Reduced Nitro-oxidative Stress and Neural Cell Death Suggests a Protective Role for Microglial Cells in $\text{TNF}\alpha^{-/-}$ Mice in Ischemic Retinopathy

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PURPOSE. Neovascularization occurs in response to tissue ischemia and growth factor stimulation. In ischemic retinopathies, however, new vessels fail to restore the hypoxic tissue; instead, they infiltrate the transparent vitreous. In a model of oxygen-induced retinopathy (OIR), $\text{TNF}\alpha$ and iNOS, upregulated in response to tissue ischemia, are cytotoxic and inhibit vascular repair. The aim of this study was to investigate the mechanism for this effect.

METHODS. Wild-type C57/BL6 (WT) and $\text{TNF}\alpha^{-/-}$ mice were subjected to OIR by exposure to 75% oxygen (postnatal days 7–12). The retinas were removed during the hypoxic phase of the model. Retinal cell death was determined by TUNEL staining, and the microglial cells were quantified after Z-series capture with a confocal microscope. In situ peroxynitrite and superoxide were measured by using the fluorescent dyes DCF and DHE. iNOS, nitrotyrosine, and arginase were analyzed by real-time PCR, Western blot analysis, and activity determined by radiolabeled arginine conversion. Astrocyte coverage was examined after GFAP immunostaining.

RESULTS. The $\text{TNF}\alpha^{-/-}$ animals displayed a significant reduction in TUNEL-positive apoptotic cells in the inner nuclear layer of the avascular retina compared with that in the WT control mice. The reduction coincided with enhanced astrocytic survival and an increase in microglial cells actively engaged in phagocytosing apoptotic debris that displayed low ROS, RNS, and NO production and high arginase activity.

CONCLUSIONS. Collectively, the results suggest that improved vascular recovery in the absence of $\text{TNF}\alpha$ is associated with enhanced astrocyte survival and that both phenomena are dependent on preservation of microglial cells that display an anti-inflammatory phenotype during the early ischemic phase of OIR. (*Invest Ophthalmol Vis Sci.* 2010;51:3291–3299) DOI: 10.1167/iovs.09-4344

Intravitreal retinal neovascularization (NV) is a sight-threatening complication of several retinal diseases including diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion. In all these diseases, vaso-obliteration leads to tissue hypoxia and a compensatory upregulation of angiogenic

growth factors in an attempt to reoxygenate the ischemic retina. The resulting new vessels, however, do not revascularize the ischemic area but appear to be inhibited from infiltrating the ischemic retina and, instead, invade the vitreal compartment of the eye. The mechanism that inhibits recovery of the ischemic tissue is poorly understood.

Recently, using an in vivo mouse model of oxygen-induced retinopathy (OIR), we investigated the effects of tumor necrosis factor ($\text{TNF}\alpha$) on pathological NV. In this model, the disease occurs at two stages. In the first phase, exposure to hyperoxia between postnatal day (P)7 and P12 causes the vaso-obliteration of existing retinal vessels. The second stage of the disease is initiated by return of the animals to room air at P12, which renders the central retina ischemic and results in preretinal NV over the now hypoxic central retina between P15 and P21.¹ In our studies, we demonstrated that $\text{TNF}\alpha$ expression is induced in response to ischemia at P13 and that inhibition of $\text{TNF}\alpha$ reduces intravitreal NV and facilitates intraretinal angiogenesis and recovery of the ischemic tissue.² These findings have recently been corroborated by others.^{3,4} This effect is similar to that observed in inducible nitric oxide synthase (iNOS) null animals⁵ and suggests that $\text{TNF}\alpha$ and nitric oxide (NO), produced from iNOS, inhibit intraretinal vascular recovery.^{2,5–8}

$\text{TNF}\alpha$ and iNOS are monocyte/macrophage-derived proinflammatory mediators that are not constitutively expressed but are often co-induced by the same stimuli and act in synergy to bring about a coordinated antimicrobial effect. Together, their effects are mediated via the production of reactive nitrogen (RNS) and oxygen species (ROS) which act as effector molecules that protect the host by ensuring efficient killing of pathogens in situations of infection. For example, $\text{TNF}\alpha$ is involved in the activation of the NADPH oxidase and iNOS to produce superoxide (O_2^-) and nitric oxide (NO), respectively.^{9–11} In the immune system, peroxynitrite (ONOO^-), the combined product of these species, is cytotoxic, which is necessary for its bactericidal activity. Unchecked, however, the generation of these radical species can be damaging to host tissue and inhibitory to tissue repair and recovery. Indeed, in the retina, microglia and Müller cells express iNOS and $\text{TNF}\alpha$ in response to inflammatory cytokines and tissue hypoxia,^{2,5,12–17} where they are involved in mediating neuronal cell death in retinal inflammatory and degenerative diseases such as diabetic retinopathy and OIR.^{6,18,19}

