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miR-30a-5p inhibition promotes interaction of Fas+ endothelial cells and FasL+ microglia to decrease pathological neovascularization and promote physiological angiogenesis.

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Abstract

Ischemia-induced angiogenesis contributes to various neuronal and retinal diseases, and often results in neurodegeneration and visual impairment. Current treatments involve the use of anti-VEGF agents but are not successful in all cases. In this study we determined that miR-30a-5p is another important mediator of retinal angiogenesis. Using a rodent model of ischemic retinopathy, we show that inhibiting miR-30a-5p reduces neovascularization and promotes tissue repair, through modulation of microglial and endothelial cell cross-talk. miR-30a-5p inhibition results in increased expression of the death receptor Fas and CCL2, to promote endothelial cell survival and microglial migration and phagocytic function in focal regions of ischemic injury. Our data suggest that miR-30a-5p inhibition accelerates tissue repair by enhancing FasL-Fas crosstalk between microglia and endothelial cells, to promote endothelial cell apoptosis and removal of dead endothelial cells. Finally, we found that miR-30a levels were increased in the vitreous of patients with proliferative diabetic retinopathy. Our study identifies a role for miR-30a in the pathogenesis of neovascular retinal disease by modulating microglial and endothelial cell function, and suggests it may be a therapeutic target to treat ischemia-mediated conditions.

Graphical Abstract

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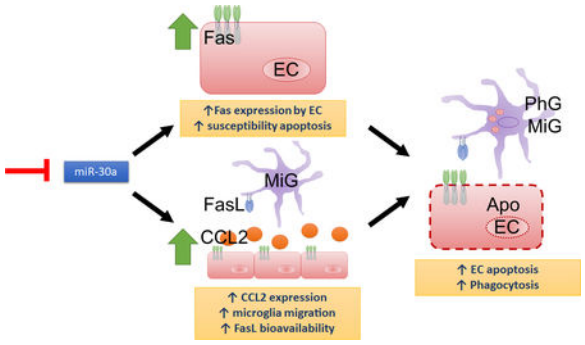
Conflict of interest statement

The authors have declared that no conflict of interest exists.



Keywords

Microglia; miR-30a-5p; CCL2; angiogenesis; ischemia

Introduction

Being highly vascular, the central nervous system (CNS) is susceptible to ischemic injury due to changes in blood vessels caused by age, genetics, lifestyle or a combination of these (Lok et al. 2015). Subsequent neuronal cell death leads to significant morbidity and mortality. The retina is a direct extension of the CNS and, as such, provides a useful model to study glial components of the CNS during hypoxic stress, ischemia and infarction (Kautzman et al. 2018; London et al. 2013; Weidemann et al. 2010). Often, these ischemic events lead to abnormal angiogenesis, a hallmark of many retinopathies, and this in turn leads to neurodegeneration resulting in visual impairment. Currently, therapies employing anti-vascular endothelial growth factor (VEGF) agents to target abnormal angiogenesis are used to treat ischemic retinal diseases including age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP) (Chawla and Darlow 2016; Duh et al. 2017; Gemenetzi et al. 2017; Stewart 2017). While anti-VEGF agents represent a major breakthrough in the treatment of retinal diseases with an angiogenic component, they have been used with limited success (Usui et al. 2015). Not all patients respond to anti-VEGF treatment (Ashraf et al. 2016; Chin-Yee et al. 2016) and increasing evidence suggests that, in some cases, prolonged treatment with anti-VEGF may lead to hypoxia, exacerbate retinal ischemia (Lee et al. 2017; Toy et al. 2016) and lead to degeneration of retinal neurons and retinal pigment epithelium (RPE) (Ashraf et al. 2016; Gemenetzi et al. 2017; Keir et al. 2017). Thus, there is a large unmet medical need to develop new, targeted therapies that will either improve efficacy or reduce off-target effects when used in combination with anti-VEGF drugs.

microRNAs (miRs) have been the focus of widespread interest (Rupaimoole and Slack 2017). These are 22–25 base pair, non-coding RNA molecules that have emerged as potent regulators of physiological processes such as angiogenesis and tissue repair (Anand et al. 2010; Gallach et al. 2014; Westenskow et al. 2013). Through sequence complementarity of their “seed sequence”, i.e. nucleotides 2–7/8, miRs bind to target mRNAs to repress protein expression through one of three mechanisms: degradation of target mRNA, destabilization of target mRNA, or inhibition of translation. One novel aspect of miRs, is that they can target

several mRNAs within the same pathway or biological process, and, thus, more potently regulate biological processes (Rupaimoole and Slack 2017), making them attractive candidates as therapeutics. For example, in this study we observe that miR-30a inhibition results in increased expression of two of its targets, Fas and CCL2. Specific miRs have been identified that play key roles in angiogenesis, ischemia, glial responses and microglial activation (Hutchison et al. 2013; Landskroner-Eiger et al. 2013; Ponomarev et al. 2013; Wang et al. 2015a; Zhang et al. 2012). Recent evidence suggests that miRNAs, contained within microvesicles, can modulate nervous tissue responses to ischemia and glial cell-to-cell communication (Blandford et al.), as is the case in oxygen-induced retinopathy (OIR) (Dellett et al. 2017). Analysis of these vesicles showed that, miR-30a-5p (miR-30a) was one of the most enriched miRs. miR-30a is particularly interesting since increased expression is associated with ischemia and vascular co-morbidities such as obesity, diabetes, stroke and subarachnoid hemorrhage (Carreras-Badosa et al. 2015; Long et al. 2013; Muller et al. 2015; Nielsen et al. 2012). Finally, miR-30a is highly expressed in proliferating endothelial cells *in vitro* (Anand et al. 2010), is enriched in microglia (Butovsky et al. 2014), and has recently been shown to be one of the 10 most abundant miRNAs in the human retina (Karali et al. 2016).

Microglia are of yolk-sac origin and the tissue resident macrophages of the brain and retina (Ginhoux and Prinz 2015). They are the first responders to CNS injury, including ischemia (Paques et al. 2010). As the tissue resident macrophages of the retina, microglia perform many of the same functions as other macrophages (Murinello et al. 2014; Perry and Holmes 2014). It is known that these cells play a critical role in retinal angiogenesis both at key developmental time points and during disease progression (Biswas et al. 2017). Fas-Fas ligand (FasL) interactions have been documented as an important mechanism to curb abnormal angiogenesis following ischemia. Specifically, interactions between Fas-expressing endothelial cells and FasL-expressing microglia have been shown to promote endothelial cell apoptosis (Barreiro et al. 2003). Microglial cells have also been implicated in retinal tissue repair following ischemic injury (Ritter et al. 2006). A key function necessary for appropriate tissue repair is phagocytosis of cellular debris and extracellular matrix, clearing tissues to facilitate appropriate revascularization and repair (Fadok et al. 1998; Khanna et al. 2010; Swift et al. 2001). Thus, in addition to targeting endothelial cells, manipulating microglia behavior is an alternative strategy to prevent pathological angiogenesis and promote repair following injury (Dorrell et al. 2010; Ritter et al. 2006; Rutar et al. 2015). We have previously shown that supporting microglia survival is instrumental in promoting retinal tissue repair and healthy revascularization following ischemic retinal damage (Ritter et al. 2006), but the mechanisms that regulate microglia function during ischemia remain incompletely understood. Certain miRNAs are known to modulate glial behavior (Amici et al. 2017; Wang et al. 2015a; Zhang et al. 2012), and miR-30a is highly enriched in microglia (Butovsky et al. 2014), although it’s precise impact on these cells remains unknown. miR-30a is conserved in humans and mice, and regulating miR-30a in microglia could have broad utility for treating a number of ischemic neuropathies.

