for F4/80 were present near or within the laser burns of WT and Tnfrsf1b−/− recipient mice (Fig. 7). The number of green cells expressing macrophage morphology and labeled with the macrophage marker F4/80 (orange) was higher in WT/gfp−/− mice compared with WT/WT mice (8.3 ± 2.8; n = 3 versus 4.2 ± 1.9; n = 4, P < 0.05). Using the monocytic marker F4/80 we observed that the majority of monocytes associated with CNV was gfp+ labeled, indicating that these cells had recently been recruited into the lesion from the circulation system (i.e., blood-derived macrophages). Resident F4/80-labeled cells (red) with dendritic shape but not gfp-positive were also observed near to the CNV regions (Fig. 7).

FIGURE 4. Representative images of CNV lesions after cross-transplantations. Laser burns on choroidal flatmounts from recipient WT and Tnfrsf1b−/− mice 2 weeks after laser treatment and after transplantation of TNF-α receptors competent and TNF-α receptor 1b−/− deficient BM-derived cells. Green fluorescence represents recruited gfp+BM-derived stem cells. Red fluorescence results from perfusion of the vasculature with rhodamine-conjugated Con A lectin. Gfp+ cells are shown invading CNV lesions in WT and Tnfrsf1b−/− recipients. Some of the green cells colocalized (orange) with the blood vessels (red) within and in the borders of the scars. Outside the laser burns, the typical structure of nondamaged choriocapillaris is visible. The degree of CNV lesions was reduced in Tnfrsf1b−/− compared with the WT recipients. Transplantation of TNF-α receptor 1b−/− deficient BM-derived cells to WT recipients (Tnfrsf1b−/−/WT) reduced CNV lesion size compared with WT/WT and also WT/Tnfrsf1b−/− recipients. Further decrease in lesion size was seen after transplantation of TNF-α receptor 1b−/− deficient BM-derived cells to Tnfrsf1b−/− recipients (Tnfrsf1b−/−/Tnfrsf1b−/−). Original magnifications, ×20. was reduced in Tnfrsf1b−/− compared with the WT recipients (WT/WT = 3.97 ± 10^4 ± 0.3; n = 6 and WT/Tnfrsf1b−/− = 2.12 ± 10^4 ± 0.13 μm; n = 7; P < 0.001) (Figs. 4, 5). This result was similar to results obtained after transplantation of BM-derived cells from gfp+ animals to WT and Tnfrsf1b-deficient recipients (gfp+/WT and gfp+/Tnfrsf1b−/−; see above and Figs. 2, 3). To investigate whether absence of TNF-α receptor 1b on the BM-derived cells can influence the recruitment of these cells and the development of CNV, we isolated BM cells from Tnfrsf1b-deficient mice and transplanted them to WT and Tnfrsf1b−/− mice (Tnfrsf1b−/−/WT and Tnfrsf1b−/−/Tnfrsf1b−/− mice). Significant reduction in CNV size was also measured in WT recipients transplanted with BM-derived cells that lack receptor 1b (Tnfrsf1b−/−/WT) compared with WT/WT recipients (1.45 × 10^4 ± 0.24 μm; n = 5 versus 3.97 ± 10^4 ± 0.3 μm; n = 6; P < 0.001) and WT/Tnfrsf1b−/− mice (1.45 × 10^4 ± 0.24 μm; n = 5 versus 2.12 × 10^4 ± 0.13 μm; n = 7, P < 0.05) suggesting that TNF-α receptor 1b expressed on BM-derived cells plays an important role in the mobilization and recruitment of these cells to the site of injury. Transplantation of TNF-α receptor 1b−/− deficient BM-derived cells to Tnfrsf1b−/− recipients (Tnfrsf1b−/−/Tnfrsf1b−/−) further reduced the CNV lesion size compared with Tnfrsf1b−/−/WT recipients (0.87 ± 0.21 × 10^4 μm; n = 5 in Tnfrsf1b−/−/WT recipients versus 1.45 ± 0.2 × 10^4 μm; n = 5 in Tnfrsf1b−/−/WT recipients; P < 0.05) (Figs. 4 and 5).

