Harnessing the angiogenic and immunomodulatory properties of Mesenchymal Stem/Stromal Cells for vascular regeneration of ischemic retinas

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List of Abbreviations

Abbreviation	Full Name
4-HDHA	4-hydroxy-docosahexaenoic acid
AGE	Advanced glycation endproduct
Akt	Protein kinase B (PKB)
Ang	Angiopoietin
Arf6	ADP-ribosylation factor 6
Arg-1	Arginase-1
bFGF	basic Fibroblast growth factor
BM	Basement membrane
BMDM	Bone-Marrow-Derived Macrophages
BRB	Blood retinal barrier
BW	Birth weight
CD	Cluster of Differentiation
CD206 (also known as MMR)	Mannose receptor
Cdc42	Cell division control protein 42 homolog
COUP-TFII	Chicken Ovalbumin Upstream Promoter Transcription Factor II
COX-2	Cyclooxygenase-2
C-X-C-motif chemokine receptor	CXCR
CXCR 4	C-X-C motif chemokine receptor 4
DAG	Diacyl glycerol
DCC	Deleted in colorectal cancer
DHA	Docosahexaenoic acid
Dll4	Delta-like ligand4
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
EC	Endothelial Cell
ECM	Extracellular matrix
EGFL7	Epidermal Growth Factor Like-Domain 7
Eph	Erythropoietin-producing human hepatocellular
Epo	Erythropoietin
ERK	Extracellular Signal–Regulated Kinase
EV	Extracellular vesicles
FABP4	Fatty acid binding protein 4
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
Flk1	Fetal liver kinase 1
Flt1	Fms-Like Tyrosine Kinase 1

- FOXC — Forkhead box C
- FSS — Fluid shear stress
- GA — Gestation age
- GAPDH — Glyceraldehyde 3-phosphate dehydrogenase
- GCL — Ganglion Cell Layer
- GFAP — Glial Fibrillary Acidic Protein
- GM-CSF — Granulocyte-macrophage colony-stimulating factor
- GSH — Glutathione; L-γ-glutamyl-L-cysteinyl-glycine
- GTPase — Guanosine triphosphotase
- GvHD — Graft versus Host Disease
- HGF — Hepatocyte Growth Factor
- HIF — Hypoxia-inducible factor
- HSC — Hematopoietic stem cells
- HypRGC-CM — Hypoxic RGC conditioned media
- HypRGC-MSCs-CM — Conditioned medium derived from RGCs previously stimulated with MSCs-CM
- IA — Intussusceptive angiogenesis
- ICAM — Intracellular adhesion molecule
- IDO — Indoleamine 2,3-dioxygenase
- IFNγ — Interferon gamma
- IGF — Insulin-like growth factor
- IGFBP — Insulin-like growth factor binding protein
- IL — Interleukin
- INL — Inner Nuclear Layer
- iNOS — Inducible Nitric Oxide Synthase
- IR — Ischemic Retinopathy
- IRE1α — Inositol-requiring Enzyme-1α
- ISCT — International Society for Cellular Therapy
- Jag — Jagged
- KDR — Kinase insert domain receptor
- KLF2 — Kruppel-like factor 2
- MAPK — Mitogen-Activated Protein Kinase
- MCP-1 — Monocyte chemotactic protein 1
- M-CSF — Monocyte colony stimulating factor
- MMP — Matrix metalloproteinase
- MSC — Mesenchymal Stromal Cells; Mesenchymal Stem Cells; Multipotent Mesenchymal Stromal Cells; Medicinal Signaling Cells
- MSCs-CM — MSCs-conditioned media
- MT1-MMP — Membrane type 1-matrix metalloproteinase
- NADPH — Nicotinamide adenine dinucleotide phosphate
- N-cadherin — Neural cadherin

- NFL — Nerve Fiber Layer
- NF-κB — Nuclear transcription factor kappa-B
- NO — Nitric oxide
- Nox — NADPH oxidase
- NPDR — Non-Proliferative Diabetic Retinopathy
- NR2F2 — Nuclear receptor subfamily 2 group F member 2
- NRARP — Notch-regulated ankyrin repeat-containing protein
- NRF2 — Nuclear factor erythroid 2-related factor 2
- Nrp — Neuropilin
- NV — Neovascularization
- OIR — Oxygen-Induced Retinopathy
- ONL — Outer Nuclear Layer
- PARP — Poly(ADP-ribose) polymerase
- PDGF-B — Platelet-derived growth factor B
- PDGFRβ — Platelet-derived growth factor receptor β
- PDR — Proliferative Diabetic Retinopathy
- PECAM-1 — Platelet endothelial cell adhesion molecule
- PEDF — Pigment epithelium-derived factor
- PGE2 — Prostaglandin E-2
- PI3K — Phosphoinositide 3-Kinase
- PKC — Protein kinase C
- PPARγ — Peroxisome proliferator activated receptor gamma
- RAGE — Receptor of advanced glycation endproduct
- RASIP1 — Ras-interacting protein 1
- RGC — Retinal ganglion cell
- ROBO — Roundabout
- ROCK — Rho-associated coiled-coil kinase
- ROP — Retinopathy of Prematurity
- RORγ — Retinoic acid receptor-related orphan nuclear receptor γ
- ROS — Reactive oxygen species
- RPE — Retinal Pigment Epithelium
- S1P — Sphingosine-1-phosphate
- SDF — Stromal derived factor
- Sema — Semaphorin
- Shh — Sonic hedgehog
- SHP-1 — Src homology-2-domain-containing phosphatise-1
- SMC — Smooth muscle cell
- T1D — Type 1 Diabetes
- T2D — Type 2 Diabetes
- TEL/CtBP — Translocation ETS leukaemia/carboxy-terminal-binding protein
- TGF — Transforming growth factor

• TLR	Toll-Like Receptor
• TNF	Tumor Necrosis Factor
• TSG	Tumor Necrosis Factor-Stimulated Gene
• Tumor necrosis factor-stimulated gene-6	TSG-6
• UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
• VCAM	Vascular cell adhesion molecule
• VE-cadherin	Vascular endothelial cadherin
• VEGF	Vascular Endothelial Growth Factor
• VEGFR	Vascular Endothelial Growth Factor Receptor

Abstract

Ischemic Retinopathies (IRs), notably retinopathy of prematurity and diabetic retinopathy, are the leading cause of blindness in children and working population. They are characterized by initial degeneration of the superficial microvascular network instituting a hypoxic state. In response to hypoxia, the retina instigates uncontrollable excessive vascular proliferation in an attempt to perfuse the avascular regions. However, the neovessels grow pathologically into the vitreous humor which can cause adverse ocular complications including hemorrhage, retinal detachment and eventually blindness. While current therapies aim to preserve vision by inhibiting neovascularization, they fail at preventing vascular decay and repopulating the damaged regions, which leaves the retina malnourished and susceptible to hypoxia-mediated vascular abnormalities. Hence, there is a need for effective therapies that simultaneously arrest vascular injury and facilitate healthy revascularization. An attractive therapeutic approach involves the use of Mesenchymal Stem/Stromal Cells (MSCs) due to their known angiogenic and immunomodulatory properties which are associated with tissue repair and regeneration.

Using a murine model of oxygen-induced retinopathy (OIR), we showed that intravitreal administration of MSCs secretome (MSCs-CM) curbed vaso-obliteration and neovascularization, thus promoting vascular repair. These vascular effects were mediated in part by replenishing levels of neuronal guidance cue Semaphorin (Sema) 3E, which plays a key role in vascular growth and its downregulation contributes to neovascularization in ischemic retinas. Intravitreal injection of OIR retinas with recombinant protein Sema3E reproduced MSCs-CM-driven vascular regeneration. Moreover, Sema3E downregulation exacerbates inflammation by increasing production of pro-inflammatory cytokines, particularly interleukin-17A (IL-17A). We found that treatment of OIR retinas with MSCs-CM curtailed production of IL-17A from retinal myeloid cells, concomitant to Sema3E upregulation. We further confirmed that neutralization of Sema3E

in MSCs-CM-treated retinas abrogated IL-17A suppression impeding revascularization. These results propose a novel paracrine mechanism by which MSCs elicit vascular repair in ischemic retinas, partly by restoring Sema3E which in turn inhibits the release of myeloid cells-derived IL-17A.

Interestingly, we also found that MSCs-CM modulated cytokine expression in myeloid cells of OIR treated retinas without impacting their density compared to vehicle treatment, suggesting that MSCs-CM controlled myeloid cell dynamic. To demonstrate this, bone-marrow derived macrophages (BMDM) polarized to pro-inflammatory phenotype M1 were treated with MSCs-CM. We showed that exposure of M1 to MSCs-CM significantly reduced production of pro-inflammatory cytokines (iNOS, IL-1β, TNF-α, IL-6) and promoted expression of M2-specific markers (CD206, Arg-1, IL-10), suggesting MSCs-CM shifted macrophage polarization to M2 phenotype. Similarly, MSCs-CM-treated OIR retinas exhibited significant reduction in M1 markers. Because inducible nitric oxide (iNOS) and arginase-1 (Arg-1) compete for the same substrate, we evaluated the ratio of iNOS to Arg-1 in myeloid cells of treated ischemic retinas to determine changes in M1/M2 balance. Treatment of OIR retinas with MSCs-CM substantially lowered the ratio of iNOS/Arg-1 indicating a shift in M1/M2 balance toward the immunosuppressive M2 phenotype.