Methods

Reagents and antibodies

Isolectin GS-IB_4 (IB4) labeled Alexa Fluor 647 (I32450) and 488 (I21411) and rat-anti mouse CD11b (MA5–16528) were purchased from Thermo Fisher Scientific. Rabbit anti-mouse/human PU.1 (2258) was obtained from Cell Signaling Technology. Brilliant Violet 421 and FITC anti-mouse CD31 (102423 and 102405), PE anti-mouse CD178 (FasL; 106605), APC and PerCP/Cy5.5 anti-mouse/human CD11b (101211 and 101227), Brilliant Violet 510 anti-mouse Ly-6G (127633), PE/Cy7 anti-mouse Ly-6C (128017), APC and PerCP/Cy5.5 anti-mouse CX3CR1 (149007 and 149009), Brilliant Violet 421 anti-human CD95 (Fas, 305623), were obtained from BioLegend. PE and Brilliant Violet 421 anti-mouse CD95 (Fas, 561985 and 562633) were obtained from BD Biosciences. LIVE/DEAD fixable near-IR dead cell stain (L34975) was obtained from Thermo Fisher Scientific. Recombinant human sFas Ligand (310–03H) and recombinant murine JE/MCP-1 (CCL2, 250–10) were obtained from PeproTech. mirVana miRNA inhibitors hsa-miR-30a-5p (4464084) and negative control #1 (4464076), were obtained from Thermo Fisher Scientific. For cell culture transfections, hsa-miR-30a-5p (MIM0091) and non-targeting_1 (MIM9001) mimic and hsa-miR-30a-5p (INH0091) and non-targeting_1 (INH9001) inhibitors were obtained from Switchgear Genomics. In situ hybridization probes for hsa-miR-30a-5p (cat VM1-10240-01) and CCL2 (cat VB1-13201-01) were obtained from Affymetrix.

Mice and animal experimental procedures

All animal protocols were approved by the IACUC committee at the Scripps Research Institute, La Jolla, California. All animals received food and water ad libitum. C57BL6 mice were obtained from The Scripps Research Institute animal facility. $CCL2^{flox}$ and LysM-cre mice on a C57BL6 background were obtained from The Jackson Laboratory. OIR was induced as previously described (Smith et al. 1994). Briefly, post-natal day 7 (p7) pups and their mothers are exposed to an environment of 75% oxygen in a hyperoxia chamber (BioSpherix ProOx P110) for 5 days and returned to room air at p12. Under these conditions, retinal neovascularization occurs between P12 and P17. Intravitreal injections of anti-miR-30a and scramble-miR control (0.5μl of a 100μM solution) or recombinant murine CCL2 (0.5μl of a 0–5000ng/ml) were performed at p12, immediately after returning the mice to normal room air. An *in vivo* phagocytosis assay was performed as previously described(Murinello et al. 2016). Mice were euthanized by cervical dislocation at varying time points, as indicated in the results and figure legends.

Human vitreous collection

Fifty-four PDR (age 57.9 ± 10.7 years, 43 male and 11 female), and 5 macular hole patients (age 67.6 ± 6.5 years, 1 male and 4 female) that underwent pars plana vitrectomy at Tokyo Medical University Hospital were enrolled in this study. All patients underwent a standard 3-port 25-gauge vitrectomy. Vitreous samples were harvested from the mid vitreous region at the start of vitrectomy. The vitreous was removed by a vitreous cutter before intraocular infusion. The undiluted samples were stored immediately at −80°C until assayed. When post-vitrectomy bleeding occurred, samples were excluded from analysis. PDR patients were divided into two groups according to the presence of fibrovascular membrane.

Cells and cell culture

HUVECs (Thermo Fisher Scientific, C-003–5C) were cultured in M200 (Thermo Fisher Scientific, M-200–500) supplemented with LVES (Thermo Fisher Scientific, A14608–01). HUVECs were seeded at 1.3×10^4 cells/cm^2 24 hours before transfection. Cells were transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientifc, 13778075) according to manufacturer's instructions, for 6 hours. After 6 hours, cells were incubated in complete media with or without 100ng/ml of recombinant sFasL, as indicated in figure legends, for 15 hours until used for downstream applications.

Proliferation Assay

Proliferation was assessed by measuring incorporation of bromodeoxyuridine (BrdU) and cell cycle phase was determined by co-staining with the total DNA stain, 7-AAD, using a flow cytometric kit from BD Biosciences (559619). 10μM of BrdU were added to transfected HUVECs for 45 minutes, after which cells were collected with accutase (Biolegend, 423201) and stained following manufacturer's instructions. Stained cell suspensions were analyzed on a three laser LSR-II flow cytometer (BD Biosciences). Quantification of BrdU and 7-AAD staining was performed in FlowJo v10 (Flowjo, LLC).

Apoptosis Assay

Apoptosis was assessed by measuring staining with annexin V. HUVECs transfected with miRNA inhibitors or mimics were collected with accutase (Biolegend, 423201), resuspended in 100 μl of annexin V binding buffer (Biolegend, cat 422201), after which PE-annexin V and of 7-AAD were added to each samples. After a 15 minute incubation at room temperature, 300μl of annexin V binding buffer were added per sample, and the samples were analyzed on a three laser LSR-II flow cytometer (BD Biosciences). Quantification of annexin V staining was performed in FlowJo v10 (Flowjo, LLC).

Tube formation assay

A 24-well plate was coated with 50ul/cm^2 Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific, A14132–02) and solidified for 30 minutes at 37°C. Cells were collected with accutase (Biolegend, 423201) and seeded onto the coated 24-well plate at 2.5×10^4 viable cells/cm^2. Cells were incubated for 16 hours and stained with 2ug/ml of Calcein AM (BD Biosciences, 564061) for 30 minutes prior to imaging on a Zeiss A10 inverted microscope A-10. Vessel percentage area, average vessel length and number of branching points were quantified using AngioTool software (National Cancer Institute).

Immunofluorescence

Eyes were collected and fixed in 4% paraformaldehyde for 4 hours at 4ºC. For staining with IB4 only, retinas were dissected out and the vitreous removed with fine brushes. Retinas were then incubated in PBS with $Ca^{2+}Mg^{2+}$ with 10 μg of fluorescently-labeled IB4 overnight. For staining with antibodies, retinas were incubated in block buffer (PBS with 5% bovine serum albumin (Thermo Fisher Scientific, BP9703100), 10% normal donkey serum (Gemini Bio-Products, 100–151) and 0.3% (v/v) Triton X-100 (Sigma, T8787)) overnight at

4°C with gentle rocking, following by a 48h incubation with primary antibodies in block buffer at 4°C with gentle rocking. Retinas were then washed 5×10 minutes in PBS and incubated overnight with fluorescently labelled secondary antibodies (Thermo Fisher Scientific) in block buffer with 0.1% (v/v) Triton X-100 at 4°C with gentle rocking. After both staining protocols, retinas were washed in PBS 4× 10 minutes and mounted with ProLong Diamond Antifade mounting medium (Thermo Fisher Scientific, P36965). Retinas were imaged using a Zeiss 710 Confocal microscope (Zeiss).

In situ hybridization

Eyes were fixed in 4% paraformaldehyde at 4°C for 20 hours, incubated in 30% sucrose at 4°C for 24 hours, embedded in OCT, and stored at −80°C. Samples were sectioned at 7μm and melted onto Superfrost Plus Microscope Slides (Fisher Scientifc, 12-550-15). Slides were dried at room temperature for 1 hour and stored at −80°C. In situ hybridization was performed using the Affymetrix ViewRNA ISH Tissue Assay kit (QVT0050) according to manufacturer's instructions with an additional fixation in 4% paraformaldehyde at 4°C for 16 hours prior to step 1 and excluding heat pre-treatment of slides. Retinas were imaged usinf a Zeiss 710 Confocal microscope (Zeiss).

RNA Isolation and real-time PCR

Single retinas were collected in 500 μl of Triazol and RNA was isolated using a PureLink RNA mini kit (Thermo Fisher Scientific, 12183020) according to manufacturer's instructions. 125 ng of RNA were used for RT-qPCR using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, 4368814). qPCR was performed using Taqman universal master mix (Thermo Fisher Scientific, 4304437) and Taqman probes on a Quantstudio 5 (Thermo Fisher Scientific). For analysis of microRNA experiments, sRNAU6 was used as the reference gene; for mouse studies beta actin was used as the gene; for human studies GAPDH was used as the reference gene.

Purification of microRNA from human vitreous

Purification of miR-30a-5p from human vitreous was performed using a TaqMan miRNA ABC Purification kit – Human Panel B (Thermo Fisher Scientific) following manufacturer's instructions.

Flow cytometry analysis of mouse retinas

A post-natal neural dissociation kit (Miltenyi, 130-092-628) was used to prepare a single cell suspension from mouse retinas, and cells were stained as previously described(Murinello et al. 2016). Stained cell suspensions were analyzed using a LSR-II flow cytometer (BD Biosciences). Fluorescence minus one (FMO) controls were used for each antibody. Analysis of acquired data was performed in FlowJo v10 (Flowjo, LLC).

Protein analysis with Mesoscale Discovery System

Protein analysis was performed using Mesoscale Discovery Systems following manufacturer's instructions. 100μg of protein homogenate in 25μl of T-Per buffer with 1x

Halt protease inhibitor cocktail (Thermo Fisher Scientific, 78429) were added to each well of a 96-well plate.