In the OIR model, iNOS expression is induced in the inner nuclear layer (INL) of the central avascular retina in response to ischemia, where it is involved in mediating neuronal cell death through the action of ONOO^- .⁵ In addition, although inhibiting iNOS function prevents degeneration and leads to improved revascularization, it is currently unknown why protecting this central ischemic area of the retina from damage can have such beneficial effects on vessel regrowth. One likely source of iNOS and $\text{TNF}\alpha$ in this region is microglial cells, the

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Supported by the Wellcome Trust and Fight for Sight UK.

Submitted for publication July 20, 2009; revised December 14, 2009; accepted December 14, 2009.

Disclosure: L. Stevenson, None; N. Matesanz, None; L. Colhoun, None; K. Edgar, None; A. Devine, None; T.A. Gardiner, None; D.M. McDonald, None

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resident immunocompetent cells of the retina. These cells have recently been shown to play a pivotal, albeit poorly defined, role in promoting vascular development and repair.^{20,21} Thus, we investigated the hypothesis that TNF α , upregulated in the ischemic retina like iNOS, contributes to ROS- and RNS-mediated cell damage and that microglial cells, the likely source of these mediators, are involved in disease.

MATERIALS AND METHODS

Murine Model of OIR

Oxygen-induced retinopathy (OIR)¹ was induced in C57/BL6 wild-type (WT) and TNF α ^{-/-} mice, also on a C57/BL6 genetic background^{2,22}, thus removing the confounding effects caused by differences in genetic strain. In this model, 7-day-old litters along with their nursing dams were exposed to 75% oxygen until postnatal day (P)12, producing vaso-obliteration of the central retina. At P12, the mice were returned to room air, after which the retina became hypoxic and the expression of angiogenic factors was upregulated, leading to preretinal NV between P15 and P21. At time points between P12 and P17, the animals were terminally anesthetized, the eyes enucleated, and the retinas removed, snap frozen, and stored at -70°C for later RNA or protein extraction, or the eyes were cryopreserved in OCT medium for subsequent cryosectioning. Several animals were routinely killed at P12, retinal flat mounts were prepared, and the blood vessels were stained with biotinylated isolectin (Sigma-Aldrich, Poole, UK) and Alexa 488 streptavidin (Invitrogen-Molecular Probes, Paisley, UK), to confirm consistent vaso-obliteration of the central capillary beds. The iNOS^{-/-} control mice used for the microglial (MG) cell quantification were purchased from Banton and Kingman (Hull, UK) and subjected to OIR. All procedures were approved by the local animal care ethics committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under license from the British Home Office.

Sample Preparation for DCF, DHE, and TUNEL Assays

Whole eyes from WT and TNF α ^{-/-} OIR-exposed mice were collected at P13, embedded in OCT compound (Sakura Fintek, Zoeterwoude, The Netherlands) and frozen in an isopentane dry-ice bath. The retinas were vertically sectioned in a cryostat set at 10 μ m, mounted on microscope slides (Superfrost/Plus; Menzel-Glaser, Braunschweig, Germany), and stored at -20°C until use. Sampling from the ischemic area was verified by the absence of lectin-positive vascular endothelial cells, and all experiments were conducted in a batch design.

Detection of Apoptotic Cells by TUNEL

Random sections from the ischemic area were fixed with 2% paraformaldehyde for 20 minutes at room temperature followed by incubation with a 0.1% Triton X-100–0.1% citrate permeabilization solution for 2 minutes on ice. The sections were then labeled with a commercial TUNEL reaction mixture (Roche, Mannheim, Germany) for 60 minutes at 37°C in a humidified atmosphere in the dark. After an intensive wash, the slides were mounted with antifade medium containing DAPI (Vectashield; Vector Laboratories, Peterborough, UK) and visualized with a confocal fluorescence microscope. Cells stained green were considered positive. The intensity of the fluorescence detected in the area of the central retina was analyzed by Image-J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). For each section, total fluorescence was calculated from three separate high-power fields and the average values from eight separate sections/eye were combined to produce a mean value.

