Vascular Biology, Atherosclerosis and Endothelium Biology

TRAIL-Deficient Mice Exhibit Delayed Regression of Retinal Neovascularization

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While it is well established that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in various cell types, the role of TRAIL in regulation of retinal neovascularization (NV) has not been described. Here we determined the role of TRAIL in retinal NV during oxygen-induced retinopathy using TRAIL deficient ($^{-/-}$) mice. TRAIL and its receptor, DR5, were expressed in wild-type retinas at all time points evaluated (postnatal days 12, 17, 21, 24) during oxygen-induced retinopathy and in age-matched room air control animals. Localization of TRAIL⁺ cells within the neovascular tufts of hyperoxia-exposed wild-type mice suggested TRAIL plays a role in oxygen-induced retinopathy. Retinal vascular development appeared normal in the TRAIL $^{-/-}$ mice, except for a small but significant difference in the capillary-free zone surrounding major arteries. A minimal difference in avascularity was observed at postnatal day 12 in the retinas of TRAIL $^{-/-}$ mice after hyperoxia-exposure compared with wild-type mice, suggesting that TRAIL does not play a major role in the vaso-obliterative phase of oxygen-induced retinopathy. However, at the peak of NV, TRAIL $^{-/-}$ mice had a significant increase in retinal neovascularization. In addition, when NV naturally regresses in wild-type mice, TRAIL $^{-/-}$ mice continued to display significantly high levels of NV. This was attributed to a significant decrease in neovascular tuft cells undergoing apoptosis in TRAIL $^{-/-}$ mice. Together, these data strongly suggest that TRAIL plays a role in the control of retinal NV. (*Am J Pathol* 2009, 175:2697–2708; DOI: 10.2353/ajpath.2009.090099)

The hallmark of ischemic retinopathies, including retinopathy of prematurity, is pathological retinal neovascular-

ization (NV). Animal models, including the mouse model of oxygen-induced retinopathy (OIR), allow for the study of this process.^{1,2} OIR is characterized by two pathological phases. In the initial vaso-obliterative phase, exposure to hyperoxia leads to vasoconstriction, vaso-obliteration, and prevention of new blood vessel growth in the immature retina. During the second phase, the return to a normoxic environment leads to relative retinal ischemia, as a result of blood vessel loss in the first phase. Subsequent production of vascular endothelial growth factor (VEGF) results in pathological retinal NV.³

Overall, OIR is controlled by a balance of pro- and anti-angiogenic factors, or anti- and pro-apoptotic factors, which regulate endothelial cell (EC) proliferation and death, respectively. While VEGF is the prototypical pro-angiogenic factor, Fas Ligand, endostatin and PEDF have been characterized as anti-angiogenic factors, possessing pro-apoptotic properties.^{4,5} Another pro-apoptotic factor, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which forms a trimerized protein in its active state, binds four known membrane-bound receptors in the human.^{6,7} DR4 and DR5 are death-inducing receptors, each with a death domain that serves as the recognition site for the proximal components of the apoptosis signaling pathway after the receptor trimerization.⁸ When TRAIL binds a death receptor, the death-inducing signaling complex assembles at the death receptor (DR)4/DR5 death domain, resulting in caspase-8 activation. Caspase-8 then initiates a series of substrate proteolytic cleavages that culminates in apoptotic cell death.^{7,9} DcR1 and DcR2 are the other two membrane-

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bound receptors that bind TRAIL, but do not induce the apoptotic signaling cascade. They have been proposed to be decoy, or perhaps more appropriately, regulatory receptors.¹⁰ In the mouse, one death-inducing TRAIL receptor has been identified that is most homologous to DR5 and was initially named mouse KILLER/DR5 [hereafter referred to as DR5].¹¹ Two decoy receptors have been identified in the mouse, termed mouse decoy TRAIL Receptor 1 (mDcTRAILR1) and mDcTRAILR2, but little is known regarding their functional properties.¹² Osteoprotegerin, a soluble TRAIL receptor, can also bind to TRAIL, preventing activation of the death inducing TRAIL receptors.¹³

TRAIL expression has been described in multiple cell lines and tissues.^{9,14,15} That TRAIL induces apoptosis in transformed cell lines and tumor cells but not in normal, non-transformed cells and tissues has been well established.^{10,16} However, the TRAIL death receptors have also been localized to a variety of normal tissues, including blood vessels.^{17,18} Furthermore, recent reports detail EC apoptosis induction by TRAIL,^{19,20} making TRAIL germane to the study of angiogenesis. Previous work in our laboratory and others has examined the roles of various death receptors and their ligands in OIR.^{4,21} In FasL mutant (*gld*) mice, the peak of NV is increased at P17 compared with wild-type, but the NV regresses to a level comparable with that of wild-type on P21.^{4,22} This suggests that although FasL plays a role in the formation of NV, it does not seem to be crucial in the process of NV regression, warranting investigation of other pro-apoptotic ligands and their receptors. In recent gene profiling studies, DR5 was found to be expressed in human retinal EC *in vivo*.²³ In the current study, the role of TRAIL in vascular regression is investigated in the mouse model of OIR, comparing TRAIL^{-/-} and wild-type mice. Our data suggests that TRAIL, through its pro-apoptotic properties, is integral in the prevention and control of oxygen-induced retinopathy in the mouse model.