Statistics

All statistical tests were performed in GraphPad Prism v6.07 (GraphPad Software, Inc). Data comparisons between two groups were performed using unpaired or paired two-tailed Student T-tests. Data comparisons between multiple groups were performed with multiple t-test with Holmes-Sidak correction or one-way ANOVA with Bonferroni correction. Data comparison between two groups with more than one variable were performed with two-way ANOVA and Bonferroni correction. Tests used for each experiment are specified in the figure legends. Data are represented as mean ± SEM. A p value of $p<0.05$ was considered significant.

Study Approvals

Human studies were approved by the institutional review committee of Tokyo Medical University Hospital. Informed consent for surgery and sampling was obtained according to the Declaration of Helsinki. All animal studies have been approved by TSRI's IACUC review board.

Results

miR-30a inhibition reduces neovascularization and promotes retinal repair following ischemia

We confirmed that miR-30a functions as an angiomiR (a miR that promotes angiogenesis) through overexpression studies using HUVECs (Supp Fig 1). Transfection of HUVECs with a miR-30a mimic led to increased cell proliferation (Supp Fig 1A and B), modestly reduced apoptosis, and promoted tube formation (Supp Fig 1C–F). Using the oxygen-induced retinopathy (OIR) model we determined that miR-30a also promotes angiogenesis *in vivo*. We examined the spatial/temporal expression pattern of miR-30a in OIR mice, and observed profound changes that positively and negatively correlated with the pathological (i.e. proliferative) and physiological (i.e. tissue repair) neovascular phases, respectively (Fig 1A–B). Using qPCR we determined that at p12 to p14 stages, when neovascularization is occurring from the superficial and inner vessels (Connor et al. 2009), miR-30a levels spike, but they rapidly drop at the onset of the repair phase (p17; Fig 1B). Intravitreal injection of a miR-30a inhibitor at p12 led to a dramatic decrease in neovascularization (Fig 1D) and vaso-obliteration (Fig 1E). These data indicate that miR-30a inhibition may have a beneficial dual action during ischemia, by both preventing neovascularization and promoting tissue repair. To test this, knock-down studies were performed.

miR-30a regulates endothelial cell apoptosis by targeting Fas

miR-30a is conserved in humans and mice (Fig 1A). In addition to targeting Fas (Fig 1B) on endothelial cells, miR-30a, which is enriched in microglial cells, may modulate microglia function and thus potentially regulate both the neovascular and repair phases of OIR. miR-30a mimics and inhibitors decreased or increased Fas mRNA expression respectively (Fig 2 C, D). Transfection with miR-30a mimics also inhibited apoptosis in HUVECs in the

presence or absence of FasL (to mimic endothelial cell and microglia interactions; Fig 2 E). However, inhibition mir-30a in HUVECS induced apoptosis, and this effect was compounded by adding recombinant FasL to the cultures (Fig 2 F). These data suggest that miR-30a can regulate endothelial cell death by modulating Fas expression in endothelial cells.

miR-30a also regulates Fas expression in endothelial cells *in vivo*. Fas mRNA levels increased in whole retinas as early as six hours post intravitreal injections of miR-30a inhibitor (Fig 2G). We next analyzed Fas expression in retinal endothelial cells isolated from eyes injected with miR-30a inhibitors, and Fas was detected in a significantly higher percentage of endothelial cells (Fig 2 H–K). Fas-mediated cell death is triggered when this receptor is cross-linked by its ligand. Finally, miR-30a did not regulate FasL in microglia (defined as $CD11b^{+}GR\text{-}1^{-}CX3CR1^{+}$ cells), but the changes in endothelial cells were sufficient to decrease endothelial cell survival *in vivo* (Fig 2 N–P). Therefore, when miR-30a levels are high, Fas levels decrease and endothelial cells exhibit increased survival rates. When miR-30a levels decrease, Fas levels increase and apoptosis of endothelial cells is promoted.

miR-30a regulates retinal microglia migration.

We hypothesized that while miR-30a does not regulate FasL in microglia, it does promote migration and this can increase FasL bioavailability, thereby influencing endothelial cell survival. To test this hypothesis, we performed loss-of-function analyses in OIR mice during neovascular stages. Distribution of microglia is uneven in OIR mice due to vaso-obliteration and neovascularization, but is normalized in eyes injected with miR-30a inhibitors (Fig 3 A and B). These microglia likely migrated from the deeper regions of the inner retina since flow cytometry analyses revealed no significant differences in total number of microglia following injection of miR-30a inhibitors (Fig 3C). Migration of microglia towards ischemic injury sites could potentiate interactions between FasL-expressing microglia and Fas-expressing endothelial cells, to promote endothelial cell apoptosis.

miR-30a modulates CCL2 expression.

CCL2 is a non-conserved target of miR-30a (Fig 4A) that promotes chemotaxis (Conductier et al. 2010) and is upregulated in several CNS disorders. Therefore, we investigated if miR-30a can modulate CCL2 in the retina. CCL2 mRNA expression showed an inverse expression pattern to that of miR-30a (Fig 4 B and C—compare with Fig 1B), during the time course of OIR, indicating miR-30a targets CCL2 in the retina. Moreover, inhibition of anti-miR-30a resulted in increased levels of CCL2 mRNA (Fig 4D) and protein (Fig 4E) in the retina. Finally, following miR-30a inhibition, CCL2 expression was predominantly in the inner, ischemic layers, consistent with the microglial migration patterns and miR30a expression patterns we observed (Fig 4F). Therefore, microglia migration may be regulated by miR-30a and CCL2 signaling during ischemia to regulate, at least in part, the neovascular and repair phases of OIR.

CCL2 partially mediates the effects of miR-30a inhibition

To further identify the cellular source of CCL2, we sorted endothelial and microglial cells following intravitreal injection of miR-30a inhibitor. While both cell-types responded to miR-30a inhibition by upregulating CCL2 mRNA, microglial cells (average C_T=28.4) expressed higher levels of CCL2 than endothelial cells (average C_T=35.7; data not shown). To confirm the role of CCL2 as a mediator of the effects of miR-30a inhibition we used a Lysm-Cre to deplete CCL2 in microglia. Inhibiting miR-30a in *LysM-cre* $CCL2^{fl/fl}$ (CCL2 KO) mice had no significant effect (Fig 5B, C). The role of CCL2 in angiogenesis is controversial, but it is traditionally viewed as a pro-angiogenic factor (Cerri et al. 2016; Fernando et al. 2016; Robbie et al. 2016). Interestingly, we found that enhancing CCL2 levels in the retina by intravitreal injection of recombinant murine CCL2 (rmCCL2) resulted in a dose-dependent reduction in neovascularization and vaso-obliteration (Fig 5 D–F). These data suggest that CCL2 could act as an inhibitor of retinal angiogenesis, a previously unknown role for this chemokine.

miR-30a regulates microglial phagocytic function by targeting CCL2

We used transcriptomic and functional assays to determine that miR-30a and CCL2 can enhance microglia phagocytic function in the retina. Inhibition of miR-30a in mouse retinas resulted in upregulation of several mRNAs involved in phagocytic responses (Fig 6 A) and in increased microglial phagocytic function *in vivo* (Fig 6B, C). CCL2 supplementation also promoted microglial phagocytic function in a dose dependent manner (Fig 6D, E). Of note, the CCL2 dose that induced the highest phagocytic function also most potently inhibited pathological angiogenesis. Thus, miR-30a inhibition, through targeting of CCL2 expression, can modulate microglial migratory and phagocytic behaviors.

miR-30a levels are increased in the vitreous of patients with proliferative diabetic retinopathy

OIR mice are used to model features of ROP and PDR. Therefore, we next measured miR-30a levels in human vitreous samples from patients with PDR, an ischemic, neovascular eye disease and compared them with control patients with macular hole (MH), an atrophic retina condition that does not have any associated vascular abnormalities (vitrectomies are almost never performed on healthy subjects). qPCR analysis of the vitreous samples showed that miR-30a-5p levels were significantly upregulated in the vitreous humor of patients with PDR, especially in those in which fibrovascular membranes (FVM) were present, compared to those with MH (just as it is during the NV phase of OIR mice; Fig 7 C), supporting a role for this miR in the pathogenesis of PDR.