Altogether, these findings present some evidence of the mechanisms by which MSCs secretome facilitated vascular regeneration in ischemic retinas, by controlling production of neuronal factors and myeloid cell dynamics. MSCs offer a multifaceted therapeutic opportunity for Ischemic Retinopathies by targeting multiple underlying pathologic pathways and providing an environment favorable for vascular regeneration.

Keywords: Ischemic Retinopathies, mesenchymal stem/stromal cells, vascular regeneration, neovascularization, semaphorin 3E, interleukin-17A, inflammation, immunomodulation, myeloid cells, polarization

Résumé

Les rétinopathies ischémiques (RI), notamment la rétinopathie du prématuré et la rétinopathie diabétique, sont la principale cause de cécité chez les enfants et la population active. Elles se caractérisent par une dégénérescence initiale du réseau microvasculaire superficiel instituant un état hypoxique. En réponse à l'hypoxie, la rétine déclenche une prolifération vasculaire excessive incontrôlable pour tenter de perfuser les régions avasculaires. Cependant, les néovaisseaux se développent pathologiquement dans l'humeur vitrée, ce qui peut entraîner des complications oculaires indésirables, notamment une hémorragie, un décollement de la rétine et possiblement une cécité. Alors que les thérapies actuelles visent à préserver la vision en inhibant la néovascularisation, elles échouent à empêcher la dégradation vasculaire et à repeupler les régions endommagées, laissant la rétine mal nourrie et susceptible aux anomalies vasculaires induites par l'hypoxie. Par conséquent, il y a un besoin de thérapies efficaces pouvant stopper simultanément les lésions vasculaires et faciliter la régénération vasculaire saine. Une approche thérapeutique attrayante implique l'utilisation de cellules souches/stromales mésenchymateuses (CSM) en raison de leurs propriétés angiogéniques et immunomodulatrices connues associées à la réparation et à la régénération des tissus.

En utilisant un modèle murin de rétinopathie induite par l'oxygène (RIO), nous avons montré que l'administration intravitréenne du sécrétome de CSM (CSM-CM) freinait la vaso-oblitération et la néovascularisation, favorisant ainsi la réparation vasculaire. Ces effets vasculaires sont médiés en partie par la reconstitution des niveaux du signal de guidance neuronal semaphorine (Sema) 3E jouant un rôle clé dans la croissance vasculaire et dont une sous-expression contribue à une néovascularisation dans les rétines ischémiques. L'injection intravitréenne de rétines RIO avec la protéine recombinante Sema3E reproduit la régénération vasculaire obtenue avec le CSM-CM. De plus, la sous expression de Sema3E exacerbe l'inflammation en augmentant la production

de cytokines pro-inflammatoires, en particulier de l'interleukine-17A (IL-17A). Nous avons trouvé que le traitement des rétines RIO avec du CSM-CM réduisait la production d'IL-17A des cellules myéloïdes de la rétine qui était concomitante à une surexpression de Sema3E. Nous avons, en outre, confirmé que la neutralisation de Sema3E dans les rétines traitées par le CSM-CM abrogeait la suppression de l'IL-17A empêchant la revascularisation. Ces résultats proposent un nouveau mécanisme paracrine par lequel les CSM provoquent une réparation vasculaire dans les rétines ischémiques, en partie en restaurant Sema3E qui, à son tour, inhibe la libération d'IL-17A dérivé de cellules myéloïdes.

Il est intéressant de noter que le CSM-CM modulait l'expression des cytokines dans les cellules myéloïdes des rétines RIO traitées sans affecter la densité des cellules myéloïdes par rapport à un traitement contrôle suggérant que le CSM-CM contrôlait la dynamique des cellules myéloïdes. Afin de démontrer ce résultat, des macrophages dérivés de la moelle osseuse (BMDM) polarisés en phénotype pro-inflammatoire M1 ont été traités avec du CSM-CM. Nous avons montré que l'exposition de M1 au CSM-CM réduisait de façon significative la production de cytokines pro-inflammatoires (iNos, IL-1β, TNF-α, IL-6) et favorisait l'expression des marqueurs spécifiques à M2 (CD206, Arg-1, IL-10), suggérant que le CSM-CM a réorienté la polarisation des macrophages vers le phénotype M2. De même, les rétines RIO traitées par CSM-CM ont montré une réduction significative des marqueurs M1. Étant donné que l'oxyde nitrique inductible (iNOS) et l'arginase-1 (Arg-1) sont en compétition pour le même substrat, nous avons évalué le ratio l'iNOS/Arg-1 dans les cellules myéloïdes de la rétine ischémique afin de déterminer le changement dans l'équilibre M1/M2. Le traitement des rétines RIO avec le CSM-CM a diminué de façon substantielle le ratio iNOS/Arg-1 indiquant un déplacement de l'équilibre M1/M2 vers le phénotype immunosuppresseur M2.

Dans l'ensemble, ces résultats apportent des éléments démontrant les mécanismes par lesquels le sécrétome des CSM facilite la régénération vasculaire dans les rétines ischémiques, en contrôlant la production de facteurs neuronaux et la dynamique des cellules myéloïdes. Les CSM offrent une opportunité thérapeutique multifacette pour les rétinopathies ischémiques en ciblant de multiples voies pathologiques sous-jacentes et en fournissant un environnement favorable à la régénération vasculaire.

Mots clés: rétinopathies ischémiques, cellules souches/stromales mésenchymateuses, régénération vasculaire, néovascularisation, semaphorine 3E, interleukin-17A, inflammation, immunomodulation, cellules myéloïdes, polarisation

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Contribution of Authors

The thesis is manuscript-based in accordance with the guidelines from the Faculty of Graduate Studies and Research of McGill University. Here is the description of the authors' contributions to both manuscripts included in the thesis, highlighting the contribution of the candidate.

Chapter 4 (manuscript 1)

Mesenchymal stromal cells promote retinal vascular repair by modulating Sema3E and IL-17A in a model of ischemic retinopathy (2021) *Frontiers in Cell and Developmental Biology*

Baraa Noueihed, José Carlos Rivera, Rabah Dabouz, Pénélope Abram, Samy Omri, Isabelle Lahaie, Sylvain Chemtob

The candidate conceptualized and designed the experiments with Sylvain Chemtob and José Carlos Rivera. The candidate performed ~80% of the experiments. Quantitative PCR was done by the candidate. Imaging was done by the candidate and José Carlos Rivera. Intravitreal injections were performed by José Carlos Rivera. In vitro experiments and western blots were done by the candidate, José Carlos Rivera, Rabah Dabouz and Pénélope Abram. Isabelle Lahaie prepared retinal samples for staining. Samy Omri provided expert advice and revised the manuscript. The candidate and José Carlos Rivera prepared the figures. José Carlos Rivera designed the scheme. The candidate, Sylvain Chemtob and José Carlos Rivera wrote the manuscript.

Chapter 5 (manuscript 2)

Immunomodulation of myeloid cell dynamics by Mesenchymal Stromal Cells in oxygen-induced retinopathy model *(in preparation for publication)*

Baraa Noueihed, José Carlos Rivera, Samy Omri, Sylvain Chemtob

The candidate, José Carlos Rivera, and Sylvain Chemtob designed the study. The candidate conducted all experiments, analyzed the data, produced the figures, and wrote the first draft of the manuscript. José Carlos Rivera performed intravitreal injections

"They are ill discoverers that think there is no land,
when they can see nothing but sea."

- Sir Francis Bacon

Chapter 1. Introduction

Ischemic Retinopathies (IRs), notably Retinopathy of prematurity (ROP) and Diabetic Retinopathy (DR), are the leading cause of blindness in children and working population, respectively[1, 2, 3]. They are characterized by an initial arrest of vessel growth followed by progressive degeneration of the microvascular bed rendering the retina hypoxic and malnourished. As a result, exaggerated vessel proliferation ensues in an attempt to overcome ischemia. Misguided, the neovessels extend aberrantly into the vitreous cavity, and over time can lead to hemorrhage, retinal detachment and eventually blindness. The incidence rate of IRs has been increasing over the past decades[4, 5], imposing a significant burden on healthcare systems, and urging effective therapies.

Our understanding of the molecular mechanisms governing pathological angiogenesis has grown substantially. Several factors were found to be implicated in aberrant vascular growth, notably vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen secreted by the neuroglial cells[6-8]. Anti-VEGF was proven to be effective in curbing neovascularization and improving the vision of retinopathy patients. However, a critical limitation of this treatment modality is its inefficacy in repopulating damaged regions of the retina[9]. An alternate treatment approach would be using adult Mesenchymal Stem/Stromal Cells (MSCs) for their remarkable therapeutic properties[10, 11]. Since their initial description, MSCs have been shown to exert reparative mechanisms primarily in a paracrine fashion. Using conditioned media of MSCs (MSCs-CM), we investigated the mechanisms facilitating vascular regeneration in an oxygen-induced retinopathy (OIR) mouse model.

Retinal microvasculature injury is mediated by an intricate cross-communication between endothelial cells (ECs), neurons, and myeloid cells. Derived from neurons, class III semaphorins Sema3A and Sema3E modulate vascular patterning[12-15] and myeloid activity[16, 17]. Sema3A exerts

its anti-angiogenic effect secondary to elevated IL-1β levels[18], whereas Sema3E acts on Plexin-D1 expressing ECs of the neovascular tufts to reorient vascular growth towards the retina[19]. Interestingly, Sema3E expression is downregulated in ischemic retinas, which can exacerbate disease severity by upregulating the release of myeloid-derived cytokines, notably of interleukin-17A (IL-17A)[20, 21]. Hence, in chapter 4, we demonstrated that MSCs-CM promoted revascularization of ischemic retinas by modulating the expression of guidance cues and inflammatory mediators. We subsequently unraveled the complex interplay between neurons and myeloid cells mediating vascular regeneration.