Quantification of MG Cell Density

MG cell density was estimated from *Bandeiraea simplicifolia* lectin-stained flat mounts (as described for visualization of retinal vessels) by

collecting images on a confocal microscope. All images were taken in the ischemic region of the central retina with a $\times 40$ objective, and a series of Z-stack images was taken throughout the entire retina 2 μ m apart, starting at the internal limiting membrane. The INL and ONL were demarcated by nuclear staining with DAPI which acted as a guide to allow quantification of MG cells localized in these layers. The images were scored for the presence of MG (see Fig. 2) by a skilled operator who was blinded to the experimental groups. One image was taken from each quadrant, and the average of four was used to calculate the mean.

Detection of Intracellular ROS and RNS Production

DCF is the oxidation product of 2',7'-dichlorodihydro-fluorescein diacetate (DHDCF) and is widely used to detect the presence of reactive oxygen and nitrogen species (ROS and RNS).^{23,24} In situ superoxide generation was also measured with the oxidative fluorescent dye dihydroethidium (DHE).^{25,26} Briefly, serial cryosections (10 μ m) were preincubated for 30 minutes at room temperature in PBS in the presence or absence of each of the following inhibitors: polyethylene glycol-superoxide dismutase (PEG-SOD, 400 U/mL), uric acid (1 mM), and *N*^G-nitro-L-arginine methyl ester (L-NAME, 1 mM). After the preincubation step, sections were incubated with DCF or DHE (10 μ M) in the presence or absence of the same inhibitors for 1 hour at 37°C in the dark. DCF or DHE images were obtained with a laser-scanning confocal microscope (Microradiance CSLM; Bio-Rad, Hercules, CA) at a magnification of $\times 200$ using identical acquisition settings for each section. Fluorescence was quantified by automated image analysis with Image-J software. For each section, mean fluorescence was calculated from five separate high-power fields per eye. At least three eyes were assayed per experimental group from three individual experiments.

Measurement of Retinal Nitrotyrosine by Immunochemical Dot-Blot Analysis

Peroxynitrite (ONOO⁻) is a short-lived molecule that nitrates protein tyrosine residues, making the detection of nitrotyrosine a useful surrogate marker for peroxynitrite.^{27,28} Retinas were homogenized in ice-cold extraction buffer and cellular protein prepared as described elsewhere.²⁹ Equivalent amounts of homogenates were blotted onto a PVDF membrane (Pall Life Sciences, Hampshire, UK). The membrane was blocked and probed overnight at 4°C with mouse monoclonal antibody to nitrotyrosine (1:100; Abcam, Cambridge, UK). Anti-mouse IgG conjugated to horseradish peroxidase (1:2000) was used as a secondary antibody. Nitrotyrosine BSA (Cayman Chemical, Ann Arbor, MI) was used as a positive loading control, to confirm the specificity of the primary antibody. Immunoreactivity was detected with chemiluminescence (Immobilon Western Chemiluminescent HRP substrate; Millipore, Billerica, MA) and images acquired by a fluorescence/chemiluminescence image-acquisition system (AutoChemi System; UVP, Cambridge, UK). Relative levels of nitrotyrosine immunoreactivity were determined by densitometry.

Western Blot Analysis

Pooled retinas were lysed in ice-cold extraction buffer as just described. Equivalent amounts of protein were resolved on a 9% SDS-polyacrylamide gel and transferred by electrophoresis to PVDF membrane. The membrane-bound protein was probed with anti-iNOS antibody (1:400 dilution; Santa Cruz Biotechnology, Santa Cruz) followed by anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2500 dilution; Promega, Madison, WI). Protein bands were visualized and quantified as described earlier.

NOS and Arginase Enzyme Activity Assay

NOS and arginase activity was determined by the conversion of radio-labeled arginine in the presence and absence of arginase inhibition with an NOS activity assay (Cayman) with several modifications.

FIGURE 1. The presence of TUNEL-positive apoptotic cells in the INL of the ischemic retina was much reduced in $\text{TNF}\alpha^{-/-}$ animals. (A) TUNEL-positive cells (green) and DAPI (blue) staining of retinal cross sections from P13 WT C57/BL6 wild-type and $\text{TNF}\alpha^{-/-}$ mice. (B) An enlargement of a portion of the image from WT P13 oxygen-treated eyes. Original magnification, $\times 40$ (C) DNase treated P13 WT retina positive control. (D) Quantification of the fluorescence intensity of TUNEL-positive cells ($n = 3$ per group from three independent OIR experiments; $*P < 0.05$).