Materials and Methods

Animals

Breeding pairs of C57BL/6 (B6) mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine) and used as wild-type controls. TRAIL^{-/-} mice, backcrossed onto a C57BL/6 background, have been previously characterized.²⁴ All mice were provided food and water *ad libitum*, and kept on a 12-hour light-dark cycle. Mice were housed and bred in the Oregon Health & Science University animal care facilities and treated in compliance with the NIH guidelines and the guidelines outlined in the Association for Research in Vision and Ophthalmology statement for "The Use of Animals in Ophthalmic and Vision Research." All protocols were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee. Using the mouse model of OIR established by Smith and colleagues,² both TRAIL^{-/-} and wild-type control pups, along with nursing females, were exposed to 75% oxygen

for 5 days beginning on P7 and then recovered in room air on P12. Hyperoxia-exposed and room air control pups were euthanized by CO₂ euthanasia or cervical dislocation on P8, P12, P17, P21, and P24. One eye was carefully enucleated from each mouse, placed in 10% neutral-buffered formalin for 3 hours, and routinely processed for paraffin embedding. These eyes were sectioned at 5- μ m intervals, mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA) and stored at room temperature until used for immunohistologic, proliferating cell nuclear antigen (PCNA), and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis. The retina of the contralateral eye was dissected for mRNA isolation and analysis as described below. Furthermore, subsets of mice were used for retinal flat-mount studies, as described below.

Reverse Transcription-PCR

Retinas were dissected on postnatal days (P)17, P21, and P24 from room air and hyperoxia-exposed wild-type mice and pooled ($n = 4$) for RNA isolation and analysis. RNA was extracted using Qiagen (Valencia, CA) RNeasy columns according to the manufacturer's instructions. Total RNA was purified by DNase treatment to remove potential genomic DNA contamination. cDNA was then synthesized using oligo(dT)-primed M-MLV reverse transcriptase (Promega, Madison, WI) for 2 hours at 37°C. PCR was then performed to demonstrate expression of TRAIL, DR5, mDcTRAIL R1, and osteoprotegerin mRNA. Primers used include TRAIL: forward 5'-ACCTCAGCTTCAGTCAGCACTTCA-3', reverse 5'-AAGCTGAGTTGCTTCTCCGAGTGA-3'; DR5 forward 5'-ACAGCTAACCCAGCCCATAATCGT-3', reverse 5'-TGCAGTTAGAGCATGACTGGCAGA-3'; mDcTRAIL R1 forward 5'-TCAGTTGGACAGAGCCCTCAAACA-3', reverse 5'-AAGTGCCCATGTGCAGAGAGAGAA-3'; and osteoprotegerin forward 5'-GGTGACCAAGACACCTTGAAGGG-3', reverse 5'-GGGTGACAGTTTTGGGAAAGTGG-3'. A primer pair for glyceraldehyde-3-phosphate dehydrogenase was included in each assay as an internal loading control; primers: forward 5'-CGGCATCGAAGGTGGAAGAGT-3', and reverse, 5'-GCATGGCCTTCCGTGTTCTTA-3'.

Western Blot Analysis

TRAIL protein levels were evaluated by Western blot analysis of whole retina extracts prepared from P8, P12, P17, and P21 room air and hyperoxia-exposed mice. Retinas were dissected and homogenized in radioimmunoprecipitation assay lysis buffer. They were incubated at 4°C for 30 minutes and then centrifuged at 10,000 rpm for 20 minutes. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein (60 μ g) were separated by SDS-polyacrylamide gel electrophoresis (10% Tris-HCl Ready Gels, BioRad, Hercules, CA) and transferred to a nitrocellulose membrane. Membranes were incubated with primary goat anti-human-TRAIL antibody (1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Membranes were washed and incubated with a secondary antibody of donkey-anti-goat IR-800 (1:5000, Odyssey, Licor, Lincoln, NE). The same membrane was also probed with anti- β -actin (Sigma, St. Louis, MO) to verify equal protein loading in all lanes.

Immunohistochemistry

For whole mounts, the retina and lens were dissected and fixed in 4% paraformaldehyde for 30 minutes at 4°C. The lens was then removed and the retina was fixed for 15 minutes in 100% methanol. Following a series of TBS washes and a blocking step with rabbit serum, retinas were incubated overnight at 4°C with Alexa Fluor 594 labeled Griffonia Simplicifolia (GS)-isolectin (Invitrogen, Carlsbad, CA), an EC marker, and a primary antibody against TRAIL (Santa Cruz Biotechnology, Inc.) or DR5 (eBioscience, San Diego, CA). An Alexa Fluor 488 (Invitrogen) labeled secondary antibody was used to localize TRAIL or DR5 expression. Retinas were then incised radially and flat-mounted with SlowFade mounting media (Invitrogen). Frozen retinal sections, 10 μ m each, were immunolabeled with a primary antibody against TRAIL alone (Santa Cruz Biotechnology, Inc.), or double labeled with an anti-F4/80 monoclonal antibody (Serotec, Raleigh, NC). Following an overnight incubation at 4°C with primary antibody, sections were incubated with appropriate AlexaFluor 594 (Invitrogen) or rat anti-mouse secondary antibodies labeled with fluorescein isothiocyanate. Additional sections were immunolabeled with a primary antibody against DR5, an Alexa Fluor 488 secondary antibody and double labeled with GS-isolectin. Sections were counterstained with 4,6-diamidino-2-phenylindole and mounted with SlowFade antifade (Invitrogen). Retinal whole mounts and cross sections were visualized by either light or fluorescence microscopy and photographed with a Leica DC500 digital camera (Leica Microsystems, Bensheim, Germany).

Assessment of Vascular Development of the Retina

A quantitative assessment of retinal vasculature was performed on wild-type and TRAIL^{-/-} mice at P3, P5, and P7. Retinal whole mount retinas were isolated and incubated with GS-isolectin as described above ($n = 6$). Retinal vessels were visualized and imaged by fluorescence and confocal microscopy (Olympus Fluoview 1000 Confocal Microscope; Olympus Corporation, San Diego, CA). Images were then viewed and analyzed using Photoshop CS4 software (Adobe, San Jose, CA). The vascular area was traced manually and calculated as a percentage of total retinal area, allowing comparison of vascular areas between wild-type and TRAIL^{-/-} mice. Data are expressed as mean $\pm$ SEM. A student's *t*-test was used to compare the two types of mice.