Inflammation plays a critical role in pathological angiogenesis. In response to ischemia, microglia become activated and secrete a myriad of pro-inflammatory cytokines, including IL-17A, IL-1β and TNF-α, which partake in vascular degeneration[22]. Because microglia respond to environmental changes, they can be classified as pro-inflammatory M1 or anti-inflammatory M2 based on the profile of secreted molecules[23]. In chapter 4, we found that MSCs-CM modulates cytokine expression of retinal myeloid cells without altering their cell density. Thus, in chapter 5, we examined the role of MSCs-CM in shifting the polarization state of ischemic myeloid cells from pro-inflammatory M1-like to M2-like anti-inflammatory phenotype. Altogether, our data suggest that MSCs-CM are effective in promoting vascular regeneration by simultaneously accelerating vascularization and inhibiting aberrant neovascularization. This beneficial effect is mediated by acting directly and indirectly on the retinal neurovascular unit, comprising of ECs, neurons, and myeloid cells, and modulating the expression of various factors, thereby creating a regenerative microenvironment favorable for healthy vascular growth.

Chapter 2. Literature Review

2.1 Ischemic Retinopathies

Ischemic Retinopathies (IRs), namely Retinopathy of prematurity (ROP) and Diabetic Retinopathy (DR), are the leading cause of blindness in children and working population, respectively. They are biphasic pathologies characterized by an initial arrest of vessel growth followed by progressive degeneration of the microvascular bed rendering the retina hypoxic and malnourished. As a consequence, exaggerated vessel proliferation ensues in the second phase of IRs in an attempt to overcome ischemia. Misguided, the neovessels extend aberrantly into the vitreous humor, which over time can lead to hemorrhage, retinal detachment and eventually blindness. Incidence rate of IRs has been increasing over the past decades, imposing a significant burden on healthcare systems, urging effective therapies.

2.1.1 Retinopathy of Prematurity

2.1.1.1 Epidemiology of ROP

Retinopathy of prematurity (ROP), an ocular condition exclusive to preterm babies (gestation age (GA) <37 weeks), emerged with the development of infant incubators. Originally known as retrolental fibroplasia, ROP was first reported in 1942 by Dr. Theodore L. Terry who described a preterm infant with a fibrous tissue formed behind the lens[24]. During 1940s-1950s in high-income countries, preterm infants <1,000g birth weight (BW) rarely survived while older, more mature babies were supplemented with intensive oxygen to overcome respiratory distress. Survival rates surged but were accompanied with significant increase in ROP incidence. This period is known as the first ROP epidemic and soon oxygen was identified as a principal causative risk factor[25-28]. Oxygen supplementation was thus restricted which reduced ROP rates as expected,

yet cases of cerebral palsy[29, 30] and neonatal death increased[31]. In 1970s, a second epidemic of ROP took place, again in industrialized countries, as premature babies with extremely low BW (<1,000g) survived[32]. Medical advances in neonatal and ophthalmic care along with the development of retinal ablative therapy helped curtail ROP incidence[33, 34]. Two decades later, ROP started emerging in middle-income countries including India, China, Eastern Europe and Latin America, as living standards and medical care improved, which resulted in increased preterm births and concomitantly ROP incidence[35]. Currently, a third epidemic is ongoing as ROP remains a major cause of childhood blindness[1].

Globally, 10% of births occur preterm which accounts for 15 million premature babies born annually[36, 37]. Although ROP afflicts preterm neonates worldwide, its prevalence depends on the affluence and socioeconomic development of the country[38]. In high-income nations, extremely low BW (<1500g) and low GA (<30 weeks) neonates develop severe ROP. Varying ROP incidence has been reported for developed countries with similar neonatal intensive care facilities. For example, in Australia and New Zealand[39], 10% of preterm infants developed severe ROP, whereas Sweden[40] and Belgium[41] reported high ROP incidence of 35% and 26%, respectively. This variation may be partly due to differences in infant mortality rates, which are highly correlated with ROP and widely variable between countries. In middle-income economies, however, moderately premature infants are at a high risk of developing severe ROP, which is often avoidable and treatable[1, 35]. Inadequate quality of neonatal and ophthalmologic care available in neonatal intensive care units, as well as lack of well-defined ROP screening, treatment and prevention programs are identified as causative factors of ROP in such countries. In low-income countries, where infant mortality rates are very high, zero cases of ROP are documented due to the short-term survival of neonates to develop severe ROP or lack of neonatal care units.

While ROP incidence has been globally increasing particularly in middle and low-income countries[4, 42-44], majority of preterm neonates diagnosed with the disease don't develop serious visual dysfunctions or blindness[45, 46]. Acute forms of ROP (stages 1 and 2) often spontaneously regress without requiring clinical intervention. Despite this favorable clinical outcome, other ocular deficiencies including poor visual acuity, refractive errors (such as myopia and astigmatism), strabismus, structural sequelae, and choroidal involution (thinning) persist as a consequence of ROP[47-49].

2.1.1.2 Etiology of ROP

Fetal development occurs in hypoxic intrauterine environment which is permissive for the growth and maturation of the retinal vascular bed. In preparation for the extrauterine life, key maternally derived factors are amply supplied to the growing fetus in the last trimester of pregnancy when rapid growth occurs. Preterm birth separates the maternal-fetal connection prematurely and thus the newborn is poorly equipped for postnatal life. Because the retinal vascular development is completed at term (40 weeks gestation)[50], the retina of a preterm baby is immature and highly vulnerable to various insults. The extent of retinal immaturity is proportional to gestational age and correlates positively with ROP severity[51]. The sudden rise in oxygen tension relative to *in utero*, coupled with deficiencies in growth factors, arrests hypoxia-driven vascular growth. Clinically, this translates to the first phase of ROP known as vaso-obliteration (representing stages 1 and 2 of ROP).

As the underdeveloped retina grows, its metabolic demand increases triggering an exaggerated angiogenic response in efforts to alleviate its hypoxic state. A surge in oxygen-regulated vaso-proliferative factors particularly vascular endothelial growth factor (VEGF)[52] and

erythropoietin (EPO)[53] is triggered. Simultaneously, oxygen-independent factor insulin-like growth factor I (IGF-1) rises gradually as the fetus develops potentiating VEGF activity[54, 55]. These mechanisms may prompt healthy revascularization of the retina and in such cases, ROP spontaneously regresses. In other instances, however, uncontrollable vascular growth occurs extending pathologically into the vitreous cavity. This is referred to as the neovascularization phase of ROP (stage 3). Left untreated, intravitreal neovascularization can impose vitreoretinal tractional forces which cause partial (stage 4) or complete (stage 5) retinal detachment, leading to permanent vision loss.

Understanding the risk factors of ROP pathogenesis is critical for implementing effective management programs and providing timely interventions. The most significant cause of ROP remains the degree of neonatal prematurity which predisposes the retina to adverse injuries[51]. Low gestational age and low birthweight[45] as well as oxygen exposure[56, 57] and loss of maternal-fetal interaction[58, 59] have been consistently associated with increased ROP risk. Other potential risk factors include inflammation[60, 61], nutrition[62-64], and maternal diabetes[65, 66].

Gestational age (GA), birth weight (BW) and postnatal weight gain:

Low GA (≤ 30 weeks) and low BW (≤ 1500g) are the most important risk factors for ROP[45]. Both factors are proportionally related to the underdevelopment of the vascular and neural networks of the retina, which are highly susceptible to injury. Loss of intrauterine factors essential for healthy development, compounded by the infant's immaturity to produce these factors at preterm birth, has profound impact on ROP occurrence and progression. The lower the GA and BW, the higher the risk of ROP. Low GA further prolongs the newborn's exposure to adverse postnatal insults from the extrauterine environment. Additionally, Low BW for GA due to *in utero*

growth restrictions may contribute to ROP[67-70]. Preterm neonates (<30 weeks) born small for their GA are at higher risk for developing ROP.

Poor postnatal weight gain is associated with ROP progression and severity. Human[71, 72] and animal[73] studies demonstrated that slow postnatal growth rate of premature neonates impedes normal retinal angiogenesis and aggravates the clinical outcomes of ROP. Several factors contribute to postnatal growth retardation such as neonate immaturity, insufficient nutrition, oxygen fluctuation, increased neonatal metabolic rate, infections, and illnesses[72, 74]; all of which reflect the subpar overall health status of the newborn and impair general as well as retinal growth. Notably, the sudden loss of maternally-derived IGF-1 at preterm birth strongly correlates with poor postnatal weight gain and impacts ROP development[58] (discussed below). On an optimistic note, postnatal weight gain serves as a reliable parameter to predict occurrence of severe ROP[75]. Several prediction models have incorporated postnatal weight gain measures to identify early infants at high risk of ROP, albeit with varying specificity and sensitivity. Developing robust well-validated weight-based prediction models remains unmet; nonetheless, they offer a non-invasive approach to screening ROP by reducing stressful eye examinations.