Pooled mouse retinas were homogenized in extraction buffer, and equivalent amounts of homogenate added to the reaction mixture containing universally labeled [^{14}C] arginine (0.05 μCi ; Amersham Biosciences, Piscataway, NJ). The assay was allowed to proceed for 1 hour at 37°C , and the reaction was terminated by the addition of ice-cold HEPES buffer containing EDTA (pH 5.5). An ion-exchange resin (Dowex; Dow Chemical Co., Midland, MI) was added to the sample to bind positively charged, unreacted arginine and ornithine and was removed by filtration through a spin column. The radioactivity corresponding to [^{14}C]-L-citrulline and [^{14}C]-L-urea was measured in the presence of scintillation fluid (Ready Safe Scintillation Cocktail; Beckman Coulter, Fullerton, CA). For measurements of NOS activity only, (S)-(2-boronoethyl)-L-cysteine-HCl (BEC; 5 μM ; Calbiochem, San Diego, CA) was added to the reaction mix and the [^{14}C]-L-citrulline levels were determined. For each sample, background was determined by incubation in the presence of L-NNA, which inhibits NOS and arginase, and was subtracted from the total counts. For determination of NOS activity, five retinas were pooled and assayed in triplicate and for the NOS plus arginase determination, five retinas from the contralateral eye of the same group were assayed.

Real-Time PCR

RNA was extracted (Tri Reagent; Sigma-Aldrich), and samples were reverse transcribed into cDNA (Superscript II Reverse Transcriptase cDNA Synthesis Kit; Invitrogen). As a negative control, the reverse transcriptase was omitted from one reaction mixture during each reverse transcription reaction. The resulting single-stranded cDNA was consequently used as a template for amplification using sequence-specific primers (Invitrogen) to arginase I and II³⁰ and 18S ribosomal RNA genes. Real-time PCR was performed (Prism 7000 Sequence Detection System; Applied Biosystems, Inc., Foster City, CA). Briefly, PCR was performed in a 10- μL volume with 0.5 μM primers, 2.5 mM MgCl_2 , dNTPs, *Taq* DNA polymerase (Hotstart; Qiagen, Crawley, UK), and green fluorescent dye (SYBR Green I; Qiagen). The specificity of the reaction was confirmed by melting-curve analysis and data quantified using the comparative method ($2^{-\Delta\Delta\text{Ct}}$).

Astrocyte Grading

Retinal flat mounts were immunostained with anti-GFAP antibody and imaged by confocal microscopy. Individual retinal quadrants were graded for the presence or absence of GFAP-positive astrocytes by an operator blinded to the genotype of the tissue. A score of 1 or 2 was assigned if the astrocytic phenotype resembled that of a room air control. A grade of 3 to 4 was given if the phenotype was markedly altered from that observed in the quadrant scored 1, and a score of 5 indicated complete absence of GFAP staining (see Fig. 8 for examples

of the scoring system). Nine retinas per group were graded from animals at P13 and P15 after OIR exposure.

Statistical Analysis

All data are presented as $\pm\text{SEM}$ from three to six individual OIR experiments unless otherwise indicated. Statistical analysis was performed by independent Student *t*-test, for comparison of P13 values, or one-way ANOVA followed by Bonferroni post hoc test ($P < 0.05$; $P < 0.01$), for multiple comparisons.

RESULTS

Effect of $\text{TNF}\alpha$ Ablation on Cell Death in the Ischemic Retina in OIR-Treated Mice

Central vaso-obliteration and subsequent return to room air results in acute ischemia between P12 and P13; therefore, we investigated the extent of hypoxia-mediated cell damage at P13, the time that corresponds to the largest increase in hypoxia-regulated gene expression. At P13 in the WT animals, there was a significant number of TUNEL-positive cells localized to the INL of the avascular area (Fig. 1). In the $\text{TNF}\alpha^{-/-}$ OIR-treated animals there were significantly fewer TUNEL-positive cells in equivalent avascular areas (Fig. 1).

Number of MG Cells in the Ischemic Region Actively Engaged in Phagocytosing Apoptotic Bodies in $\text{TNF}\alpha^{-/-}$ and Control Mice

MG cells are highly ramified with long dendritic processes that radiate through many different retinal planes, making them difficult to identify in serial sections. Because of this difficulty, quantification was performed on images taken from flat-mounted retinas following Z-series capture on a confocal microscope. In the $\text{TNF}\alpha^{-/-}$ group there were significantly more MG cells in the ischemic central retina than in the corresponding WT group (Fig. 2). This finding was similar to those of the $\text{iNOS}^{-/-}$ control group. Most of the MG cells were localized throughout the plexiform, inner and outer nuclear layers, where they displayed a dendritic appearance, with many apparently swollen processes clearly containing DAPI-positive apoptotic bodies.