Measurement of Capillary-Free Zones

A quantitative assessment of the capillary-free zone surrounding the retinal arteries in wild-type and TRAIL^{-/-} mice was performed at P5 and P7. Whole mounted retinas were isolated and incubated with GS-isolectin as described above. Retinas were also stained with α -smooth muscle actin (Sigma). Photographs of the arteries within the retina were obtained by confocal microscopy (Olympus). Arteries were identified by presence of α -smooth muscle actin staining as well as by the presence of a capillary-free zone. A standard 500 μ m length of artery beginning at the edge of vascularization, closest to the optic nerve, was approximated. The avascular area around that artery, for the approximated 500 μ m, was traced and calculated using Photoshop software in a masked fashion. The actual length of artery was then measured and a ratio of area (μ m²) per 100 μ m of arterial length was calculated and compared between wild-type and TRAIL^{-/-} mice. Six retinas were examined at each time point for each animal strain ($n = 12$ to 19 arteries per time point per strain). Data are expressed as mean $\pm$ SEM. A student's *t*-test was used to compare the two types of mice.

Assessment of Avascular Area

A quantitative assessment of retinal vasculature was performed on hyperoxia-exposed mice at P8, P12, P17, and P21 as described above. Retinal vessels were visualized by fluorescence microscopy and photographed with a Leica DC500 digital camera (Leica). Images were then analyzed using Image Pro Plus software (Media Cybernetics, Bethesda, MD). In this software, the avascular area is calculated²⁵ and compared between wild-type and TRAIL^{-/-} mice. Data are expressed as mean $\pm$ SEM.

Neovascular Nuclei Quantification

NV was quantified by counting the vascular nuclei that extended anterior to the inner limiting membrane in H&E stained sections. Retinal tissue sections were routinely deparaffinized and rehydrated. Retinal vascular nuclei were counted in a masked fashion and averaged, avoiding hyaloid vessel nuclei near the optic disk and lens (At all time points, $n = 6$ to 10 eyes, 15 sections per eye). Data are expressed as mean $\pm$ SEM.

TUNEL Assay

At P17 and P21 after hyperoxia exposure, ($n = 6$ to 8 eyes, 10 sections per eye), retinal tissue sections, from both wild-type and TRAIL^{-/-} mice, were routinely deparaffinized and rehydrated before antigen retrieval by proteolytic digestion with proteinase K for 5 minutes at room temperature. Sections were labeled with the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY) according to the manufacturer's instructions. After labeling the exposed 3'-OH ends of DNA fragments, apoptotic cells were visualized with diaminoben-

zidine substrate and counterstained with methyl green. Representative sections, avoiding the optic nerve were assessed for TUNEL-positive (TUNEL⁺) cells located anterior to the inner-limiting membrane and quantified in a masked fashion. Data are expressed as mean percentage of TUNEL⁺ neovascular nuclei $\pm$ SEM. In addition, TUNEL⁺ cells were quantified in the area that included the inner plexiform, inner nuclear and outer plexiform layers. Finally, TUNEL⁺ cells were quantified in the outer nuclear layer.

PCNA Assay

At P17 and P21 after hyperoxia exposure ($n = 6$ to 12 eyes, 6 sections per eye), retinal tissue sections, from both wild-type and TRAIL^{-/-} mice, were routinely deparaffinized and rehydrated. Antigen retrieval was performed with a 20 minute pepsin digest followed by incubation with 3% hydrogen peroxide to quench endogenous peroxidases. Cross sections were then blocked with goat serum for 60 minutes followed by incubation overnight with rabbit anti-proliferating cell nuclear antigen (PCNA; Abcam, Cambridge, MA) at 4°C. Sections were then incubated with Biotinylated goat anti-rabbit IgG for 60 minutes. ABC-HRP (Vector Laboratories, Burlingame, CA) was applied to the retinas and visualized with diaminobenzidine substrate. Methyl green was used to counterstain. Proliferating cells anterior to the inner limiting membrane were quantified in a masked fashion. Data are expressed as mean percentage of PCNA-positive neovascular nuclei $\pm$ SEM.

Induction of Apoptosis by TRAIL in Human Retinal Endothelial Cells

ACBRI 181 Primary Human Retinal Microvascular Endothelial Cells (Applied Cell Biology Research Institute, Kirkland, WA) were grown to 80% confluence in the presence of MCDB131 media (Sigma) plus 10% to 20% fetal calf serum, with 1 μ g/ml amphotericin B and endothelial growth factors (Clonetics, Cambrex Bioscience, Walkersville, MD; EGM-2 SingleQuot, without addition of gentamicin, hydrocortisone, and fetal bovine serum). Once at 80% confluence, cells were kept in low nutrient (0.1%) serum overnight. After this overnight serum starvation, serum and other growth factors were reintroduced in eight different conditions, as follows: 1) No serum or growth factors; 2) Fresh, fully supplemented media; 3) VEGF (10 ng/ml), with no serum; 4) Low-dose TRAIL (10 ng/ml) plus VEGF; 5) High-dose TRAIL (100 ng/ml) plus VEGF; 6) 6X-His (10 μ g/ml) plus VEGF; 7) Low-dose TRAIL plus 6X-His and VEGF; 8) High-dose TRAIL plus 6X-His and VEGF. 6X-His is an antibody that cross-links TRAIL to simulate the trimerization of TRAIL, which is necessary to bind DR5 *in vivo*.^{15,26} After 24 hours of stimulation with these serum and growth factor combinations, a TUNEL assay, as described above, was performed and apoptotic cells quantified ($n = 3$ plates, four areas counted from each plate). Differences in numbers

of apoptotic cells were analyzed using one-way analysis of variance.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM. Statistical significance was determined using the Student's *t*-test for comparison between two groups or one-way analysis of variance for multiple group comparison (Prism, Graphpad, San Diego, CA). Statistical significance was set at $P < 0.05$ in all statistical analyses used.