Discussion

Understanding the role of miRs in health and disease has been of interest to many researchers. Since they can fine-tune biological processes by modulating expression of several genes in a particular pathway, they are being tested in various disease contexts (Christopher et al. 2016; Garden and Campbell 2016). In fact, modulators of miRs including miR-155, miR-29, miR-122 and miR-34a are being studied and tested in clinical trials (Rupaimoole and Slack 2017). In this study we describe the role of miR-30a in modulating

ischemic responses by focally controlling endothelial cell survival in precise temporal/spatial patterns. Inhibition of miR-30a not only reduced pathologic angiogenesis by promoting endothelial cell apoptosis, but also promoted tissue repair and physiological revascularization by modulating microglia behavior. Moreover, miR-30a levels were increased in the vitreous humor of ischemic PDR patients, suggesting it may be implicated in this disease.

In this study, miR-30a inhibition both decreased pathological neovascularization (NV) and increased physiological revascularization (i.e. decreased vaso-obliteration). This paradoxical effect may potentially be explained by its different effects on multiple cells types. The death receptor Fas has been previously shown to be an important regulator of angiogenesis in ocular tissue (Barreiro et al. 2003; Kaplan et al. 1999). Endothelial cells of both the retinal and choroidal vasculatures express Fas and their cell fate is regulated by interaction with FasL expressed by microglia or RPE, respectively (Barreiro et al. 2003; Kaplan et al. 1999). In particular, FasL-expressing macrophages can migrate to areas of tissue injury to promote endothelial cell apoptosis and thus reduce pathologic neovascularization (Barreiro et al. 2003; Kelly et al. 2007). Here we show that miR-30a inhibition can simultaneously promote Fas expression by endothelial cells and promote migration of FasL-expressing microglia towards the sites of ischemic injury. Thus, by promoting endothelial and microglial cell interactions, miR-30a inhibition can lead to increased endothelial cell death and, thus, directly decrease neovascularization.

Microglia are of yolk sac origin and the tissue resident macrophages of the CNS, including the retina (Ginhoux et al. 2010). While they are known to perform a variety of functions that are essential for tissue homeostasis, their precise involvement and contribution to diseases of the CNS is still unresolved (Colonna and Butovsky 2017; Perry and Holmes 2014; Usui et al. 2015). An overwhelming amount of literature supports that microglia contribute to pathological changes through secretion of pro-inflammatory cytokines leading to cytotoxicity (Chen and Xu 2015; Madeira et al. 2015; McVicar et al. 2015; Murinello et al. 2014; Perry and Holmes 2014; Zhang et al. 2012). However, few recent studies have shown that, at least in certain instances, microglia activation can be protective (Chuang et al. 2016; Holtman et al. 2017; Le et al. 2016; White et al. 2017). With regard to ischemic injury of the CNS, microglia depletion has been shown to induce more severe ischemic neuronal damage (Szalay et al. 2016). These results are consistent with our previous findings that maintaining microglial numbers in the retina protects against ischemic damage in the OIR model (Ritter et al. 2006). Moreover, a recent study by Biswas et al, has demonstrated that microglia activation and interaction with the mural basement membrane can promote physiologic angiogenesis, promoting retinal tissue repair (Biswas et al. 2017). Here we show that miR-30a inhibition leads to increased coverage of avascular areas by microglia and is associated with increased revascularization. It is possible that microglia are promoting revascularization through mechanisms described previously (Biswas et al. 2017). Furthermore, we demonstrated that miR-30a inhibition results in increased phagocytic function by microglia. Phagocytosis of cellular debris is critical in ensuring the microenvironment is primed for appropriate revascularization and tissue repair (Bulow 1978; Jin and Yamashita 2016). In response to miR-30a inhibition, microglia migrate to areas of tissue injury, where they may phagocytose dead or compromised endothelial cells, as well as

extracellular matrix, to facilitate tissue repair. Based on cell morphology and flow cytometric analysis of cell-surface markers, our data indicate that the observed migrating $CD11b^+$ cells are mostly microglia. However, we cannot completely rule out the hypothesis that some bone-marrow derived cells may infiltrate the retina and contribute to the observed effects. Through its impact on these cells, miR-30a inhibition may promote physiological neovascularization, thus leading to a reduction in vaso-obliteration.

The work presented here shows that miR-30a inhibition has a profound effect on microglia and endothelial cells, thus suppressing pathologic neovascularization and promoting physiologic re-vascularization. Protection of retinal neurons has also been shown to promote re-vascularization in the OIR model (Wei et al. 2015). Our ISH data shows that miR-30a is widely expressed across the retina, suggesting it may also be expressed by retinal neurons. Of note, miR-30a inhibition in cortical neurons has been shown to protect against ischemia-induced cell death (Wang et al. 2015b). Thus, it is possible that in addition to curbing pathological angiogenesis and promoting re-vascularization through its effects on endothelial and microglial cells, miR-30a inhibition may also result in protection of retinal neurons from ischemic damage.

Like microglia, the role of CCL2 in CNS injury remains controversial. High levels of CCL2 are often associated with disease and several mouse models have demonstrated a deleterious effect of CCL2 (Cerri et al. 2016; Fernando et al. 2016; Hughes et al. 2002; Robbie et al. 2016). Thus, the prevailing view is that CCL2 plays a major role in many brain and retinal diseases. It was surprising to us that elevated CCL2 was associated with reduced angiogenesis and increased tissue repair. However, there is some evidence that, at least under certain circumstances, CCL2 may be protective. Ischemic injury in the brain can be prevented by ischemic preconditioning. In this paradigm, a brief mild ischemic episode is triggered and this initiates a cascade of molecular and cellular events that protect the brain from damage when subsequent severe ischemic events are triggered. CCL2 has been shown to be a major component of this protective effect and CCL2 depletion completely ablates it (Stowe et al. 2012; Wacker et al. 2012). High levels of CCL2 have also been detected in diabetic wounds, and it has been thought that CCL2 would prevent wound repair. However, recent studies showed that, instead, CCL2 is required for appropriate tissue repair following epidermal wounding (Wood et al. 2014) and ischemic kidney injury (Stroo et al. 2015). Accordingly, one study showed that, in a rat model of cerebral hemorrhage, while CCL2 ablation reduced initial injury size, it significantly slowed recovery (Yao and Tsirka 2012). In the retina, some studies have shown that CCL2 depletion aggravates laser-induced choroidal neovascularization and accelerates neurodegeneration (Luhmann et al. 2009; Yu et al. 2015). These studies suggest that CCL2, consistent with our results, is a key player in protection following ischemia and in mediating tissue repair. Furthermore, we show that CCL2 can promote microglia phagocytic function, a potential mechanism by which this chemokine may promote tissue repair.

High levels of CCL2 have been previously demonstrated in the vitreous of patients with PDR and AMD (Koss et al. 2011), and so it has been hypothesized that CCL2 may contribute to the pathology in these conditions. In light of our results, it is interesting to speculate that the high levels of CCL2 may reflect a failed attempt of the retina to “heal

itself". While this is difficult to test in humans, a recent clinical trial using an anti-CCL2 antibody for idiopathic lung fibrosis seems to support this idea (Raghu et al. 2015). Elevated levels of CCL2 have also been detected in idiopathic lung fibrosis, which led to the use of the anti-CCL2 antibody Carlumab in clinical trials. Results from a phase II clinical trial showed that, unexpectedly, not only did anti-CCL2 therapy not improve the condition, but all treated patients had faster disease progression when compared to placebo control and over 50% of the patients treated with the highest dose of anti-CCL2 suffered severe side effects (Raghu et al. 2015). These results caution against the use of anti-CCL2 antibodies to treat ocular disease, which is now being considered (Das 2016).

Vitreous can be readily obtained when patients undergo ocular surgical procedures. Analysis of vitreous samples from patients affected with PDR showed that miR-30a levels are elevated in PDR, suggesting miR-30a may be involved in the pathogenesis of PDR. Of note, while miR-30a levels are elevated in the vitreous of PDR patients, the levels of miR-30a dramatically drop at p17, the peak of the proliferative phase in OIR. We believe this disparity in our data may be explained by the different tissue responses to ischemic injury in humans and mice. Unlike PDR in humans, the mouse retina can repair itself following ischemic injury in the OIR model. While histologically the proliferative phase peaks at p17, this is also when tissue repair responses begin (Connor et al. 2009). Thus, we believe the drop in miR-30a levels at p17 may act as a signal to trigger tissue repair responses. In contrast, in humans with PDR, miR-30a levels remain elevated, potentially hindering the onset of repair responses. Though more work is necessary to determine the role of miR-30a in ischemic pathologies, its elevated levels suggest that miR-30a may be involved in the pathology of PDR. Since miR-30a targets several mRNAs, it is possible that miR-30a inhibition would be an effective therapy against ischemic neovascular disorders, by targeting endothelial Fas and microglial behavior.