Hypoxia-Induced Peroxynitrite and Superoxide Generation in the Retinas of $\text{TNF}\alpha$ Knockout Mice Subjected to OIR

Peroxynitrite and superoxide generation was assessed with DCF and DHE fluorescence (Figs. 3, 4, respectively). Shown are

FIGURE 2. Preservation of the MG cell population in the ischemic retina of TNFα^{-/-} and iNOS^{-/-} animals. Microglia were identified by staining with *B. simplicifolia* isolectin (red) in PFA-fixed retinal flat mounts, and sequential Z-series images were captured with a confocal microscope. (A) Representative images of MG cells in the ischemic retina full of apoptotic bodies (arrow). (B) Enlarged image (blue DAPI nuclear stain, arrow). (C) The number of MG cells was quantified in the INL and ONL, corresponding to the site of maximum TUNEL-positive staining; *n* = 6 to 8 retinas/group; **P* < 0.05. Results are typical of two independent OIR experiments.

representative sections from P13 WT and TNFα^{-/-} mice. In the WT animals, there was a significant amount of peroxynitrite and superoxide at P13, which was inhibited with uric acid, PEG-SOD, and L-NAME, demonstrating that one of the enzymatic sources of this ROS was NOS derived. Results indicated that in the absence of TNF-α, there was a marked reduction in both red and green fluorescence intensity and therefore a decrease in the levels of free radical formation. DCF fluorescence was strongest in the inner nuclear and plexiform layers

which corresponded to the site of MG cell localization. DHE intercalates with genomic DNA, resulting in a nuclear localization. Accordingly, red fluorescence was bright in the INL, ONL, and GCL of both the WT and TNFα^{-/-} mice, which again was much reduced in the TNFα^{-/-} group.

Nitrotyrosine Staining in TNFα^{-/-} Mouse Retinas at P13 Post-OIR

In the WT animals, there was a significant increase in nitrotyrosine immunoreactivity between P12 and P13 (Fig. 5), which was not observed in the TNFα-depleted animals, indicating a decrease in the levels of peroxynitrite formation.

Difference in iNOS Protein Expression in the Retinas of WT and TNFα-Knockout Mice Subjected to OIR

iNOS protein expression was detected in the retinas of WT and TNFα^{-/-} mice by Western blot analysis of whole retinal homogenates (Fig. 6). An induction of iNOS from P12 to P13 was observed in WT and TNFα^{-/-} mice but was not significantly different between groups.

NOS and Arginase Activity in TNFα^{-/-} Mice

In the absence of the specific arginase inhibitor, BEC, radiolabeled arginine was converted to citrulline plus urea, which is indicative of total NOS and arginase enzymatic activity (Fig. 7). In the absence of the arginase inhibitor there was no significant difference in the conversion rate between the two groups. In contrast, in the presence of inhibitor there was a significant difference between the two groups, indicating a decrease in NOS activity in the TNFα^{-/-} group and suggesting a greater proportion of arginine conversion was due to arginase activity.

FIGURE 3. Peroxynitrite generation in WT and TNFα^{-/-} mouse retinas. Peroxynitrite generation in mouse retina was assessed by using the oxidative fluorescent dye DCF in 10-μm fresh cryosections from WT and TNFα^{-/-} mice exposed to hyperoxia for 5 days. P13 retinal sections were measured in the presence and absence of the inhibitors; PEG-SOD, L-NAME, and uric acid. (A) Representative retinal sections showing that, in the absence of TNFα, there was a marked reduction in the green fluorescence intensity, indicating a decrease in the levels of peroxynitrite formed. Data are representative of three individual OIR experiments. (B) Quantified specific retinal DCF fluorescence is expressed for sections in arbitrary units (AU) for each group; ***P* < 0.01.

FIGURE 4. Superoxide generation in WT and $\text{TNF}\alpha^{-/-}$ mice mouse retinas. O_2^- generation was assessed with the oxidative fluorescent dye DHE in cryosections. P13 retinal sections were measured, and corresponding controls showed that the reaction was reduced in sections treated with PEG-SOD and L-NAME. In the absence of $\text{TNF}\alpha$, there was a marked reduction in the red fluorescence intensity indicating a decrease in the levels of O_2^- formed compared with the WT group; ** $P < 0.01$.

Arginase II mRNA Expression in $\text{TNF}\alpha$ -Knockout Mice Subjected to OIR

To substantiate the results of the arginine conversion assay, we compared the expression profile of the two known arginase I and II isoforms by real-time PCR (Fig. 7C). Arginase II (arg II) mRNA expression was significantly greater in the $\text{TNF}\alpha^{-/-}$

mice than in the WT group. In contrast, arginase I transcript levels were unaltered between groups (data not shown).