Results

Retinal TRAIL Expression in Wild-Type Mice

We initially analyzed TRAIL mRNA expression in normal and diseased mouse retina from wild-type mice using RT-PCR. TRAIL mRNA expression was observed on P17, P21, and P24 in room air-control mice, as well as hyperoxia-exposed mice (Figure 1 A, top). Western blot analysis of TRAIL protein expression in wild-type mice at P8, P12, P17, and P21 was observed with no alteration of expression in the hyperoxia-exposed retinas at any time point (Figure 1A, bottom). Using immunohistochemistry, TRAIL protein expression was then evaluated in fresh-frozen retinal sections and in retinal whole mounts from control and hyperoxia-exposed mice. In hyperoxia-exposed mice, TRAIL immunoreactivity was localized in and around the neovascular tufts at both P17 and P21 (Figure 1B, arrows). In contrast, TRAIL-positive cells were restricted to intraretinal regions in room air control mice on P17 (Figure 1B, arrow). In retinal whole mounts, TRAIL-labeled cells (green, arrows) were associated with GS-isolectin-stained neovascular tufts (red) on P17 in hyperoxia-exposed mice (Figure 1C). To further characterize the TRAIL-positive cells, retinal sections from wild-type mice were double-labeled with anti-TRAIL and anti-F4/80 monoclonal antibody, a specific macrophage and microglial cell marker, and counterstained with 4,6-diamidino-2-phenylindole (DAPI). We found that TRAIL colocalized with a subset of F4/80-positive cells in the neovascular tufts (Figure 1D, arrows).

Retinal DR5 Expression in Wild-Type Mice

DR5 mRNA expression was observed on P17, P21, and P24 in normal and diseased mouse retina from wild-type mice using RT-PCR (Figure 2A). Additionally, mDcTRAILR1 and osteoprotegerin were expressed at the same time points (data not shown). DR5 mRNA expression has been noted to be wide-spread.²⁷ Therefore, we characterized DR5 protein expression with immunohistochemistry. At P17 under room air conditions, DR5 expression was observed in whole mounted retinas, predominantly in the superficial vascular network (Figure 2B-E). While both veins and arteries stained positive for DR5 (data not shown), expression was more intense on arteries, particularly at primary branch points (Figure 2C, arrow). In addition, weaker DR5 expression was observed

Figure 1. Expression of TRAIL in the mouse retina. TRAIL expression was observed at all time points as analyzed by RT-PCR (**A; top**) and Western blot (**A; bottom**). *N* = normoxia, *O*₂ = hyperoxia-exposed. GAPDH and β -actin were used as internal loading controls for RT-PCR and Western blot analysis respectively. **B:** Cryo-sections from P17 and P21 hyperoxia-exposed and P17 room air (RA) control retinas were immunolabeled with anti-TRAIL antibody. Positive staining in magenta is indicated by **arrows**. **C:** Retinal wholemounts were labeled with GS-isolectin (red) and anti-TRAIL antibody (green) and the images were merged, demonstrating TRAIL expression within neovascular tufts (**C, arrows**) at P17. **D:** Cryo-sections were double labeled with anti-TRAIL (red) and anti-F4/80 (green), a marker for macrophages and microglial cells, and counterstained with DAPI (blue) at P17. The merged image demonstrates F4/80-positive cells (**arrow** and **arrowheads**) within neovascular tufts. A portion of these F4/80-positive cells are also TRAIL-positive and visualized as yellow (**arrows**). Original magnification $\times 400$ (**B–D**).

on blood vessels in the transitional vascular layer and minimal expression was appreciated in the deep vascular network (Supplemental Figure 1, see <http://ajp.amjpathol.org>). At P17, after hyperoxia conditions, intense DR5 positive immunoreactivity was observed within the neovascular tufts in whole mounted retinas (Figure 2F–I). In addition, DR5 expression in the transitional and deep vessels, increased compared with the room air control retinas at the same time point (Supplemental Figure 2, see <http://ajp.amjpathol.org>). In cross section, DR5-positive cells were seen in both the neovascular tufts and deep vasculature (Figure 2J). In both control and hyperoxia-exposed mice DR5 expression was observed on non-vascular cells (Figure 2C, asterisk).

Normal Retinal Vascular Development in Wild-Type and *TRAIL*^{−/−} Mice

Wild-type and *TRAIL*^{−/−} mice were used to investigate the functional role of TRAIL protein in the retina during

NV. We also investigated normal vascular development of the retinas in these two strains of mice, to determine whether a baseline difference between the two strains existed. Retinas were immunolabeled with GS-isolectin at P3, P5, and P7 from mice kept in room air conditions and analyzed for percentage of vascularized retina. A small but statistically significant difference was seen at P3 (Figure 3, A and B) between the wild-type and *TRAIL*^{−/−} mice, but no further difference was seen at P5 (Figure 3, C and D) or P7 (Figure 3, E and F). At P3, a mean $17.3 \pm 1.7\%$ of the retina was vascularized in the wild-type mice compared with $27.6 \pm 3.5\%$ in *TRAIL*^{−/−} mice ($P = 0.032$; $n = 7$ to 9 eyes). At P5, in the wild-type mice, $56.3 \pm 6.3\%$ was vascularized compared with $44.1 \pm 2.0\%$ in *TRAIL*^{−/−} mice ($P = 0.059$; $n = 10$ to 12 eyes). Finally, at P7, $69.3 \pm 0.9\%$ was vascularized in the wild-type mice compared with $73.8 \pm 2.6\%$ in the *TRAIL*^{−/−} mice ($P = 0.184$; $n = 8$ to 12 eyes). Overall, though there may be some early differences in vascularization of the retina, there is no difference by P7, the time at which the