Oxygen:

Oxygen plays an important etiological role in ROP[26, 56, 57]. *In utero*, low oxygen levels (hypoxia) drive vascular development which is disrupted by the sudden transition to the hyperoxic extrauterine environment at preterm birth. Hyperoxia secondary to oxygen supplementation (typically between 85-100%) results in cessation of vascular growth and obliteration of newly formed microvessels. This is exacerbated by the newborn's lack of oxygen delivery autoregulation system which restricts ocular blood flow to nourish the immature retina[76]. Multiple clinical trials

have examined the effect of different oxygen saturation levels (SpO_2) on ROP risk, in efforts to determine the ideal target range[45, 57]. The results from these studies collectively highlight the protective effects of limiting oxygen exposure against ROP. However, the optimal SpO_2 remains uncertain, particularly since the use of low target range is associated with increased neonatal mortality and morbidity.

At the molecular level, oxygen is a key regulator of angiogenic mediators. Hyperoxia instigates ROP by destabilizing hypoxia-inducible factor (HIF) which leads to downregulation of pro-angiogenic, pro-survival factors VEGF[52, 77, 78] and Epo[53, 79, 80]. Both oxygen-sensitive factors are critical for normal vascular development of the retina and thus their suppression as a result of hyperoxia contributes to vessel loss. Furthermore, hyperoxia favors the upregulation of anti-angiogenic factors such as thrombospondin-1[81] and tumor necrosis factor-alpha (TNF-α)[82] which induce vascular dropout by promoting endothelial cell apoptosis.

Elevated tissue oxygenation further shifts the redox state towards an oxidative environment and results in excessive generation of reactive oxygen species (ROS)[83, 84]. Because polyunsaturated fatty acids exist abundantly in the membrane phospholipids of the retina, they are prone to peroxidation by ROS. Accumulation of lipid peroxidation products (trans-arachidonic acid, thromboxane A_2, platelet-activating factors, and lysophosphatidic acid) compromises endothelial cell membrane structural integrity and function culminating in vascular degeneration. The resulting oxidative damage is exacerbated by the lack of antioxidant defense mechanisms in preterm infants and their subsequent inability to mount an effective antioxidant response[85].

Maternally derived factors:

Preterm birth severs the maternal-fetal interaction at a critical period of fetal development. Rapid growth of the brain and retina takes place during the third trimester of pregnancy when accretion of essential maternally provided factors, notably IGF-1 and Docosahexaenoic acid (DHA), is at maximum[86, 87]. Loss of IGF-1 and DHA maternal supply have profound impact on an infant's health as well as on the development of ROP and other neonatal morbidities[58, 59].

IGF-1, a polypeptide protein hormone, is crucial for fetal development throughout gestation but particularly during the third trimester[86]. Infants born prematurely exhibit significant and rapid drop in IGF-1 levels which persist for many weeks as opposed to full-term neonates who experience a surge in circulating IGF-1 within the first 15 days of life[58, 88]. Persistent low IGF-1 levels are directly correlated with the development of ROP and other neonatal morbidities, slow postnatal weight gain, and poor neurodevelopment[58]. Experimental animal models have elucidated the importance of IGF-1 for normal retinal vascular growth. Mice deficient in IGF-1 are substantially smaller at birth compared to wild-type controls, present delayed retinal vascularization similar to the clinical features observed in ROP infants and exhibit impaired VEGF-mediated EC survival[54, 55, 89]. The strong association between the loss of maternally derived IGF-1 and ROP occurrence suggests that IGF-1 supplementation may prevent or at least attenuate ROP. This is supported by animal studies demonstrating that exogenous administration of IGF-1 or its binding protein (IGFBP3) reduced considerably ROP severity[90, 91]. Clinically, the use of fresh-frozen plasma (a rich source of IGF-1) or recombinant human IGF-1 and IGFBP3 in extremely premature neonates augmented IGF-1 levels within the intrauterine range[92-94]. A recent phase II randomized trial evaluated whether infusions of rhIGF-1 complexed with rhIGFBP-3 in extremely preterm infants could prevent ROP. Clinical data, however, reported that IGF-1

replacement failed to lower ROP rates but decreased the development of severe bronchopulmonary dysplasia[95].

DHA, an ω-3 long chain polyunsaturated fatty acid, is an essential lipid for vision and neurodevelopment[96]. Because it can't be synthesized *de novo*, DHA is obtained directly from dietary sources such as fish oil. *In utero*, DHA is transferred from mother to fetus and occurs primarily during the third trimester of pregnancy. Preterm birth, consequently, results in DHA deficit, which is associated with ROP occurrence and poor neurodevelopment, particularly in very low BW neonates (<28 weeks GA)[59, 87]. Because the retina contains the highest DHA content of all tissues, it's no surprise that DHA supplementation of preterm infants decreased ROP severity[97-99] and improved visual acuity[100, 101]. In animal models of ROP, lactating mothers fed with ω-3 enriched diet protected nursing mouse pups against pathological angiogenesis[102-105]. These protective effects are mediated by anti-inflammatory ω-3-derived metabolites (resolvins D series and neuroprotectins) which suppress TNF-α production[102]. Conversion of DHA to 4-HDHA (4-hydroxy-docosahexaenoic acid) via lipid enzyme 5-Lipoxygenase elicits an anti-angiogenic effect by activating peroxisome proliferator activated receptor gamma (PPARγ) in ECs[104]. Furthermore, dietary intake of ω-3 polyunsaturated fatty acid increases adiponectin serum levels which are low in preterm babies and positively correlate with postnatal weight gain and GA[105, 106]. Adiponectin potentiates DHA beneficial effects in reducing abnormal microvessel formation via TNF-α suppression[107] and in preserving visual function by promoting photoreceptor cell survival[108].

2.1.2 Diabetic Retinopathy

2.1.2.1 Epidemiology of DR

Over the past decades, the global prevalence of diabetes in adults aged 20-79 years has been steadily rising. The International Diabetes Federation reported that 366 million had diabetes in 2011[109] increasing to 463 million in 2019[5] and estimates 578 million individuals will have diabetes by 2030 and 700 million by 2045[5]. With increasing incidence trends, diabetes is certainly a major public health problem imposing a substantial economic burden. In 2015, the global health expenditure on diabetes and its complications was USD 1.31 trillion and could reach more than USD 2 trillion by 2030[110, 111]. Diabetes is a metabolic disorder characterized by high systemic glucose levels (hyperglycemia) due to either insulin deficiency (type 1 diabetes; T1D) or insulin resistance (type 2 diabetes; T2D). T2D accounts for 90% of diabetic cases and its incidence is attributed to increased life expectancy, consumption of calorie-dense/low-nutritional value diets, urbanization, and sedentary lifestyle[5].

Chronic hyperglycemia jeopardizes the vascular system and disposes patients to significant long-term macrovascular complications such as stroke and cardiovascular disease, as well as microvascular complications such as retinopathy and nephropathy. Diabetic retinopathy (DR) is the most common complication befalling one-third of diabetic patients, of which one-third develop severe vision-threatening conditions such as proliferative DR (PDR) and/or diabetic macular edema (DME)[112, 113]. The severity and progression of DR are impacted by the duration of diabetes. Nearly all T1D patients develop DR within 20 years of diagnosis in contrast to 60% of T2D[114]. Of late, DME, which is frequently observed in T2D, has become the most common cause of diabetes-related blindness as its prevalence has almost doubled in comparison to PDR[115, 116].

Recent epidemiologic studies documented a 2-3 fold decrease in the incidence of PDR and PDR-related blindness in the last 3 decades, particularly in developed countries[117]. This decline is attributed to improved glycemic control, enhanced public awareness, as well as effective screening and treatment regimens. In developing countries, however, a different trend was reported. In 2019, 79% of individuals living with diabetes resided in low and medium-income countries where medical and socioeconomic resources are scarce[5].

With the alarming increase in diabetes, it is worrisome that the incidence of diabetes-associated vision impairment will follow a similar pattern. By 2030, an estimated 191 million individuals will be globally diagnosed with DR and 56.3 million will have some form of vision-threatening DR[118]. Furthermore, 50% of individuals living with diabetes were unaware of their condition in 2019[5], suggesting that opportunities for early diagnosis and timely intervention are most likely to be missed in such cases. Given an imminent epidemic in diabetes, concerted public health efforts are in motion to increase public awareness and implement effective management strategies, especially in developing countries where healthcare systems and eye-care services are inadequate or unavailable.

2.1.2.2 Etiology of DR

Diabetes impairs the retinal neurovascular unit which is composed of an interconnected network of neurons (retinal ganglion cells (RGCs), bipolar, horizontal and amacrine cells), glial cells (astrocytes, Müller cells, and microglia) and vascular cells (ECs and pericytes)[119]. These intimate intercellular connections enable the retina to employ an adaptive physiological process, termed neurovascular coupling, by which blood flow is regulated in response to neural metabolic

demands. Perturbations in this process secondary to diabetes disrupt neural and vascular functions leading to DR.

DR is no longer regarded merely as a microvascular disease of the retina but rather a neurovascular pathology[120]. Accumulating evidence indicates that neurodegeneration precedes vascular lesions and could contribute to diabetic vasculopathy[121]. Neural apoptosis and reactive gliosis, which are key histological features of neurodegeneration, have been observed in animal models and post-mortem human studies[122-125]. Furthermore, neuroprotective agents such as pigment epithelial-derived factor (PEDF)[126] and somatostatin[127] are downregulated in DR, hence compromising neurons integrity against the hostile diabetic milieu and accelerating their loss leading to reduction in neuroretinal thickness[128-130]. Clinically, this translates to a spectrum of visual deficiencies including reduced color vision and contrast sensitivity, impaired dark adaptation and delayed electroretinogram responses (which measure neuroglial function)[131-134]. Thinning of the retina can now be detected via ophthalmoscopic examinations in diabetic patients without overt vasculopathy, though such patients are not diagnosed with DR until the first clinical signs of vascular damage are visible[135-137].