Preservation of the Astrocytic Template in $\text{TNF}\alpha^{-/-}$ Mice Exposed to OIR

At P13, we did not observe any measurable difference in astrocyte phenotype between the groups, and most of the

FIGURE 5. Peroxynitrite generation in mouse retinas using dot-blot analysis. In the absence of $\text{TNF}\alpha$, there was a reduction in the presence of nitrotyrosine indicating a reduction in peroxynitrite formation. (A) Peroxynitrite generation in the retinas of WT and $\text{TNF}\alpha^{-/-}$ OIR-treated mice was assessed by dot-blot analysis of nitrotyrosine immunoreactivity. (B) Relative levels of nitrotyrosine immunoreactivity were determined by densitometry and the values plotted represent changes from P12WT. Histogram data represent $\pm$ SEM of changes in nitrotyrosine in retinal homogenates from five individual OIR experiments; each with $n = 6$ retinas/time point (* $P < 0.05$).

FIGURE 6. iNOS protein expression is increased at P13 in WT and $\text{TNF}\alpha^{-/-}$ mice OIR-treated retinas. (A) The expression of iNOS was assessed by Western blot analysis and β -actin was used as a loading control. (B) Densitometric band analysis was performed and the data were corrected for equal loading to β -actin. Values are expressed as changes relative to P12 WT levels and represent $\pm$ SEM from $n = 4-5$ individual OIR experiments; each with $n = 6$ pooled retinas per time point. An induction of iNOS from P12 to P13 in both WT and $\text{TNF}\alpha^{-/-}$ mice was observed.

FIGURE 7. iNOS activity was significantly reduced in TNF $\alpha^{-/-}$ mice and correlated with an increase in arginase expression and activity. Arginine-citrulline conversion in the (A) presence or (B) absence of a specific arginase inhibitor (BEC); $n = 5$ retinas/group. (C) Real-time PCR expression determined with arg II-specific primers. Data are normalized to 18S ribosomal RNA, are plotted as the change relative to WT levels, and represent changes $\pm$ SEM from three individual OIR experiments with $n = 6$ retinas/time point (* $P < 0.05$).

retinas demonstrated a score of 1 to 3. By P15 however, there was a large increase in quantifiable damage to the astrocytes in the WT group that was significantly reduced in the absence of TNF α (Fig. 8).

DISCUSSION

In ischemic retinopathies, it is still unknown why angiogenic growth signals fail to facilitate revascularization of the chronically hypoxic tissue. Using an in vivo model of this disease process, we have shown that ablation of the inflammatory cytokine, TNF α , allows recovery of the ischemic retina.² This effect is similar to that observed in iNOS null animals.⁵ Together, these findings suggest that TNF α and NO produced from iNOS inhibit vascular repair, and in the present work, our intent was to investigate the mechanisms that lead to this outcome.

In the OIR model iNOS and TNF α are induced in Müller glia and MG cells in response to tissue ischemia.^{2,3,16,17} In the acute hypoxic phase (P13), their expression is found predominantly within the central avascular zone. Accordingly, our study focused on the effect of iNOS and TNF α in this region.

NO produced from iNOS has been shown to be associated with an increase in apoptotic cell death in the INL of the ischemic retina during OIR, which coincided with the site of iNOS induction.^{5,16} Therefore, initially we investigated whether TNF α had a similar effect by comparing the number of TUNEL-positive cells in WT and TNF $\alpha^{-/-}$ animals. We found that in the TNF $\alpha^{-/-}$ retinas there was a significantly reduced number of TUNEL-positive cells in the INL, as described for the iNOS $^{-/-}$ animals.¹⁶ The INL consists predominantly of tightly packed neural cells and Müller cell processes. Isolated Müller cells are believed to express TNF α and iNOS in response to hypoxia; however, another possible cellular source of these inflammatory cytokines is MG cells, whose contribution to the consequences of OIR remains to be properly defined. These cells normally reside in the plexiform layers on either side of the inner and outer nuclear layers but can migrate to sites of injury on activation, where they can either exacerbate cellular damage through the increased expression of proinflammatory cytokines such as iNOS and TNF α ,³¹⁻³³ or they can be involved in repair by the removal of apoptotic debris.³⁴⁻³⁶ Therefore, we next investigated the possibility that MG cells were contribute to disease.