Figure 2. Expression of DR5 in the mouse retina. RT-PCR analysis of DR5 demonstrated expression at all time points in both normoxic (N) and hyperoxia-exposed (O_2) retinas (A). Whole-mounted retinas at P17 were collected from both room air control- and hyperoxia-exposed mice and labeled with GS-isolectin (red), anti-DR5 (green), and DAPI (blue). **B–I:** Representative micrographs of maximum intensity projections of confocal z-stacks (~20 z-sections at 0.5- μ m sections) from room air control animals (**B–D**). DR5 expression was seen predominantly in the superficial vascular layers, with an increase in intensity at primary branch points (**C**, arrow). Weaker DR5 expression is also observed in transitional vessels (Supplemental Figure 1, see <http://ajp.amjpathol.org>). DR5- and DAPI-stained image of single 0.5 μ m z-section, focused on the top of the main artery (**E**). DR5 expressing cells are seen in the neuronal layers of the retina, as well (**C**, asterisk). In the hyperoxia-exposed animals, DR5 is readily seen in the neovascular tufts (**F–I**; maximum intensity projection) and is also expressed in the transitional and deep vessels (Supplemental Figure 2, see <http://ajp.amjpathol.org>). Again, DR5 is seen to be expressed in the neuronal cells (**F**). DR5- and DAPI-stained image of a single 0.5 μ m z-section through the middle of the neovascular tuft (**I**). In paraffin-embedded cross-sections, at P17 in the hyperoxia-exposed animals, DR5 is again localized to neovascular tufts (**J**, arrow), as well as deeper retinal vessels (**J**, arrowhead).

animals enter oxygen exposure and begin the disease process (Figure 3G).

Arterial Capillary-Free Area in Normal Development of Wild-Type and *TRAIL*^{-/-} Mice

Because of the high oxygen environment around arteries, it is typical to see a capillary-free area surrounding the artery.²⁸ This area was assessed at P5 and P7. At P5, the wild-type retinas had a mean of $15,125 \pm 578 \mu\text{m}^2$ capillary-free area per 100 μm of artery length compared with $15,077 \pm 1308$ in the *TRAIL*^{-/-} retinas, a reduction in capillary-free area of less than 1% ($P = 0.970$; $n = 12$ to 19 arteries). At P7, there was a mean of $15,580 \pm 455$

μm^2 capillary-free area per 100 μm of artery length in the wild-type retinas compared with $13,760 \pm 476$ in the *TRAIL*^{-/-} retinas, a reduction in capillary-free area per 100 μm of arterial length, of 11.7% ($P = 0.0095$; $n = 17$ arteries) (Figure 3H). Overall, there was a small but significant decrease in the capillary-free area surrounding the artery at P7 (Figure 3I).

Retinal Avascularity after Oxygen Exposure in *TRAIL*^{-/-} Mice

Retinas were evaluated for avascular area after oxygen exposure at various time points, to assess differences

Figure 3. Retinal development and capillary-free zone in TRAIL^{-/-} and wild-type (WT) mice. Whole mount retinas were labeled with GS-isolectin, a marker for endothelial cells, as well as for α -smooth muscle actin. The area of vascularization was quantified at P3 (**A**, **B**; $n = 7$ to 9), P5 (**C**, **D**; $n = 10$ to 12), and P7 (**E**, **F**; $n = 8$ to 12) in wild-type and TRAIL^{-/-} mice. A significant difference was seen between the mouse strains at P3 but not at P5 or P7 (**G**). Additionally, the capillary-free zone surrounding the arteries in these retinas was quantified in the two mouse strains. Representative arteries are shown from P7 (**H**). A small but significant reduction in the capillary-free zone was observed in the TRAIL^{-/-} mice at P7, but not at P5 (**I**). * $P < 0.05$.

in the vaso-obliterative phase of OIR in TRAIL^{-/-} animals as compared with wild-type. Retinas from both wild-type and TRAIL^{-/-} mice were isolated and stained with GS-isolectin at P8, P12, P17, and P21 after hyperoxia exposure. At P8, there was a mean $39.4 \pm 2.6\%$ avascular area in the wild-type mice as compared with $40.8 \pm 3.4\%$ in the TRAIL^{-/-} mice ($P = 0.76$, $n = 6$ to 8). At P12 (Figure 4, A and B, asterisks), there was a mean $32.5 \pm 1.1\%$ avascular area in the wild-type mice, as compared with $28.1 \pm 0.8\%$ in the TRAIL^{-/-} mice ($P = 0.01$; $n = 6$ to 8). At P17 (Figure 4, C and D, asterisks), the mean avascular area of the wild-type mice was $19.6 \pm 1.2\%$ compared with $22.9 \pm 0.9\%$ in the TRAIL^{-/-} mice ($P = 0.06$; $n = 6$). At P21 (Figure 4, E and F), wild-type mice had a mean avascular area of $1.0 \pm 0.8\%$ versus the TRAIL^{-/-} mice with $1.8 \pm 1.6\%$ avascular ($P = 0.64$; $n = 6$). Overall, except for a statistically significant difference at P12, there is no difference in the avascular area between the two types of mice (Figure 4G).