DR develops asymptomatically and insidiously. Over the course of the disease, irreversible microvascular changes emerge as DR progresses from non-proliferative diabetic retinopathy (NPDR) to proliferative diabetic retinopathy (PDR). NPDR, characterized by the presence of microaneurysms and intraretinal hemorrhages, develops from mild to moderate to severe, and subsequently turns into PDR with capillary closures and loss of pericytes. Neovascularization, which is the hallmark on PDR, proceeds whereby leaky blood vessels grow pathologically into the vitreous body resulting in vitreous hemorrhages and retinal detachment[138]. At any stage of DR, diabetic macular edema (DME) may develop due to blood retinal barrier (BRB) breakdown

resulting in vascular leakage and plasma accumulation at the macula (the focal point of the retina)[139].

An early microvascular event of DR is thickening of the basement membrane (BM) lying between ECs and pericytes[140]. Overproduction of ECM proteins (fibronectin, laminin and collagen IV)[141-143] constituting the BM weakens EC tight junctions and disrupts EC-pericyte interactions causing pericyte detachment and migration away from the capillary wall[144-146]. Such changes render the vessels dysfunctional and leaky which clinically manifest as microaneurysms and dot intra-retinal hemorrhages[147, 148] – the earliest detectable signs of NPDR. Local inflammation gradually sets in as production of VEGF and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, MCP-1)[149-152] considerably increase creating a chronic inflammatory milieu, which subsequently activates expression of adhesion molecules (ICAM-1 and P-selectin) on ECs and promotes adhesion of circulating leukocytes to the vascular wall (leukostasis) leading to BRB breakdown[153-155]. As a consequence, intravascular components consisting mainly of lipids and proteins leak into the retina and form hard exudates which can be detected by fundoscopy[114]. Formation of hard exudates in the macula signify the development of DME which progresses from mild to severe proportional to the severity of BRB breakdown. Secretion of vasoconstriction agents (thromboxane A2 and endothelin) ensues driving occlusion and degeneration of capillaries thus creating a hypoxic state. At this point, NPDR is in its severe form and may progress to PDR. The resulting hypoxia prompts uncontrollable proliferation of ECs primarily via VEGF overproduction in an attempt to repopulate the non-perfused retina, yet the neovessels form paradoxically into the vitreous cavity[156, 157]. These extra-retinal vessels are brittle, fragile and prone to rupture causing vitreous hemorrhage and eventually fibrovascular scarring which can lead to blindness.

DR is a complex disease resulting from a series of interdependent mechanisms which are triggered by several factors. Hyperglycemia remains the principal risk factor associated with DR onset[158]; hence it's not surprising that early interventions focus on glycemic control to mitigate any resulting neurovascular complications. Oxidative stress and inflammation are also considered high risk factors in DR pathogenesis as described below[159]. Other risk factors such as hypertension and dyslipidemia may also contribute to DR development and progression[160].

Hyperglycemia:

Glucose is an essential metabolite and its adequate supply to the retina is requisite for proper visual function. In early course of diabetes, metabolic homeostasis is disrupted as excessive amounts of plasma glucose is delivered to the inner retina via the vascular endothelium[161]. The resulting local hyperglycemic milieu triggers a series of pathologic events such as metabolic dysregulation, oxidative damage, and inflammation, which culminate in neuronal dysfunction and vascular lesions. Because glucose plays a central role in DR etiology, early intensive glycemic control has been shown to attenuate DR progression in diabetic patients[158, 162, 163]. Notwithstanding, progression to DR is eventually inevitable with the accumulation of subtle irreversible changes over time leading to neurovascular abnormalities.

Mechanisms of hyperglycemia-mediated damage has been linked to the activation 4 metabolic pathways branching off from glycolysis[159]: (1) advanced glycation endproducts (AGEs), (2) protein kinase C (PKC), (3) sorbitol/polyol and (4) hexosamine.

(1) <u>Advanced glycation endproducts (AGEs):</u> Reducing sugars such as glucose and other glycating molecules react non-enzymatically with proteins, lipids and nucleic acids to produce AGEs[164]. These toxic endproducts are actively generated in diabetes and accumulate irreversibly

in the retina. AGEs aggregate in ECM and alter its composition by cross-linking structural proteins leading to vascular stiffness and resistance to degradation[165, 166]. Loss of ECM degradation coupled with exaggerated ECM protein synthesis promotes BM thickening and BRB breakdown[167-169]. Concurrent with AGE accumulation, a prominent effect of hyperglycemia is overexpression and activation of AGE receptor (RAGE)[170-172]. AGE-RAGE binding instigates a cascade of intracellular pathways resulting in NF-κB-driven transcription of leukostasis-mediating adhesion molecules (endothelin, ICAM-1, VCAM-1) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α)[164, 173]. Furthermore, AGEs drive pathological angiogenesis in part by suppressing neuroprotective and anti-angiogenic agent pigment epithelium-derived factor (PEDF), and conversely by promoting autocrine actions of pro-angiogenic VEGF[169, 174-177], which is a key mediator in the development of PDR and DME[178].

(2) Protein Kinase C (PKC): Glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are converted to diacyl glycerol (DAG) which upregulates PKC pathway. PKC exists in multiple isoforms and have been shown to be implicated in DR pathogenesis by modulating the expression of several downstream target genes to adversely affect vascular integrity[179]. PKC-β activation mediates VEGF-driven neovascularization and disruption of EC tight junctions leading to BRB breakdown[180-182]. Hyperglycemia-induced PKC-δ phosphorylation activates p38α MAPK pathway increasing SHP-1 (Src homology-2-domain-containing phosphatise-1) which in turn inactivates PDGFR-β (platelet-derived growth factor receptor-β) resulting in loss of PDGFR-β-mediated survival factors and pericyte apoptosis[183]. Clinically, these biochemical changes manifest as microaneurysms. Moreover, PKCζ promotes vascular permeability, leukostasis, and BRB dysfunction in diabetic retina by stimulating expression of iNOS, TNF-α, COX-2 and ICAM-1 via NF-κB[184, 185].

(3) Sorbitol/Polyol pathway: High intracellular glucose accelerates polyol pathway to reduce excess glucose to sorbitol via aldose reductase. Because sorbitol is a highly hydrophilic molecule with little ability to diffuse through cell membranes, it accumulates intracellularly causing vascular injury by increasing osmotic pressure and leading to cell death[186, 187]. Moreover, increased aldose reductase activity is implicated in the early events of DR including BRB dysfunction, pericyte loss, VEGF-mediated vascular leakage, membrane thickening and gliosis[188-192].

(4) Hexosamine pathway: Glycolytic intermediate fructose-6-phosphate is channeled through hexosamine pathway to yield the end product uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which serves as a substrate for post-transcriptional protein modification whereby N-acetylglucosamine (GlcNAc) binds to serine/threonine residues of transcription factors causing functional changes[193, 194]. For example, modification of transcription factor SP3 reduces its binding to glucose-sensitive GC box of Ang-2 promotor, leading to augmented expression of Ang-2[195], which in turn plays a critical role in pericyte loss via α3β1 integrin signaling pathway leading to vascular regression[196]. Overstimulation of hexosamine reaction also elicits retinal neurodegeneration by inhibiting the neuroprotective effects of insulin-Akt signaling cascade[197] as well as by enhancing O-GlcNAcylation of NF-κB resulting in retinal ganglion cell death[198].

Oxidative Stress:

Oxidative stress plays an important etiological role in DR. The cumulative metabolic insults (AGE, polyol, hexosamine, PKC) triggered by hyperglycemia translate to a dysregulated oxidant milieu which ultimately causes irreversible vascular damage. AGEs impose structural and functional changes on the mitochondrial electron transport chain resulting in increased reactive oxygen species (ROS) and superoxide formation, which lead to pericyte dropout by activating

caspase-3-dependent cell death[164, 199]. Moreover, AGE-RAGE binding activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family which consists of multiple isoforms, of which Nox 1, 2 and 4 are abundantly expressed in vascular cells and are associated with diabetes-driven injury by exhausting NADPH as an electron donor to yield superoxide ions and hydrogen peroxide (H_2O_2)[200]. Nox 1-induced ROS production contributes to apoptosis of ECs and RGCs[201, 202], and to mitochondrial oxidative damage triggering metabolic memory in DR[203, 204]. Activation of Nox 2 accelerates EC senescence[205], upregulates retinal apoptotic factors[206] and augments vascular permeability[207]. In addition, Nox 2-derived ROS stimulates VEGF and ICAM-1 overexpression thus increasing leukocyte adhesion and BRB dysfunction[208]. High Nox 4 expression is involved in early course of DR by facilitating BRB breakdown and vascular permeability[209], as well as in late disease phase by eliciting retinal neovascularization via H_2O_2/VEGFR2/ERK axis[210]. PKC-δ activation also contributes to mitochondrial dysfunction and ROS overproduction by inactivating complex IV of the electron transport chain[211]. Furthermore, increased flux of polyol pathway exacerbates NADPH depletion and reduce its bioavailability to regenerate the free radical scavenger glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine)[212]. This results in buildup of free radicals and other reactive species which disrupts redox balance and comprises protective antioxidant responses, thus contributing to neurovascular lesions and eventually to DR development.