We discovered that in the TNF $\alpha^{-/-}$ animals compared with the WT control animals there were significantly more MG cells that were clearly involved in phagocytosing apoptotic bodies. These findings suggest that MG cells migrate to the INL in response to signals from apoptotic cells and have a role in aiding repair in a manner similar to their role in promoting photoreceptor survival in retinal degenerative diseases.^{37,38} With regards to iNOS, Sennlaub et al.⁵ speculated that the

cellular source of iNOS mediated nitrotyrosine activity was localized to Müller glial cells. However, close examination of their staining pattern shows that nearly all the staining is punctate and located in the INL, suggesting that it may be MG cell derived. Therefore, we sought to investigate the presence of MG cells in the ischemic retina of OIR-treated iNOS $^{-/-}$ animals in confocal images of flat mounts. We found that the phenotype of OIR in these animals closely resembled that in TNF $\alpha^{-/-}$ mice with a large increase in MG cells present in the INL and ONL. Thus, combining our results with those of Sennlaub et al. we found that in TNF $\alpha^{-/-}$ and iNOS $^{-/-}$ animals there is an increase in MG cells in the ischemic retina actively engaged in removing apoptotic debris.

Macrophages actively involved in the phagocytosis of neighboring apoptotic bodies are known to alter their phenotype to one that is anti-inflammatory, accompanied by a decrease in iNOS-mediated free radical production.^{30,39-41} Therefore, we next sought to determine whether such a correlation could be demonstrated in retinal microglia during OIR with an alteration in the production of free radical species—the downstream effector molecules of TNF α and iNOS function. At low levels, these free radicals are important signaling molecules involved in VEGF function,⁴² but they are damaging at high concentrations.

Using the in situ fluorescent probes, DCF and DHE, in retinal sections from TNF $\alpha^{-/-}$ mice, we demonstrated a marked reduction in the levels of ONOO $^-$ and O $_2^-$ formation in comparison to that in WT control retinas. These findings were further supported by nitrotyrosine dot-blot analysis, which again demonstrated that at P13 there was a decrease in nitrotyrosine in the TNF $\alpha^{-/-}$ group.

Next, because of the observed decrease in ONOO $^-$ and the apparent phenotypic similarity between the TNF $\alpha^{-/-}$ and iNOS $^{-/-}$ models, we focused our attention on the effects of TNF α depletion on iNOS function. The regulation of iNOS and TNF α is often interdependent. For example, iNOS expression can be induced by TNF α .⁴³ Considering the relationship between these two mediators and the fact the iNOS-deficient animals also show improved revascularization of the ischemic retina,¹⁶ it is possible that the beneficial effects of TNF α ablation were due, in part, to an alteration of iNOS expression. Accordingly, we sought to determine whether the decrease in RNS and ROS was a result of a decrease in iNOS expression in the absence of TNF α . We demonstrated that there was a similar hypoxia-mediated induction of iNOS expression in both TNF $\alpha^{-/-}$ and WT groups which was maximal between P12 and P13.

NO is synthesized from NOS by the oxidation of the N-terminal guanidino group of L-arginine. The reaction is a complex one that is dependent on the availability of various cofactors and substrates, including arginine. Another enzyme that also uses arginine is arginase, which competes with iNOS for

FIGURE 8. Enhanced preservation of astrocytes in $\text{TNF}\alpha^{-/-}$ mice after OIR. Retinal flat mounts were immunostained with GFAP (red) and images captured with a confocal microscope. GFAP staining was graded between 1 and 5 (examples shown in **A**), and the relative frequency of each grade was used for statistical comparisons (**B**); $n = 9$ retinas/group (** $P < 0.01$). (**C**) Example of new vessels infiltrating the avascular retina of $\text{TNF}\alpha^{-/-}$ mice at P15, guided by a normal astrocytic template.

arginine bioavailability. Thus, an increase in the expression of this enzyme reduces NO production. Indeed, the switch in macrophage phenotype from one that is pro- to one that is anti-inflammatory has been shown to be associated with an increase in arginase expression with a concomitant decrease in NOS activity.³⁰ Therefore, next we measured the enzymatic activity of NOS and arginase. We demonstrated that NOS activity, determined by the conversion of radiolabeled arginine to citrulline in the presence of an arginase inhibitor was significantly lower in the $\text{TNF}\alpha^{-/-}$ group than in the control animals. In contrast, in the absence of arginase inhibition, there was no difference in conversion rates between the two groups, suggesting that a greater proportion of the arginine conversion in these animals is accountable to arginase. Indeed, this was confirmed by real-time PCR, which demonstrated that there was an increase in arg II expression in $\text{TNF}\alpha^{-/-}$ mice. In some cell types, an increase in arginase expression without a decrease in NOS protein can lead to the uncoupling of NOS, which would increase peroxynitrite production and exacerbate oxidative stress.⁴⁴ However, in other circumstances, the resulting decrease in arginine availability caused by increased arginase can impair iNOS protein synthesis.^{45,46} Although we did not observe a decrease in global iNOS production, the finding that the $\text{TNF}\alpha^{-/-}$ group had a larger number of MG cells suggests that, on an individual cellular basis, there may