Retinal Neovascularization in TRAIL^{-/-} Mice

Neovascular nuclei anterior to the inner limiting membrane of the retina (Figure 5, A–F) were quantified in both wild-type (Figure 5, A–C) and TRAIL^{-/-} (Figure 5, D–F) hyperoxia-exposed mice. A normal neovascular response to hyperoxia was observed in wild-type mice with the peak in NV at P17 with 25.1 ± 1.2 neovascular nuclei per section ($n = 10$). At P21, some regression was observed with 9.8 ± 2.5 neovascular nuclei per section ($n = 10$). Near-total resolution was seen at P24 with 3.1 ± 0.8 neovascular nuclei per section ($n = 6$). Alternatively, in the hyperoxia-exposed TRAIL^{-/-} mice at P17, a prominent neovascular response was appreciated with 34.8 ± 2.6 neovascular nuclei per section ($n = 10$). The peak in NV in TRAIL^{-/-} mice was not seen until P21, at which time there were 38.6 ± 7.5 neovascular nuclei per section ($n = 9$). Resolution began by P24, at which time 10.9 ± 0.9 neovascular nuclei per section were observed in the TRAIL^{-/-} mice ($n = 8$). The TRAIL^{-/-} mice had a signif-

Figure 4. Quantification of avascular areas of the retina. Whole mount retinas, after hyperoxia exposure were labeled with GS-isolectin, a marker for endothelial cells, and displayed at P12 (**A, B**; $n = 6$ to 8), P17 (**C, D**; $n = 6$), and P21 (**E, F**; $n = 6$) from both wild-type (WT) (**A, C, E**) and TRAIL^{-/-} (**B, D, F**) mice. Avascular areas (asterisk) were quantified and compared (**G**). Except at P12, when a statistically significant but small difference was observed, no significant differences between the two strains of mice were appreciated at any time point. **Arrows** denote neovascular tufts. (* $P = 0.01$) Original magnification $\times 25$.

icant increase in neovascular nuclei per section at all three time points compared with wild-type. The TRAIL^{-/-} mice had both a more pronounced neovascular response to hyperoxia and a delay in the regression of retinal NV (Figure 5G).

Retinal Proliferation and Apoptosis in TRAIL^{-/-} Mice

To assess the extent to which the increased NV in the TRAIL^{-/-} mice was due to increased proliferation or decreased apoptosis, immunostaining for PCNA and a TUNEL assay were performed. The PCNA staining revealed that after hyperoxia exposure, P17 wild-type mice had $41.2 \pm 6.4\%$ of the neovascular nuclei proliferating as compared with $17.5 \pm 4.4\%$ in the TRAIL^{-/-} mice ($P =$

0.0084 , $n = 10$ to 12). At P21, no difference was seen between the two strains of mice. Wild-type mice showed $22.3 \pm 6.9\%$ of the neovascular nuclei to be proliferating while TRAIL^{-/-} mice had $22.2 \pm 5.4\%$ proliferating ($P = 0.99$, $n = 6$ to 8). Overall, the TRAIL^{-/-} mice were observed to have fewer proliferating neovascular nuclei than the wild-type mice at P17, and no difference was observed at P21 (Figure 6, A–D, and I).

TUNEL analysis revealed significant differences in the amount of apoptosis, in pre-retinal neovascular nuclei, in the two strains of mice after hyperoxia exposure. In P17 wild-type mice, $9.8 \pm 1.1\%$ of the neovascular nuclei were apoptotic as compared with the TRAIL^{-/-} mice in which $2.0 \pm 0.2\%$ of the neovascular nuclei were apoptotic ($P < 0.0001$, $n = 6$ to 8). At P21, the wild-type mice showed $15.0 \pm 4.3\%$ of the neovascular nuclei to be apoptotic compared with $5.5 \pm 1.4\%$ in the TRAIL^{-/-} mice ($P = 0.035$, $n = 6$ to 8). Thus, there was significantly decreased apoptosis in neovascular tufts at both time points in the TRAIL^{-/-} mice, as compared with the wild-type mice (Figure 6, E–H, and J).

Additionally, apoptosis was evaluated in the inner plexiform, inner nuclear, and outer plexiform layers and compared between the two strains of mice. At P17 there were 4.78 ± 1.50 apoptotic nuclei per section in the wild-type mice, as compared with 1.47 ± 0.16 apoptotic nuclei in the TRAIL^{-/-} mice ($P = 0.08$; $n = 6$ to 8). At P21, there were 2.18 ± 0.17 apoptotic nuclei per section in wild-type mice, as compared with 2.53 ± 0.36 in TRAIL^{-/-} mice ($P = 0.45$; $n = 6$ to 8). TUNEL-positive cells were not appreciated in intraretinal vessels in the inner and outer plexiform layers.

Apoptosis was also evaluated in the outer nuclear layer where at P17 there were 13.9 ± 2.53 apoptotic nuclei per section in wild-type mice, as compared with 22.46 ± 3.22 in the TRAIL^{-/-} mice ($P = 0.055$; $n = 6$ to 8). At P21, the wild-type mice demonstrated 18.73 ± 2.81 apoptotic nuclei per section, as compared with 32.13 ± 8.63 in the TRAIL^{-/-} mice ($P = 0.22$; $n = 6$ to 8). Overall, apoptotic cells were observed in the neovascular nuclei and neuronal layers, but not in the intraretinal blood vessels.

TRAIL-Induced Apoptosis of Human Retinal Endothelial Cells

Human retinal EC (HRECs) were stimulated under various conditions that included the presence of VEGF (to mimic *in vivo* conditions), varying amounts of TRAIL, and the presence or absence of 6X-His Ab, an antibody that cross-links TRAIL and significantly enhances its apoptosis inducing ability.^{15,26} Once the stimulation was complete, a TUNEL assay was performed and apoptotic cells quantified. The introduction of TRAIL (10 ng/ml) plus 6X-His Ab and VEGF (Figure 6M) resulted in 243.3 ± 95.6 apoptotic cells per $\times 100$ field, as compared with 21.2 ± 6.5 apoptotic cells per $\times 100$ field in those cells receiving VEGF alone (Figure 6L, $P < 0.01$). Quantification of apoptosis is represented graphically (Figure 6K).