FIGURE 5. Quantification of CNV lesion size (μm²) on choroidal flatmounts after cross-transplantations (see Fig. 4). The total CNV area per flatmount was measured. Increased total CNV area per eye (μm²) is measured in WT recipients transplanted with WT-BM-derived stem cells (WT/WT) compared with WT recipients transplanted with TNF-α receptor 1b−/− deficient BM-derived cells (WT/Tnfrsf1b−/−) compared with WT/WT recipients (***P < 0.001 versus WT/WT). Significant reduction in CNV size is measured also in WT recipients transplanted with BM-derived cells that lack receptor 1b (Tnfrsf1b−/−/WT) compared with WT/WT recipients (***P < 0.001) and WT/Tnfrsf1b−/− mice (*P < 0.05). Transplantation of TNF-α receptor 1b−/− deficient BM-derived cells to Tnfrsf1b−/− recipients (Tnfrsf1b−/−/Tnfrsf1b−/−) reduced further the CNV lesion size (*P < 0.05, versus Tnfrsf1b−/−/WT mice (see also Fig. 4).
Recruited near and within the laser burns of WT and Tnfrsf1b−/− co-workers showed abundant blood-derived macrophages. The cryosections were stained with CD31 antibody to label the vessels. WT recipients had larger CNV lesions compared with Tnfrsf1b−/− mice and this correlated with the number of recruited gfp+ cells. Gfp+ labeled cells have stellar or amoeboid shapes. Our work demonstrates that gfp+ BM-derived cells were recruited near and within the laser burns of WT and Tnfrsf1b−/− recipients and were very sparse in the nondamaged choroid. Gfp+ cells were part of the CNV lesions, and many of them colocalized with the blood vessels. The most of the gfp+ cells recruited to the site of injury had a dendritic form and were positive for the macrophage marker F4/80. Gfp+ labeled cells positive for F4/80 were also observed in retinal regions adjacent to the CNV lesions. In contrast, choroidal and retinal regions far from the CNV formation were almost completely devoid of gfp+ cells. Our study shows for the first time that the absence of TNF-α receptor 1b in host leads to a reduced mobilization and homing of blood-derived inflammatory cells to the site of injury. Our study shows for the first time that the absence of TNF-α receptor 1b in host leads to a reduced mobilization and homing of blood-derived inflammatory cells to the site of injury. Our work demonstrates that gfp+ BM-derived cells were recruited near and within the laser burns.

**DISCUSSION**

Our work demonstrates that gfp+ BM-derived cells were recruited near and within the laser burns of WT and Tnfrsf1b−/− recipients and were very sparse in the nondamaged choroid. Gfp+ cells were part of the CNV lesions, and many of them colocalized with the blood vessels. The most of the gfp+ cells recruited to the site of injury had a dendritic form and were positive for the macrophage marker F4/80. Gfp+ labeled cells positive for F4/80 were also observed in retinal regions adjacent to the CNV lesions. In contrast, choroidal and retinal regions far from the CNV formation were almost completely devoid of gfp+ cells. Our results showed extensive gfp+ cell recruitment from the blood flow to the damaged choroid and adjacent retina 2 weeks after laser photocoagulation. The studies of Espinosa-Heidmann and co-workers and Caicedo and co-workers also showed abundant blood-derived macrophages in experimental CNV using also gfp+-chimeric mice. Moreover, Caicedo and co-workers showed that almost 70% of F4/80-positive cells were also gfp+ labeled in a similar model of gfp+ chimerism and laser-induced CNV. Gfp+ chimeric mice are a useful tool to analyze the role of resident and circulating macrophages in the development of CNV. Recruited macrophages are described as having a more destructive potential than resident microglia. They might regulate the severity of the CNV by secreting factors that promote the growth and invasion of new vessels such as TNF-α and VEGF. We have shown previously that the TNF-α protein content was strongly enhanced in RPE/choroid layers near the laser-induced scars and that the application of TNF-α antagonists reduced the development of CNV in mice. In our previous study, we reported about a reduced macrophage density and a decreased CNV severity in TNF-α receptor 1b-deficient mice in comparison with either WT or TNF-α receptor 1a-deficient mice and emphasized the different role of both TNF-α receptors in the development of CNV. To clarify whether the absence of TNF-α receptor 1b reduced the mobilization and recruitment of blood-derived inflammatory and other cell types to the site of injury, we generated gfp+ chimeric mice. At the first set of experiments, we transplanted gfp+ BM-derived cells (competent for both TNF-α receptors) to WT and Tnfrsf1b−/−-deficient mice. In general, the recruitment of gfp+ cells with dendritic form from the blood to the injured retinal/choroidal regions was reduced in Tnfrsf1b−/− gfp+-chimeric mice compared with WT gfp+-chimeric mice. Furthermore, we found that the reduced recruitment and density of gfp+-inflammatory cells in Tnfrsf1b−/− recipients correlated with reduced CNV sizes in these animals compared with the WT gfp+ chimeras. In line with our findings, it has been previously shown that the depletion of circulating macrophages is able to reduce CNV severity. Therefore, we suggest that lack of TNF-α receptor 1b expression in RPE/endothelial cells of Tnfrsf1b−/−-deficient mice reduced the degree of CNV formation probably via reduced mobilization and homing of blood-derived inflammatory cells to the site of injury. Our study shows for the first time that the absence of TNF-α receptor 1b in host leads to a reduced mobilization and homing of blood-derived inflammatory cells to the site of injury after laser photocoagulation and that this was accompanied with decreased CNV formation in Tnfrsf1b−/− gfp+-chimeric mice compared with the WT gfp+ chimeras. On the other hand, gfp+ /Tnfrsf1b−/− chimeric mice received TNF-α receptor-competent BM cells from the gfp-transgenic donors, which can respond to TNF-α signals. In this respect, is not clear whether TNF-α receptor 1b expressed on the RPE/endothelial cells or on blood-derived hematopoietic cells is involved stronger in the mobilization and recruitment of inflammatory cells after laser photocoagulation. Besides TNF-α receptor 1b expressed on RPE/endothelial cells, it has been shown that macrophages are the crucial effector cells, which respond to TNF-α via TNF-α receptor 1b expressed on their cell surface. Zhao and co-workers showed that large amounts of macrophages