In summary, the results described here identify miR-30a as an important regulator of angiogenesis in retinal ischemia. While more studies are warranted to fully understand the scope of miR-30a's action, we have been able to demonstrate that Fas and CCL2 are two important mediators of the anti-angiogenic and protective effects of miR-30a. Given the broad expression of miR-30a and CCL2 in ischemic disease, these results may have implications for other diseases of the CNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

miR-30a inhibition potently inhibits angiogenesis by targeting the death receptor Fas.

miR-30a also regulates microglia migration and phagocytosis by targetting CCL2.

miR-30a levels are elevated in the vitreous of diabetic patients with retinopathy.

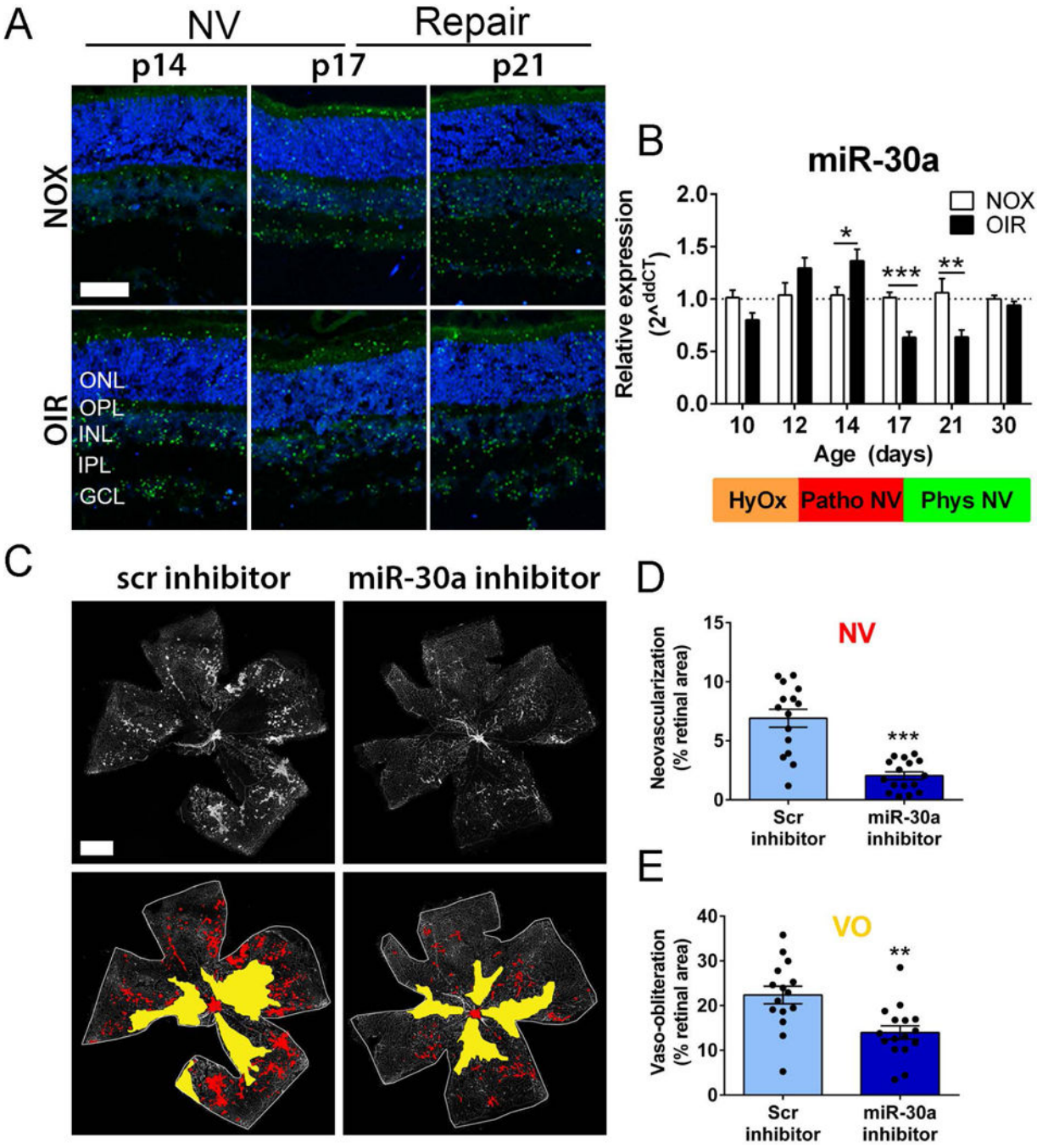

Fig 1.

miR-30a promotes angiogenesis and prevents tissue repair in the OIR model of ischemic retinopathy. (A) miR-30a was detected in retinal sections with *in situ hybridization*. ONL – outer nuclear layer, OPL – outer plexiform layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer. (B) Retinal miR-30a levels were quantified by qPCR in retinas from normoxic and OIR mice (n=6–17 mice per group and representative of at least two independent experiments). Patho NV – Pathological NV, Phys NV – Physiological NV. Data are mean + SEM. P-values were calculated using two-way ANOVA

with Bonferroni correction, *p=0.0221, **p=0.0049, ***p=0.0005. (C) Immunofluorescent staining with isolectin B4 (IB4) was performed to detect blood vessels in OIR retinas at p17. Top panels shows representative images, bottom panels show neovascular areas pseudo-colored in red and vaso-obliterated areas pseudo-colored in yellow. (D-E) Quantification of neovascular (D) and vaso-obliterated (E) areas (n=15–16 mice per group, from 4 independent experiments). P-values were calculated using a two-tailed Student's *t*-test, **p=0.0019, ***p<0.0001. Data are mean + SEM. Scale bars - 50μM.

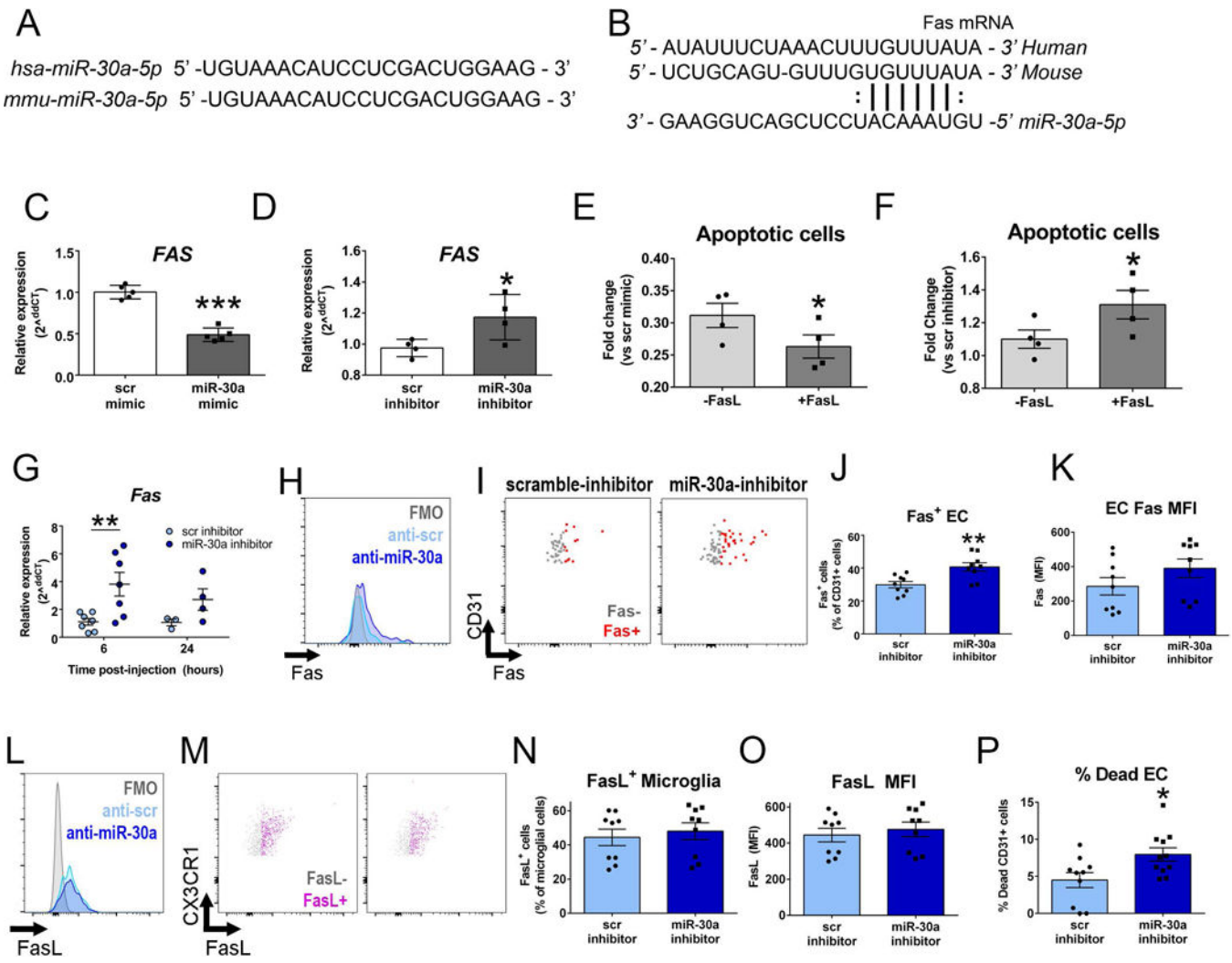

Fig 2.