Figure 5. Increased neovascularization with delayed resolution in TRAIL^{-/-} mice. Retinal cross sections from hyperoxia-exposed mice at P17 (A, D), P21 (B, E), P24 (C, F) from both wild-type (WT) (A–C) and TRAIL^{-/-} mice (D–F) were stained with H&E. Nuclei extending anterior to the inner limiting membrane (arrows) were quantified (G). Significant differences were seen at all time points between the two strains of mice. The peak of neovascularization was delayed until P21 and resolution of neovascularization was also delayed in the TRAIL^{-/-} mice as compared with wild-type. (**P* = 0.0034; *n* = 10, ***P* = 0.0014; *n* = 9 to 10, ****P* < 0.0001; *n* = 6 to 8) Original magnification ×400.

Discussion

We have established that TRAIL and DR5 protein are both expressed in the wild-type mouse retina. We have also demonstrated that TRAIL-expressing cells are co-localized with neovascular tufts in the wild-type mouse retina after hyperoxia exposure at both the peak of disease and during resolution. Interestingly, the absence of TRAIL results in increased NV and delayed resolution of this NV, with an associated decrease in vascular tuft apoptosis in the mouse model of OIR. Furthermore, we have demonstrated the ability of soluble TRAIL to induce apoptosis in HREC *in vitro*. Together, these data reveal that TRAIL is expressed in the mouse retina and plays an important role in control of OIR through apoptosis.

TRAIL has been previously described by multiple groups to be expressed in many cell and tissue types, including the mouse and human eye.^{9,29–31} TRAIL receptor mRNA is also widely expressed in various tissues.²⁷ We demonstrate TRAIL and DR5 mRNA expression throughout the OIR time course in the retinas of wild-type animals both exposed and unexposed to hyperoxia. TRAIL protein expression was also appreciated by Western blot analysis during the OIR time course, although expression was not altered by oxygen exposure, possibly due to the use of whole retinas. However, TRAIL was localized to neovascular tufts on P17 and P21 in both cross sections and flat-mount retinas using immunohistochemistry, while TRAIL positive cells were restricted to the intraretinal regions in the control retinas. TRAIL positive cells were co-localized with a subset of macrophages/microglial cells within the neovascular tufts in wild-type mice. Macrophages/microglial cells have been previously characterized to co-localize with neovascular tufts in the mouse model of OIR.^{32,33} Furthermore, mice deficient in MCP-1 (CCL2), a potent chemoattractant of macrophages, exhibit reduced macrophage infiltration and an associated decrease in vascular tuft apoptosis,³⁴ suggesting the contribution of macrophages in neovascular tuft EC apoptosis. Previous studies have demonstrated TRAIL expression by many cells of the immune system, including macrophages.³⁵ Griffith et al reported the ability of IFN-stimulated macrophages to induce TRAIL-mediated apoptosis in several human tumor cell lines.³⁶ Other groups have shown that TRAIL-expressing

macrophages induce apoptosis in various tissues, including the induction of neuronal apoptosis in a mouse model of HIV-CNS infection.³⁷ More specifically, Herold et al³⁸ have demonstrated that TRAIL-expressing macrophages, in particular, are responsible for inducing apoptosis in lung epithelium in a mouse model of influenza virus infection. The TRAIL-positive macrophages were recruited to the lung in a CCL2/CCR-2-dependent manner, while resident alveolar macrophages did not express TRAIL. In addition, epithelial cell apoptosis was attenuated in CCR-2^{-/-} mice as a result of reduced infiltration of TRAIL-positive macrophages. Additional studies to further characterize the subpopulation of TRAIL⁺ F4/80 cells in the OIR model could be accomplished by breeding TRAIL^{-/-} mice with MCP-1^{-/-} or CCR-2^{-/-} mice. Supporting the hypothesis that TRAIL-positive macrophages/microglia have the potential to directly regulate neovascular tuft apoptosis, retinal ECs are known to express DR5.²³ In the current investigation, DR5 expression was noted in the superficial vascular network in the retina of both oxygen-exposed and room air control mice. We did appreciate strong DR5 expression in neovascular tufts with an associated increased expression in transitional and deep network vessels after oxygen-induced injury. Although both control and oxygen-exposed retina express DR5 in the retinal vasculature, expression may not correlate with sensitivity to TRAIL-induced apoptosis. Susceptibility to TRAIL-induced apoptosis is modulated by a variety of mechanisms, including expression of decoy receptors and intracellular anti-apoptotic proteins.³⁹ There is also recent evidence that DR5 may have post-translational modifications by O-glycosylation,⁴⁰ suggesting that increasing death-receptor O-glycosylation promotes TRAIL-stimulated clustering of DR5, mediating activation of caspase-8.

To further examine the *in vivo* contribution of TRAIL to retinal NV and regression, we investigated TRAIL^{-/-} mice in the OIR model. Interestingly, we show here that after hyperoxia exposure, TRAIL^{-/-} mice have increased NV at P17 and delayed NV regression at P21. Consistent with a pro-apoptotic role in this pathological process, we observed decreased number of cells undergoing apoptosis within neovascular tufts at both P17 and P21. This contrasts with our prior findings that FasL mutant (*gld*)