ROS can also directly induce vascular damage by instigating the 4 metabolic pathways discussed above[213]. In diabetic conditions, elevated levels of intracellular glucose lead to overproduction of glycolytic end product pyruvate which subsequently overwhelms the mitochondrial electron transport chain (via Krebs cycle) and results in the accumulation of superoxides. These highly reactive ions, in turn, activate poly(ADP-ribose) polymerase (PARP)

by introducing DNA strand breaks[214]. Activation of PARP inhibits glyceraldehyde 3-phosphate dehydrogenase (GAPDH) via ADP ribosylation creating a bottleneck effect in glycolysis pathway and leading to the accumulation of upstream glycolytic intermediates, which are subsequently diverted into pathogenic pathways of hyperglycemic injury.

Perturbations in redox homeostasis activate master regulator of detoxification nuclear factor erythroid 2-related factor 2 (Nrf2) to initiate antioxidant defence systems[215]. In response to oxidative stress, Nrf2 translocates to the nucleus and triggers expression of antioxidant and cytoprotective genes (such as superoxide dismutase, heme oxygenase-1 and GSH reductase) to neutralize ROS and restore redox balance. However, in chronic oxidative conditions as in DR, Nrf2 machinery is impaired resulting in ROS accumulation[216-218]. Moreover, Nrf2 contributes to hyperglycemia-associated inflammation by regulating NF-κB, a redox-sensitive transcription factor and a key modulator of immune function[219, 220]. Reduced Nrf2 activity triggers NF-κB signaling cascade to elicit an inflammatory response by increasing production of pro-inflammatory mediators including IL-1β, IL-6, TNF-α, and ICAM. The coordinated crosstalk between Nrf2 and NF-κB precipitates a vicious cycle between oxidative stress and inflammation in DR pathogenesis.

Inflammation:

Inflammation is a major underlying mechanism in DR pathogenesis. The first evidence implicating inflammation as an etiological factor came from treating diabetic patients with salicylates for rheumatoid arthritis and coincidentally lowering their DR incidence rate[221]. Since then, elevated levels of inflammatory mediators (cytokines and adhesion molecules) have been detected in retinas and vitreous fluid of diabetic patients[154, 222-225]. Expression of such mediators

were found to remain persistently high in diabetic patients even after reinstituting normal glycemic levels[226], confirming a causal role of inflammation in DR pathogenesis beyond hyperglycemia.

In ischemic retinas, inflammation is locally synthesized primarily by microglia which are the chief responders to injury. Upon activation, these resident immune cells proliferate and acquire an ameboid morphology, which was reported in retinas of early DR patients and animal models[149, 227, 228]. Hyperglycemia-induced metabolic products are thought to be involved in microglial activation early in the course of DR by binding to surface receptors that recognize pathogen-associated molecular patterns and initiating the innate immune response. Under high glucose conditions, PKC-α and PKC-δ augment the expression and activity of Toll-like receptors TLR2 and TLR4, respectively, which in turn trigger downstream signaling cascade leading to NF-κB translocation and induction of pro-inflammatory cytokines[229, 230]. Inhibition of these receptors arrested NF-κB-mediated inflammatory response. In addition, glycated products (AGEs), which accumulate irreversibly in diabetic retina, bind to their receptor RAGE on microglia resulting in ROS-mediated activation of NF-κB and MAPK pathways, and subsequent TNF-α release[170, 231, 232].

Once activated, microglia amplify the inflammatory response by releasing a myriad of pro-inflammatory cytokines, notably IL-1β and TNF-α, which play a central role in microvascular degeneration[224, 233]. IL-1β propagates inflammation by activating NF-κB which in turn sustains IL-1β expression and upregulates production of other pro-inflammatory cytokines[234, 235]. Blocking IL-1β actions via inhibiting its converting enzyme (caspase-1) or deleting its receptor (IL1R1) prevented capillary degeneration in diabetic mice[236]. TNF-α is involved in EC apoptosis and in BRB breakdown by downregulating expression of tight junction proteins (claudin and zo-1) on ECs via PKCζ/NF-κB pathway, hence increasing vascular permeability[237,238]. In addition to

microglia, Muller cells are activated secondary to elevated glucose concentrations and release pro-inflammatory factors which further aggravate the inflammatory response and promote neurovascular degeneration in diabetic retinas[239]. Moreover, high levels of cytokines regulate the influx of immune cells to ischemic retina by modulating the expression of adhesion molecules, particularly ICAM-1, on ECs[154]. Adhesion of circulating leukocytes via CD18-ICAM-1 interaction mediates Fas-FasL-dependent EC death resulting in the formation of acellular capillaries and eventually BRB breakdown[153, 240]. Neutralization of ICAM-1 or CD18 prevented leukocyte-mediated EC injury and BRB dysfunction[153, 155].

2.1.3 Oxygen-Induced Retinopathy mouse model

Discovery of oxygen as a causal factor in ROP prompted researchers to develop animal models that reproduce the clinical features observed in preterm neonates and help delineate the underlying pathological mechanisms. One of the earliest models used in the 1950s was kitten, developed by Ashton *et al*[241]. Exposure of kitten to high oxygen environment (hyperoxia) led to vascular decay of newly formed capillaries, and their subsequent return to ambient air triggered neovascularization[241, 242]. This landmark study established the two-phase theory of ROP and led to the development of oxygen-induced retinopathy (OIR) model in other species including rat[243], mouse[244] and beagle puppy[245]. Because the OIR model replicates the process of neovascularization, its use has been extended to study the pathogenesis of the proliferative phase of DR[246]. To date, the OIR model has contributed tremendously to better understanding the etiology of ischemic retinopathies and to the development of various therapeutic modalities.

The OIR mouse model was developed by LE Smith *et al*[244] in an effort to recapitulate the vascular abnormalities manifested in human ROP neonates (Figure 1). Postnatal day P7 mice pups

are exposed to a high oxygen environment (75% O_2) for 5 consecutive days to arrest vascular growth and induce vaso-obliteration. This is known as the first phase of OIR which mimics the vasoconstrictive effects of oxygen and leads to degeneration of the superficial vascular bed as in human ROP retinas. However, vascular decay occurs in the center of the mouse retina, whereas in humans it is localized at the periphery. On day 5, P12 mice pups are returned to room air to initiate the second phase of OIR – neovascularization. At this point, the retina is in a relative hypoxic state from resulting vessel loss and prompts uncontrollable vascular proliferation in an attempt to perfuse the damaged regions. Rather, the neovessels grow at the junction of vascular and avascular retina and extend pathologically into the vitreous. In humans, neovascularization occurs at the mid-periphery of ischemic retina and could exert tractional forces that may lead to retinal detachment, which is not observed in the OIR mouse model (Figure 2).

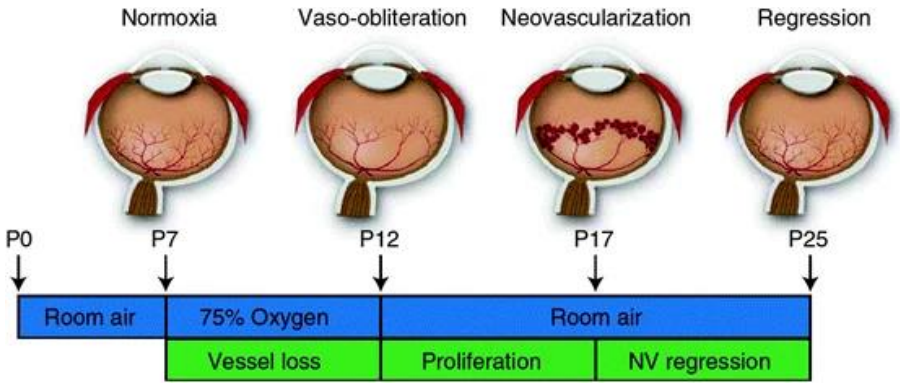

Figure 1. Oxygen-Induced Retinopathy Mouse Model. A schematic representation of the OIR mouse model reproducing the biphasic stages observed in human ROP neonates. P7 mice pups are exposed to hyperoxia induces vaso-obliteration and are then returned to room air at P12 triggering neovascularization, which peaks at P17. Neovascular regression ensues promoting vascular repair by P25. (Figure adapted from Connor *et al.*[247])