miR-30a inhibition upregulates Fas expression and promotes endothelial cell death. (A) miR-30a is conserved in humans and mice and (B) targets Fas mRNA. (C-D) Transfection of HUVECs with miR-30a mimic or inhibitor resulted in a (C) decrease or (D) increase, respectively, in FAS mRNA (n=5 wells per group, from three independent experiments). *p=0.044, ***p<0.0001 (E-F) Cells transfected with miR-30a mimics were treated with 100 ng/ml of recombinant soluble FasL (FasL) and stained with annexin V. (E) FasL treatment of HUVECs transfected with miR-30a mimic mildly reduced numbers of apoptotic cells. (F) FasL treatment of HUVECs transfected with miR-30a inhibitor modestly increased numbers of apoptotic cells. n=4 wells, from 4 independent experiments. Data is mean + SEM. P-values were calculated with a paired Student's *t*-test, *p<0.03. (G) miR-30a inhibition resulted in increased levels of retinal Fas mRNA (n=3–7 mice per group). Data are mean + SEM. P-value was calculated using two-way ANOVA with Bonferroni correction. (H-K) Expression of Fas protein by retinal endothelial cells was measured by flow cytometry. (H) Representative flow chart showing Fas fluorescence minus one (FMO) control, miR-30a and scr inhibitor histograms. (I) Representative flow chart showing endothelial cells expressing

Fas (red). (J-K) Quantification of percentage endothelial cells expressing Fas (J) and absolute Fas protein expression levels (n=9 mice per group, from three independent experiments). P-value was calculated using a two-tailed Student *t*-test, **p=0.001. (L-M) Retinal expression of FasL protein was examined by flow cytometry. (L) Representative flow chart showing FasL FMO control, miR-30a and scr inhibitor histograms. (M) Representative flow charts showing FasL-expressing microglia (purple). (N-O) Quantification of FasL expression after miR-30a inhibition. (P) The percentage of endothelial cell death. n=9 mice per group, from three independent experiments. Data are mean + SEM. P-value was calculated using a two-tailed Student's *t*-test, *p=0.019.

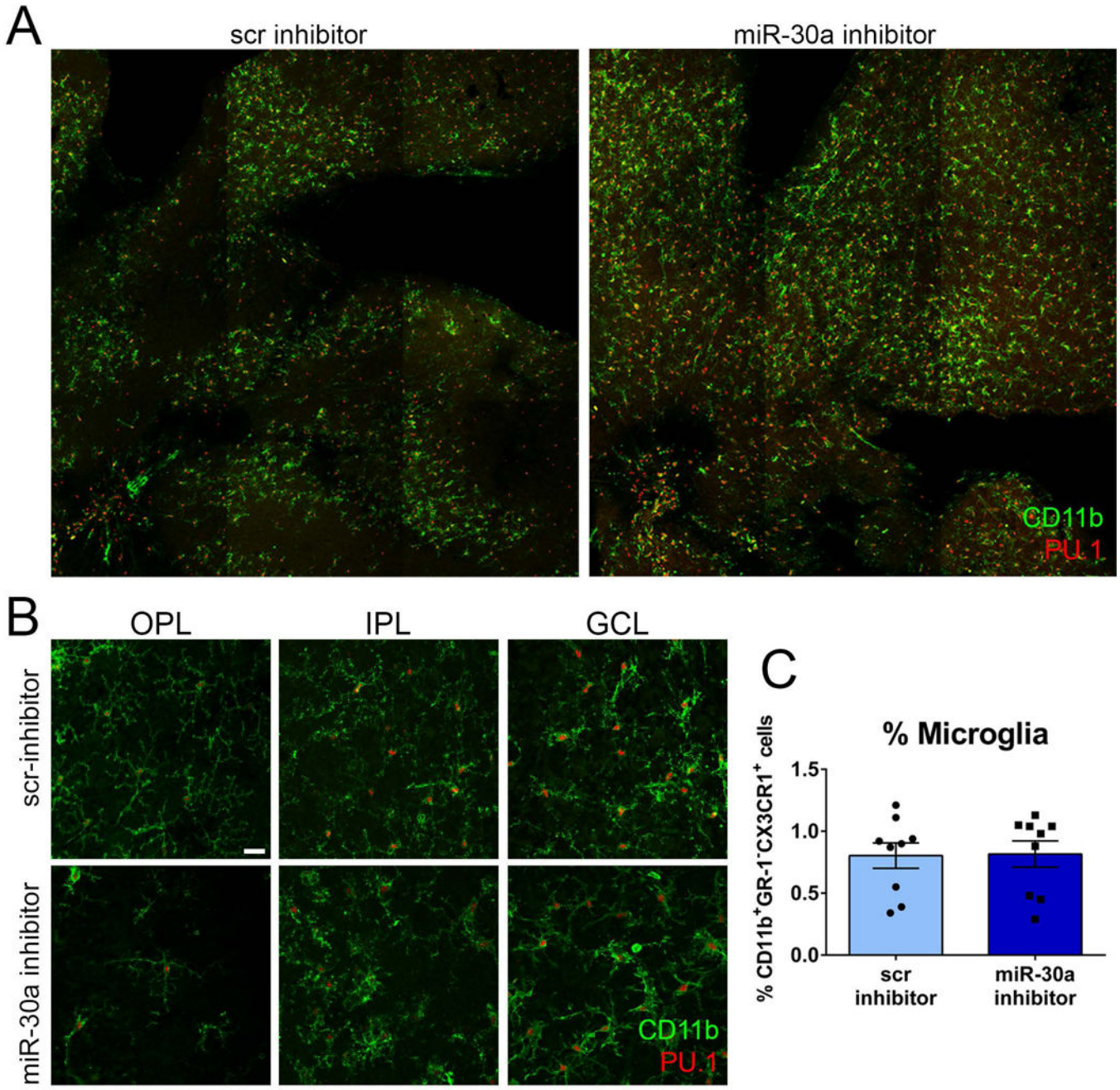

Fig 3.

miR-30a inhibition results in increased microglia migration. Immunofluorescent staining of microglia in OIR retinas with the markers CD11b (green) and PU.1 (red) 48 hours after intravitreal injection of miR-30a inhibitor. Microglia distribution was drastically changed after miR-30a inhibition, with greater coverage of avascular areas by microglia. (B) Changes in microglia cell coverage in the superficial retina appeared to be due to migration of microglia from the deeper layers (OPL) towards the ischemic, more superficial layers (IPL and GCL) as seen in retinal flat mounts. Images are representative of three independent experiments. OPL – outer plexiform layer, IPL – inner plexiform layer, GCL – ganglion cell

layer. Scale bar −20 μm (C) Flow cytometric analysis of microglia (CD11b $^{+}$GR-1^{-}CX3CR1^{+} cells) showed that the percentage of retinal microglia cells was not altered after miR-30a inhibition (n=9 mice per group, from three independent experiments).