indeed be a decrease in iNOS protein expression. In addition, taken together with our observation of a decrease in RNS/ROS, our findings suggest that in the $\text{TNF}\alpha^{-/-}$ group the increase in arginase expression acted to lower iNOS activity and decrease NO, O_2^- , and ONOO^- . Together, our findings suggest that MG cells in the absence of $\text{TNF}\alpha$ display an anti-inflammatory phenotype.

One of the biological consequences of increased RNS/ROS production is an increase in the production of VEGF. In addition, these free radicals are important downstream mediators of VEGF function. For example, ROS and ONOO^- causes increased expression of VEGF through activation of STAT3, acting in part through NADPH oxidase and NOS.⁴⁷⁻⁴⁹ Indeed, in the ischemic retina, increased NADPH oxidase-derived O_2^- plays a significant role in VEGF-stimulated, pathologic retinal NV, and inhibition of this enzyme normalizes VEGF expression and reduces NV.²⁶ Physiological angiogenesis and vascular recovery are also VEGF-driven processes. Moreover, VEGF is an important trophic factor essential for the survival of astrocytes and neurons. During development, new vessel growth is guided by a network of astrocytes that produce highly localized VEGF gradients to aid endothelial cell infiltration. Furthermore, any disturbance of this structure has the potential to alter normal vessel growth.^{50,51} Indeed, it has been shown that exposure to hypoxia during OIR disrupts this template and results in a switch in the source of VEGF production from the astrocytic bed to alternative cells, such as neural and Müller glial cells. This shift is associated with an increase in intravitreal NV.^{52,53} In agreement with this finding, preservation of the astrocytic template increases intraretinal recovery after OIR.^{54,55} Together, these conclusions demonstrate the importance of the spatial and temporal expression of VEGF in determining normal versus abnormal vascular growth.

Therefore, considering the importance of localized VEGF gradients and also the fact that the position of the MG cells in the central ischemic retina is distant from the site of the remaining vascular front, we next investigated the effect of $\text{TNF}\alpha$ depletion on the survival of the astrocytic bed. In the $\text{TNF}\alpha^{-/-}$ mice there was a significant increase in the survival of astrocytes. Thus, it is possible that MG cells, recruited to the INL in response to ischemia, release neurotrophic factors that protect the overlying astrocytes from damage. The presence of these astrocytes, in turn, acts as a template for the advancing vascular front into the ischemic region and eventual reoxygenation of this tissue. Conversely, in the WT group, the enhanced VEGF production in the absence of an astrocyte monolayer is likely to lead to uncontrolled intravitreal NV tuft formation. Together, these results suggest that identifying the putative neuroprotective agents released from MG cells would aid in identifying therapeutic targets capable of restoring vasculature to the ischemic retina.

Other studies have shown that resident MG cells or exogenously administered myeloid precursors localize to the ischemic central retina between P13 and P14, where their presence is associated with promotion of physiological revascularization.²¹ The mechanism by which this beneficial effect occurs remains to be fully determined. In the present study, we have extended these findings and have shown that MG cells are specifically localized to the INL of the ischemic avascular retina after OIR. In addition, we have provided evidence that MG cells, in this location, are involved in clearing apoptotic debris suggesting an important role for these cells in promoting tissue repair. Furthermore, we have also provided evidence to show that, in the absence of $\text{TNF}\alpha$, these cells display an anti-inflammatory phenotype. In macrophages, this phenotype correlates with the release of proresolving type cytokines or growth factors^{56,57} and neurotrophins such as NGF and BDNF,^{58,59} suggesting that MG cells in the ischemic retina of the $\text{TNF}\alpha^{-/-}$ mice may have a similar function, as

revealed by the enhanced survival of the astrocytic template. Overall, this proposed mechanism may explain the accelerated intraretinal vessel recovery observed in these animals and indicates that the absence of TNF α and iNOS facilitates early resolution which, in turn, prevents the exaggerated NV response characteristic of proliferative retinopathies.

In summary, our results demonstrate that vascular recovery is associated with the preservation of an anti-inflammatory or neuroprotective MG cell population in the ischemic TNF α -deficient retina. These findings provide further evidence that altering the proinflammatory milieu of the ischemic retina would have a beneficial effect on restorative angiogenesis and suggest an important role for MG cells in mediating this process.

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