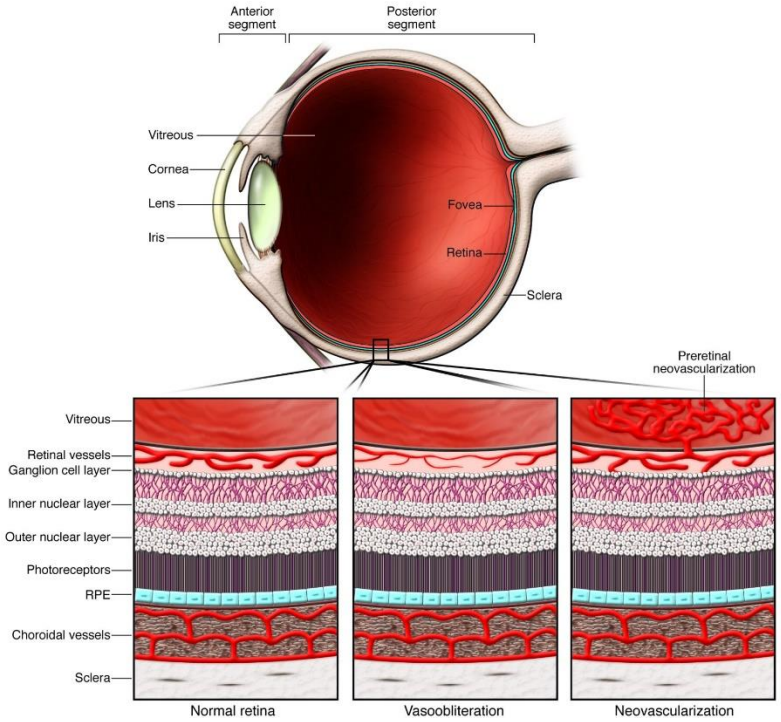

Figure 2. Pathogenesis of Retinopathy of Prematurity. A schematic illustration of the retinal vasculature depicting the vascular decay of the superficial vascular bed secondary to hyperoxia (vaso-obliteration), followed by compensatory yet exaggerated pathological growth of neovessels into the vitreous (neovascularization). (Figure adapted from Sapieha *et al.*[248])

The mouse model of OIR is used most frequently as it offers several advantages. First, it is a robust and straightforward model. The response to oxygen tension in both phases is reproducible, consistent, and easily measurable[246, 249]. Second, unlike humans, the retinal vascular network in mice forms postnatally which permits the visualization of vascular changes during development and in response to experimental manipulations[250, 251]. In addition, the ability to genetically engineer mice to selectively express or suppress a certain gene provides valuable insight into its specific functions[252, 253]. Finally, animal costs, time-efficient procedures (mouse gestational period is 3 weeks), and availability of species-specific antibodies and recombinant proteins render mouse model favourable.

2.2 Retinal Vascular Development

2.2.1 Structure of the retina and its vascular supply

Retina, the innermost part of the human eye, is a transparent light-sensitive structure responsible for detecting light and converting it into electrical signals, which are then relayed to the brain via the optic nerve[254]. It is a multilayered and highly organized network comprising of first, second and third-order neurons of the visual pathway which are arranged in the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) respectively and are intercalated by 2 plexiform (synaptic) layers where synapses of neurons interconnect (Figure 3). Retinal vasculature emerges from the central retinal artery which branches into three parallel, interconnected capillary layers: the primary (superficial) plexus in the NFL, and the intermediate and deep plexus along each side of the INL.

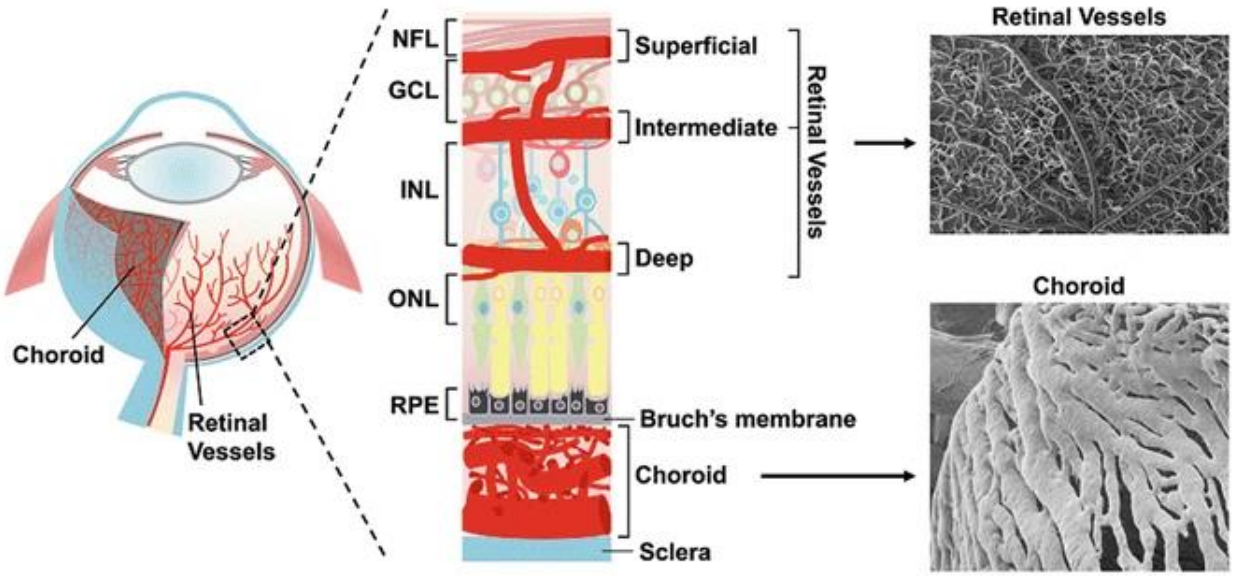

Figure 3. Retina and its vascular supply. A schematic illustration of the retina and its vascular network. *Left:* A schematic cross-section of the eye demonstrating the 3 vascular retinal layers and choroidal vascular network. Right: A cross-section of retinal and choroidal vessels. NFL=Nerve fiber layer. GCL=Ganglion cell layer. INL=Inner nuclear layer. ONL=Outer nuclear layer RPE=Retinal pigment epithelium. (Figure adapted from Chen *et al.*[255])

Embryologically, the retina is a projection of the brain and shares the brain high metabolism. Yet, the retina is considered the most oxygen-consuming structure requiring roughly 10ml O_2/100ml tissue per minute to maintain vision, which is more than double the metabolic demands of the brain[256]. The high energy cost of vision requires efficient blood supply matching the tissue's metabolic needs. To meet these critical requirements, the adult human retina is vascularized with two independent circulatory systems: the choroidal vasculature which supplies the outer one-third of the retina including the photoreceptor and retinal pigment epithelium (RPE) layers, and the retinal vasculature which nourishes the inner two-thirds of the retina.

During embryonic stages of the developing eye, the ocular vascular systems develop in an orchestrated, synchronous fashion and undergo massive remodeling driven by oxygen demands of the growing tissue. Any perturbations during development or in response to pathologic insults might lead to deficits in blood supply resulting in visual defects, including blindness. In the first trimester, when the retina is thin and avascular, its oxygenation is ensured by choroidal vessels and the hyaloid artery. Choroidal vessels start forming, prior to retinal network, at 6-7 weeks gestation (WG) encircling the entire optic cup and maturates by 21-23 WG[257, 258]. The hyaloid artery, a transient embryonic vascular system, invades the vitreous body at 7 WG and partakes in the development and maturation of the lens, formation of the primary vitreous and nourishment of the immature retina[259]. As the lens matures, the hyaloid artery undergoes progressive and complete regression, coinciding with the onset of retinal vasculature development[260]. Retinal vasculature is the last major vascular supply in the human eye to develop. Its development begins *in utero* at 14-16 WG and is completed by the end of the 3rd trimester (38-40 WG)[261].

2.2.2 Vascular biology

Vessel formation is achieved via a 3-step process starting with de novo formation of the primitive vascular plexus via vasculogenesis[262], followed by sprouting of new capillaries from pre-existing ones via angiogenesis, and remodeling of endothelial cells into an organized architecture by a process known as arteriogenesis.[263, 264].

2.2.2.1 Vasculogenesis:

Vasculogenesis is the *de novo* formation of the first, primitive blood vessels from mesoderm-derived endothelial precursor cells (EPCs) or angioblasts [262]. These precursor cells originate from hem angioblast which is a bipotential competent cell capable of giving rise to blood or endothelium depending on local environmental cues[265]. Vascular endothelial growth factor (VEGF) and its receptor VEGFR2 (also known as kinase insert domain receptor (KDR) or as fetal liver kinase 1 (Flk1)) play a pivotal role in vasculogenesis. Secreted by endoderm, VEGF acts on mesodermal cells expressing VEGFR2 to promote their differentiation into angioblasts. Thereafter angioblasts migrate extensively within the embryo. They align and gradually coalesce into vascular cords as they differentiate *in situ* into endothelial cells (ECs) to generate the primary capillary plexus[266]. *In vitro* studies underscored the critical role of VEGF in mediating normal vascular development, as mice deficient in a single VEGF gene died mid-gestation due to major vascular defects[267, 268]. Moreover, VEGFR2-deficient mice failed to undergo vasculogenesis due to defective hematopoietic and angioblastic lineages and are thus embryonic lethal[269].

As the vascular cords form, ECs become specialized to form a hierarchically organized network of arteries, veins and capillaries before blood flow begins. The decision to acquire an arterial or venous fate is governed by genetic mechanisms which preferentially direct EC

specification[270, 271]. Notch pathway plays a key role in the arteriovenous decision-making process whereby most of its components are confined to arteries. Its receptors Notch1, Notch3 and Notch4, and its ligands Delta-like ligand4 (Dll4), Jagged1 (Jag1) and Jagged2 (Jag2), are expressed in an artery-specific manner via forkhead box transcription factors FOXC1 and FOXC2. In veins, however, the expression of Notch signaling proteins is suppressed by the orphan nuclear receptor Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII; also known as nuclear receptor subfamily 2 group F member 2 (Nr2f2)), which functions as a master regulator of venous specification[272]. Mutation or deletion of Notch pathway genes results in embryonic lethality due to severe defects in vascular remodeling and arterial development. Conversely, constitutive Notch activation induces ectopic expression of arterial markers and leads to arteriovenous shunts. Moreover, Notch pathway controls the expression of ephrin-B2/EphB4 complex which belongs to ephrin-Eph receptor tyrosine kinase family and differentially labels arterial and venous ECs[270, 272]. Ephrin-B2, a membrane-tethered ligand, marks arteries in response to Notch whereas its cognate receptor EphB4 is largely restricted to veins. Ephrin-Eph signaling is required for the establishment and maintenance of arteriovenous boundaries. Deletion of ephrin-B2 or EphB4 gene results in abnormal intercalation and defective communication between arteries and veins without disrupting arterial-venous formation.