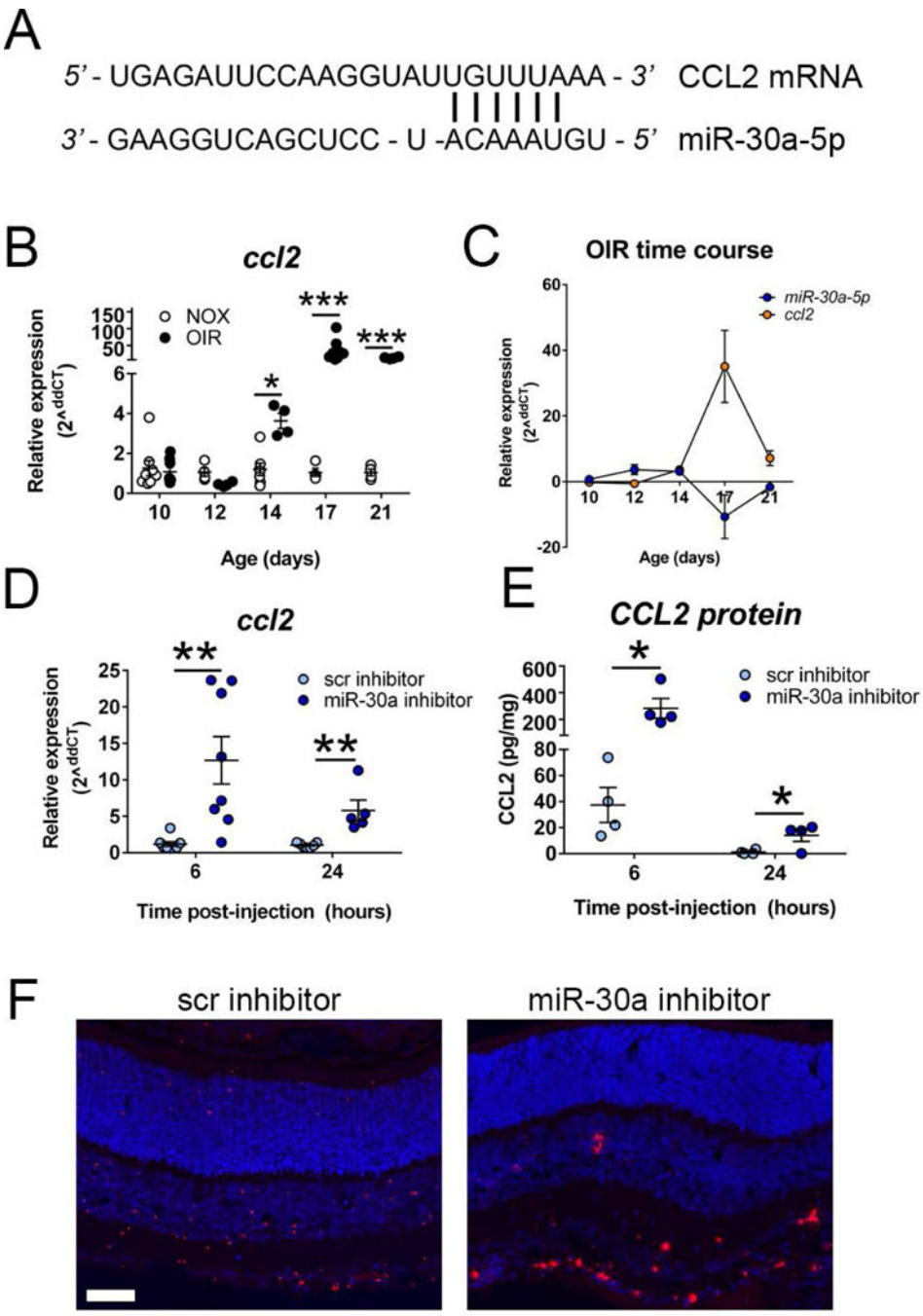

Fig 4.

miR-30a inhibition leads to increased levels of CCL2. (A) CCL2 is a non-conserved target of miR-30a. (B-C) CCL2 expression in OIR retinas occurs in an opposite pattern to that of miR-30a. (B) CCL2 mRNA levels are increased during the repair phase, contrasting to those of miR-30a (n=4–8, from at least two independent experiments). Data are mean + SEM. P-values were calculated using two-way ANOVA with Bonferroni correction, *p=0.012, ***p<0.0001. (D-E) Inhibition of miR-30a resulted in increased levels of CCL2 mRNA (D), measured by qPCR, and protein (E), measured by MSD (n=4 retinas per group, from two to

three independent experiments). Data are mean + SEM. P-values were calculated using two-way ANOVA with Bonferroni correction, *p<0.05, **p<0.001, ***p<0.0001. (F) *In situ hybridization* showed that increased levels of CCL2 (red) following miR-30a injection are mostly observed in the superficial layers, consistent with microglial migratory patterns observed (n=4 mice per group). Images are representative of two independent experiments. Scale bar −50 μm

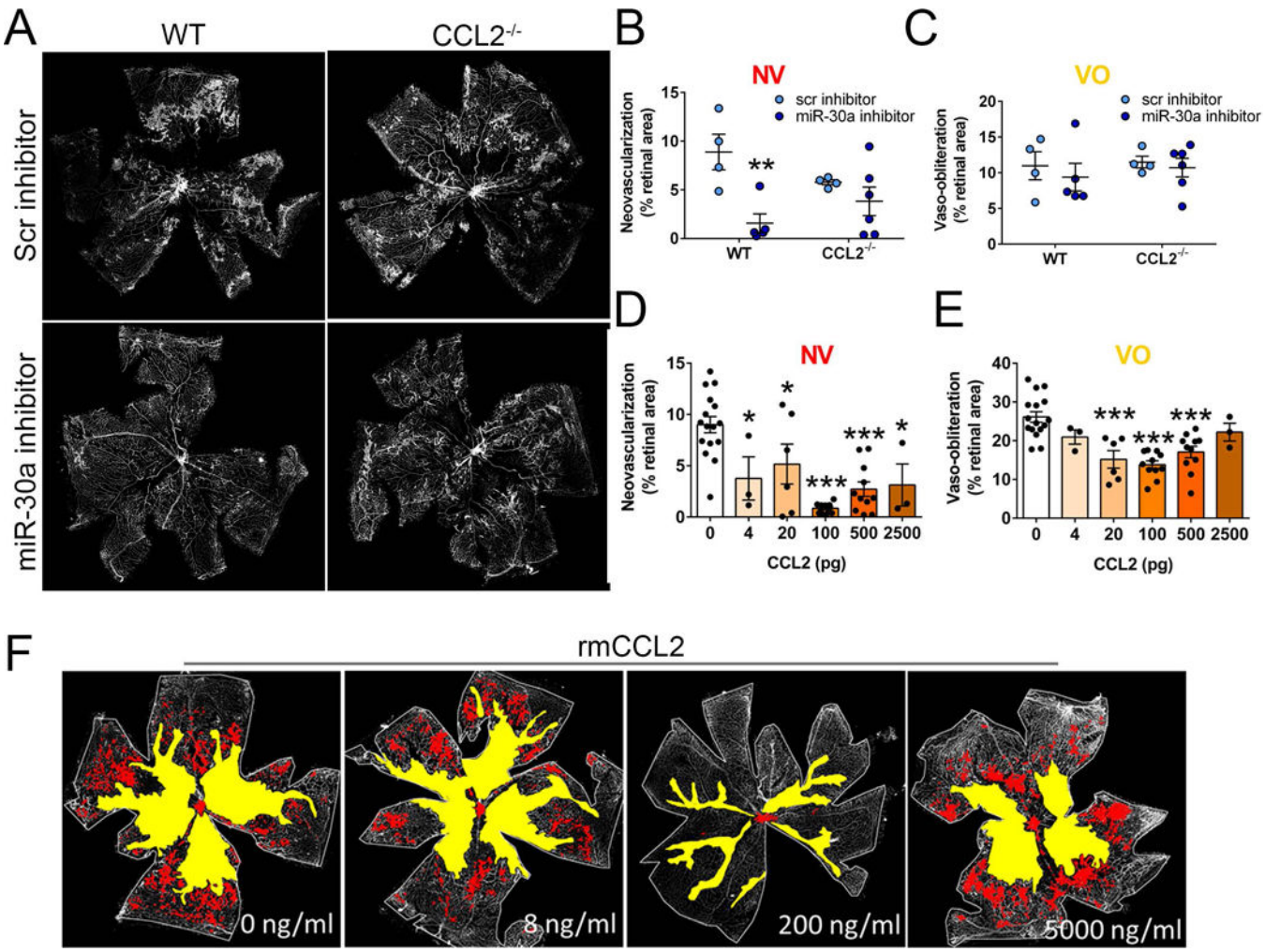

Fig 5.