Figure 6. Decreased neovascular tuft apoptosis in $TRAIL^{-/-}$ mice. PCNA staining was performed on paraffin-embedded retinal cross-sections from hyperoxia-exposed mice at P17 (**A, C**; $n = 10$ to 12) and P21 (**B, D**; $n = 6$ to 8) in wild-type (WT) (**A, B**) and $TRAIL^{-/-}$ (**C, D**) mice. Percentage of proliferating neovascular nuclei (arrows) was quantified and displayed in graphical form (**I**). There was a significant decrease in proliferation in the $TRAIL^{-/-}$ mice as compared with wild-type at P17 ($*P < 0.0084$) and no difference between the two strains of mice at P21. TUNEL assay for apoptotic cells (arrows) was performed on paraffin-embedded retinal cross-sections from mice that were hyperoxia-exposed at P17 (**E, G**) and P21 (**F, H**) in both wild-type (**E, F**) and $TRAIL^{-/-}$ (**G, H**) mice. The percentage of apoptotic neovascular nuclei was quantified and reported in graphical form (**J**). A significant decrease in the percentage of apoptotic neovascular nuclei was noted at both P17 and P21 in the $TRAIL^{-/-}$ mice, as compared with wild-type mice ($**P < 0.0001$; $n = 6$ to 8, $***P = 0.035$; $n = 6$ to 8). HRECs were cultured and introduced to various conditions before a TUNEL assay was performed to identify apoptotic cells. HRECs exposed to VEGF alone (**L**) had significantly fewer apoptotic cells as compared with those exposed to low-dose TRAIL, 6X-His cross-linking antibody, and VEGF (**M**). A graphical comparison of all conditions tested (**K**) shows the significant difference in the number of apoptotic cells found in **L** as compared with **M** ($*P < 0.05$). Original magnification $\times 400$ (**A-H**), $\times 100$ (**L, M**).

mice had an increase in the peak of NV at P17, but that NV resolved normally by P21,⁴ suggesting that TRAIL plays a greater role in resolution of pathological NV. To confirm that retinal EC are a functional target for TRAIL-induced apoptosis, HREC were cultured with TRAIL *in vitro*. We found that TRAIL induced HREC apoptosis, further supporting the hypothesis that the TRAIL pathway plays a role in retinal EC homeostasis. This finding is consistent with previous work that demonstrated the ability of recombinant human TRAIL to induce EC apoptosis in human cell lines, including human umbilical vein endothelial cells, and in blood vessels in human skin xenografts.^{20,41}

In addition to the reduced apoptosis observed in the neovascular tufts, a significant decrease in vascular tuft proliferation was also observed in hyperoxia-exposed $TRAIL^{-/-}$ mice at P17, compared with wild-type mice. Although reduced EC proliferation would not explain the observed increase in retinal NV on P17 and P21 in the $TRAIL^{-/-}$ mice, there is data to support the concept that TRAIL can modulate cell proliferation via activation of NF- κ B,^{42,43} ERK1/2, or Akt.⁴⁴ The intracellular signaling may also be regulated in some way by the concentration of TRAIL, as low TRAIL concentrations induce proliferation and high concentrations preferentially induce apoptosis.⁴⁵ Despite the reduction in neovascular tuft proliferation on P17, we observed increased retinal NV, showing the critical role that apoptosis plays in the development of retinal NV. As previously noted, work from our laboratory and others has shown the pro-apoptotic effects of the Fas/FasL system and its important role in controlling retinal NV.^{4,22} A study examining the role of the anti-apoptotic factor Bcl-2 in the mouse model of OIR has also provided evidence of the importance of apoptosis in controlling the development of retinal NV.⁴⁶ In contrast to $TRAIL^{-/-}$ mice, Bcl-2^{-/-} mice exhibited significantly reduced retinal NV compared with control mice, indicating that VEGF-A is unable to counterbalance the endogenous pro-apoptotic factors due to the lack of functional Bcl-2. Retinal NV was also significantly reduced, while EC apoptosis was increased, in the mouse model of OIR by transgenic overexpression of the pro-apoptotic thrombospondin-1.⁴⁷

Apoptosis also contributes to remodeling of the retinal vasculature during development and to vascular regression during hyperoxia-mediated vaso-obliteration.^{48,49} We observed a modest increase in retinal vascularization on P3 in the $TRAIL^{-/-}$ mice, as compared with control, but by P7, there was no difference in normal retinal vascular development in wild-type and $TRAIL^{-/-}$ mice in terms of vascular area. However, there was a small ($\sim 12\%$) but significant reduction in capillary-free zone in the $TRAIL^{-/-}$ mice on P7. Correlating with the small reduction in capillary-free zone, there was also a small but significant reduction in avascularity in the $TRAIL^{-/-}$ mice, as compared with controls on P12 after hyperoxia exposure. These data suggest that TRAIL plays a very modest role in retinal vascular remodeling during development and in response to hyperoxia. Ishida et al⁵⁰ has proposed that the Fas/FasL pathway contributes to vascular pruning in the retina during development and disease. How-

ever, Ferguson et al concluded that Fas/FasL only play a role during the NV phase in OIR, but not during the phase of vaso-obliteration.⁵¹ There is evidence that thrombospondin-1 is a major contributor to vaso-obliteration via apoptosis because thrombospondin-1-deficient mice are markedly less sensitive to hyperoxia-mediated vaso-obliteration.⁵² In contrast to activation of death receptors, reduction of the anti-apoptotic protein Bcl-2 by hyperoxia-induced down-regulation of VEGF is likely an important component of the EC death machinery during vaso-obliteration.^{53,54} Supporting the growth factor withdrawal hypothesis as a major mechanism of vaso-obliteration, we have also noted a significant reduction in hyperoxia-mediated vaso-obliteration in Bim^{-/-} mice (unpublished observations). Bim is an intracellular pro-apoptotic protein that is an efficient cell killer and is strongly induced by growth factor withdrawal.^{55,56}

In conclusion, we have shown that TRAIL^{-/-} mice have an increase in pathological NV of the retina and delayed resolution of NV in this model of OIR. Additionally, our data supports the hypothesis that TRAIL induces apoptosis, specifically in retinal ECs. It is the careful balance of many pro-angiogenic and pro-apoptotic factors that determines whether an EC survives. Likely, it will be some combination of increased pro-apoptotic and decreased pro-angiogenic factor expression that will prove most efficient in controlling pathological NV. However, potential combination therapy with more than one pro-apoptotic agonist could also prove efficacious in the controlling pathological retinal NV, although systemic approaches could be limited by potential toxicities.

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