![Figure 6](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933460/)  
**FIGURE 6.** Cryosections through the laser burns from WT and Tnfrsf1b−/− recipients transplanted with gfp+-labeled BM-derived stem cells. The cryosections were stained with CD31 antibody to label the vessels (red). WT recipients had larger CNV lesions compared with Tnfrsf1b−/− mice and this correlated with the number of recruited gfp+ cells. Gfp+ labeled cells have stellar or amoeboid shapes. Gfp+ cells were localized within and near the laser burns.

![Figure 7](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933460/)  
**FIGURE 7.** Cryosections through the CNV lesions from WT and Tnfrsf1b−/− recipients. The cryosections were stained with rat anti-RPE/endothelial cells (red fluorescence) to identify whether the gfp+ cells are macrophages and to distinguish them from the resident F4/80-positive cells. CNV lesions (×20 magnification) in WT mice were larger compared with Tnfrsf1b−/− mice, and the number of Gfp/F4/80 double-positive cells (orange) was increased.
infiltrate regressing tumors in Tnfrsf1a-deficient mice. Therefore, we investigated whether transplantation of TNF-α receptor 1b-deficient BM-derived cells can influence the development of CNV in both WT recipients and Tnfrsf1b−/− recipients. Transplantation of TNF-α receptor 1b-deficient stem cells to the WT mice significantly decreased the severity of CNV lesions compared with WT recipients that received TNF-α receptor 1b-competent BM-derived cells, suggesting that signals through TNF-α receptor 1b on the BM-derived cells can induce mobilization and recruitment of inflammatory cells to the site of injury. The important role of TNF-α receptor 1b expressed not only on the BM-derived cells but also in RPE/endothelial cells of the host animals was confirmed with the experiments, in which transplantation of BM-derived cells that lack TNF-α receptor 1b to Tnfrsf1b−/− recipients was performed. In these recipients the size of CNV lesions was further significantly reduced compared with all other experimental groups. In contrast to the low, constant, and universal expression of TNF-α receptor 1a under normal physiological conditions, the expression of TNF-α receptor 1b is inducible and restricted to hematopoietic cells and endothelial cells. This suggests that TNF-α signals through receptor 1b expressed on the BM cells is important for the recruitment of these cells to the site of injury. These results support the hypothesis that TNF-α via receptor 1b might mediate macrophage activation and increased invasion of these cells to the site of injury. Our results are in agreement with findings demonstrating that TNF-α stimulates the migration of bovine adrenal capillary endothelial cells and their formation into capillary-like-tube structures. Under inflammation and wound repair conditions, TNF-α could augment repair by stimulating new blood vessel growth.

The traditional paradigm of angiogenesis assumes that vascular cells in CNV are derived only from preexisting resident cells from the adjacent normal vascular bed. However, many research groups has demonstrated that circulating BM-derived vascular precursor cells also contribute significantly to CNV composition. Most studies have focused on the role of endothelial cells. In our hands, abundant blood-derived macrophages are observed in all recipients. However, only a few gfp+ cells within the laser burns were not stained with CD31 antibody. These results are not in agreement with previous findings that showed significant involvement of vascular endothelial cells derived from BM precursors in the development of CNV lesions. One explanation might be the fact that we transplanted unpurified total BM-derived stem cells without selection to lin− cells that have been used from above cited groups.

Taken together, our study shows that signals through TNF-α receptor 1b expressed on BM-derived inflammatory cells mediate increased inflammatory cell invasion and enhanced angiogenic response after laser-induced rupture of Bruch’s membrane. These data indicate that analysis of circulating blood monocytes and their behavior and influence via TNF-α signaling is relevant to the pathogenesis of CNV and support the idea that preventing their recruitment is a potential therapeutic target.

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References