CCL2 partially mediates the effects of miR-30a inhibition. (A-C) Intravitreal injection of miR-30a inhibitor in *LysM-cre* $CCL2^{fl/fl}$ (CCL2 KO) OIR mice prevented the protective effect of miR-30a inhibition on neovascularization (B) and vaso-obliteration (C) (n=4–6 mice per group, from two independent experiments). Data are mean + SEM. P-values were calculated using two-way ANOVA with Bonferroni correction, **p=0.0035. (D-F) Injection of recombinant CCL2 is sufficient to reduce neovascularization (D) and avascular areas (E) in OIR in a dose dependent manner (n=3–12 mice per group, from two to three independent experiments). Data are mean + SEM. P-values were calculated using one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.001, ***p<0.0001.

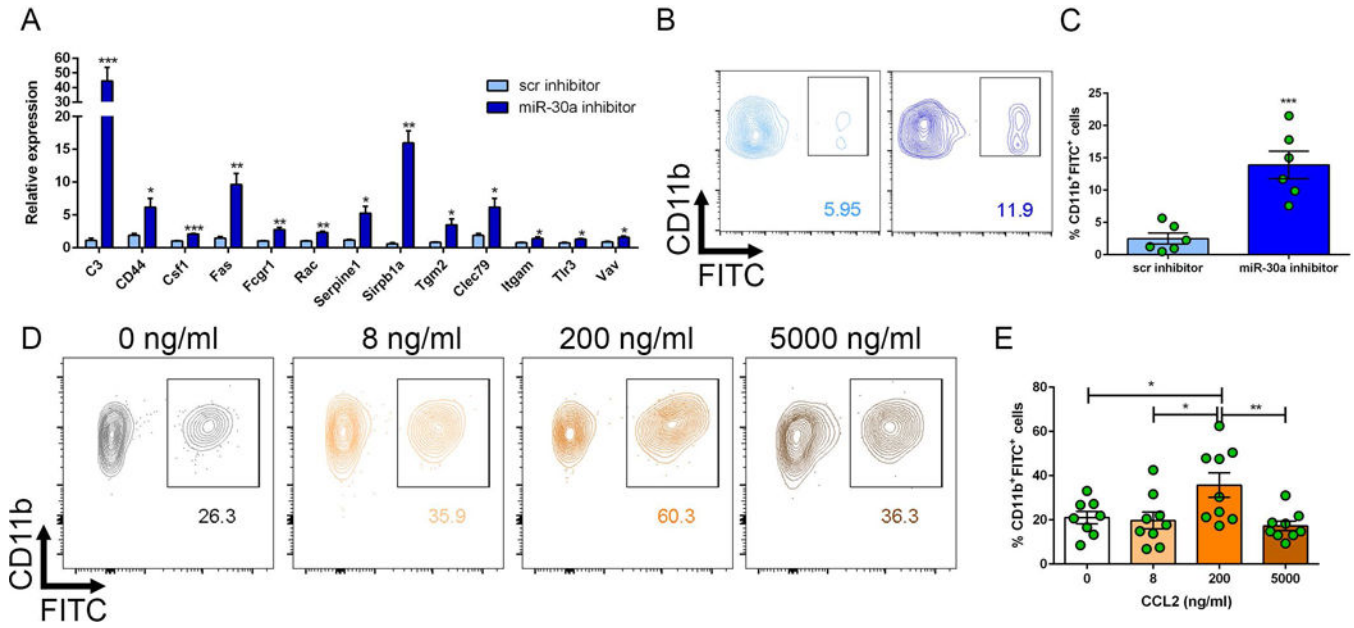

Fig 6.

miR-30a inhibition and CCL2 expression promote microglia phagocytic function. (A) Intravitreal injection of miR-30a inhibitor resulted in upregulation of several mRNAs involved in phagocytic responses. n=3, data is representative of two independent experiments (B, C) Inhibition of miR-30a resulted in increased phagocytic function of microglia cells *in vivo*, as seen by increased uptake of fluorescent beads (n=6 mice per group, from two independent experiments). Data are mean + SEM. P-value was calculated using two-tailed Student's *t*-test, ***p<0.0001. (D, E) Injection of recombinant CCL2 led to an increase in phagocytic function in a dose dependent manner (n=9 mice per group, from three independent experiments) Data are mean + SEM. P-values were calculated using a one-way ANOVA with Bonferroni multiple correction, *p<0.05, **p<0.001, ***p<0.0001.

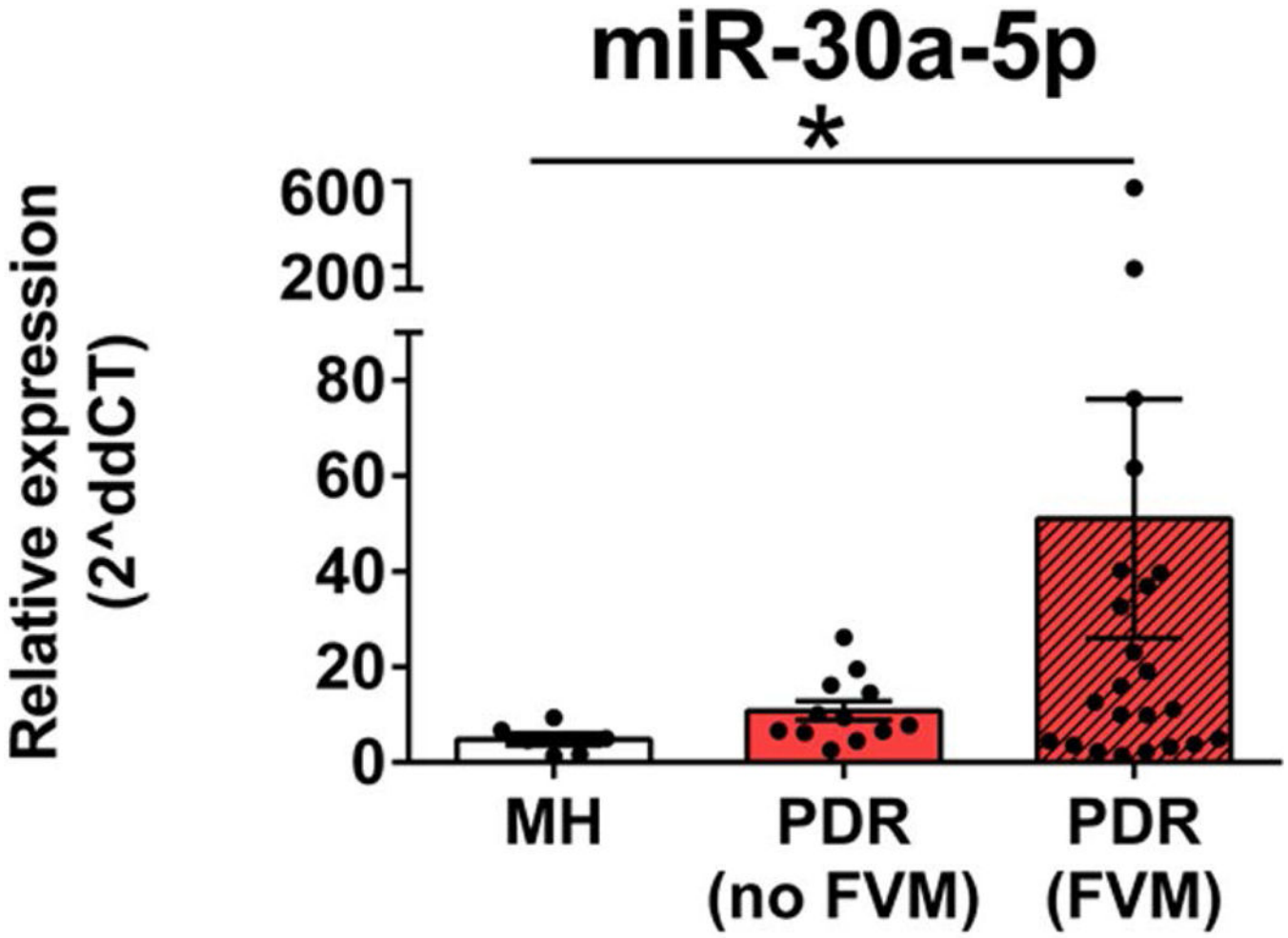

Fig 7.

miR-30a are increased in the vitreous of patients with proliferative diabetic retinopathy. miR-30a levels in the vitreous humor of patients were measured by qPCR (n=6–23 per group). Data are mean + SEM. P-values were calculated using one-way ANOVA with Bonferroni correction, *p=0.04, ***p<0.0001.