Upstream of Notch, sonic hedgehog (Shh), a member of the Hedgehog family of secreted morphogens, initiates arterial-specification cascade indirectly by inducing VEGF expression in adjacent cells [270]. Alternatively, Shh can directly induce arterial phenotype in zebrafish via calcitonin receptor-like receptor, independently of VEGF [273]. VEGF-A is secreted into three protein isoforms in mice VEGF120, VEGF164, and VEGF188 which differ in their diffusion kinetics. VEGF120 diffuses freely, VEGF188 is tightly bound to cell surface by heparan sulfate

proteoglycans, whereas VEGF164 possesses both of these properties. Mice engineered to express only VEGF120 or VEGF188 exhibit impaired myocardial angiogenesis and defective vascular branching in kidney and retina[274-276]. Interestingly, mice expressing VEGF164 alone display no vascular defects and present normal arterial and venular outgrowth indicating that this isoform drives arterial specification[274]. VEGF-A transduces its arterial effect by activating downstream transcription factors FOXC1and FOXC2 to induce Dll4 expression[277]. Furthermore, VEGF-A activates extracellular signal–regulated kinase (ERK) pathway which in turn induces Notch expression and signaling and upregulates ephrin-B2 transcription[278, 279]. This effect is facilitated by VEGF co-receptor Neuropilin-1 (Nrp1) which is abundantly expressed by arterial ECs during development and acts in concert with VEGFR2 to activate downstream arterial transcription mechanisms. In cells lacking Nrp1, VEGF-A triggers opposite differentiation outcome directing EC commitment to a venous fate via phosphoinositide 3-kinase (PI3K) pathway.

Hemodynamic forces can also influence arteriovenous EC identity[271, 280]. Studies in mice and chick embryos demonstrated that expression of Notch pathway components ephrin-B2 and Nrp1 are regulated by blood flow[281, 282]. Reversing blood circulation in chick yolk sac arteries resulted in downregulation of arterial markers and reprogramming of EC specification to a venous destiny[281]. These observations highlight the important role of hemodynamic forces in maintaining arteriovenous EC phenotype, though the molecular processes remain ill defined.

Once the primary capillary plexus is formed and ECs have acquired their arterial or venous identity, the nascent vasculature begins expanding into a highly branched network by a process termed angiogenesis.

2.2.2.2 Angiogenesis:

Angiogenesis is the formation of new vessels from pre-existing ones. There are two types of angiogenesis: sprouting angiogenesis which is the outgrowth of a new branch from an existing vessel and intussusceptive angiogenesis (IA) which is the splitting of a parent vessel into two[263]. Many of the studies have focused on delineating the molecular processes regulating angiogenic sprouting, whereas the mechanisms of intussusception are not well understood.

Sprouting angiogenesis:

For angiogenic sprouting to begin, dormant, quiescent vessels must first be destabilized. ECs undergo fundamental changes in their behavior in response to alterations in tissue hemeostasis[263]. Several factors, including hypoxia, inflammation and shear stress, stimulate the emergence of ECs from their resident sites by inducing the release of matrix metalloproteinases (MMPs)[283]. MMPs degrade the basement membrane shared between ECs and mural cells and liberate pro-angiogenic growth factors (such as VEGF, basic fibroblast growth factor (bFGF)) sequestered in the extracellular matrix (ECM). Mural cells encasing the vessel are then rapidly detached by the release of EC-derived Angiopoietin-2 (Ang2), an antagonist of Tie2 kinase activity, rendering ECs exposed and receptive to local angiogenic cues[284, 285].

VEGF-A initiates vessel sprouting by activating one EC, referred to as tip cell, to lead the growing vessel rather than moving en masse; whereas neighboring ECs adopt stalk cell phenotype[286] (Figure 4A). Tip cells are characterized by their position at the vascular front, and by their invasive, migratory behaviour guided by their numerous long filopodia. In contrast, stalk cells trail behind tip cells and support vessel elongation by proliferating, maintaining tight junctions, and forming lumen. Tip-stalk cell selection is dynamic and transient whereby ECs constantly

compete for the tip cell position and frequently shuffle between the two phenotypes[287]. This is driven by the differential expression of VEGFR2 versus VEGFR1 (also known as Fms-Like Tyrosine Kinase 1 (Flt1)) which displays high turnover during vessel branching. ECs with higher levels of VEGFR2 gain a competitive advantage to become tip cells via VEGF-A stimulation[286, 287]. Stalk cells, however, express primarily VEGFR1 which has weak tyrosine kinase activity and acts as a decoy receptor competitively binding to and trapping VEGF-A, hence impairing VEGF-driven tip cell formation [286, 288].

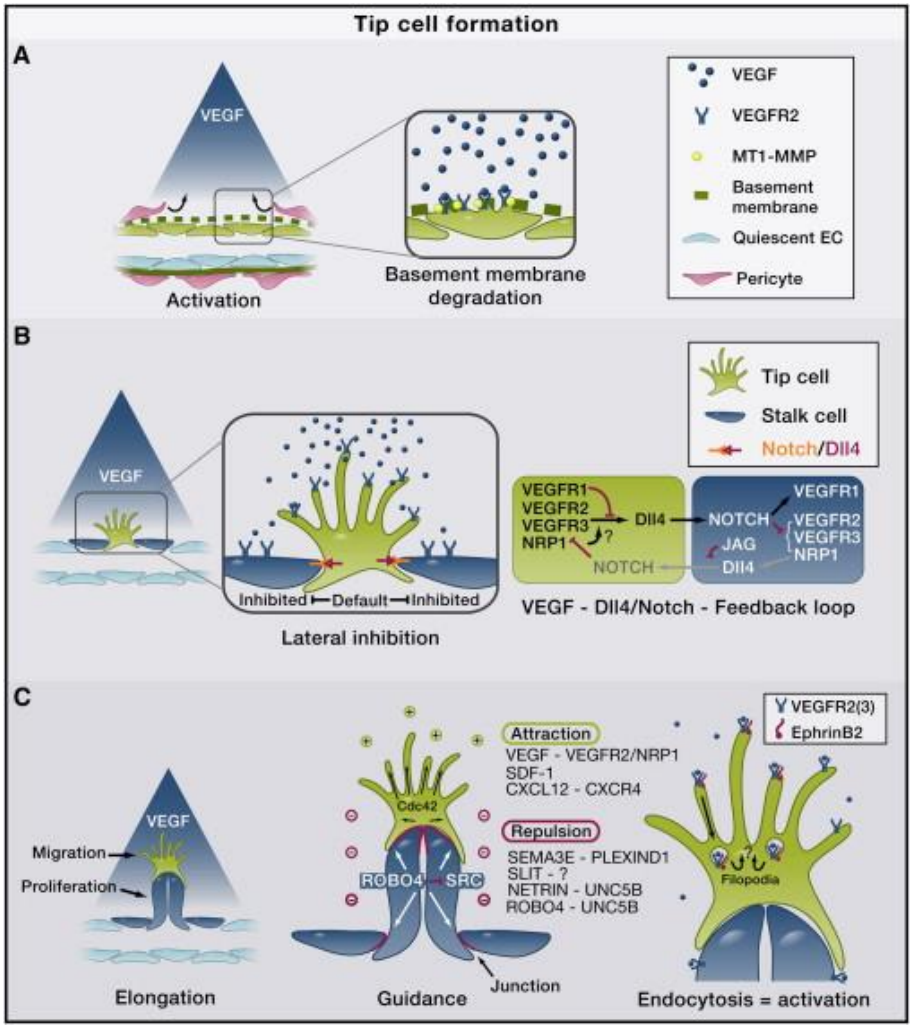

Figure 4 Formation of tip cells at the vascular front. (A) VEGF and other pro-angiogenic factors promote basement membrane degradation and pericyte detachment. (B) Notch signaling dictates tip cell selection by upregulating Dll4 expression and VEGFR2 expression on tip cells.

(C) Filopodia extending from tip cells probe the environment and steer the growing vessel in response to guidance cues. (Figure adapted from Potente *et al.*[263])

Notch orchestrates vessel growth by coordinating tip-stalk cell behaviour via VEGF-Dll4-Notch feedback loop (Figure 4B). In response to VEGF-VEGFR2 signaling in tip cells, Dll4 surface expression is upregulated by disrupting the transcriptional repressor complex TEL/CtBP (translocation ETS leukaemia/carboxy-terminal-binding protein) at the *Dll4* promoter[289]. Dll4, in turn, binds to its Notch receptors on adjacent ECs and activates Notch signaling to laterally inhibit tip cell behaviour. High Notch activity precipitates stalk cell phenotype by upregulating VEGFR1 and downregulating VEGFR2, VEGFR3 and Nrp1[286, 290]. Interestingly, Notch also induces the expression of its own inhibitor Notch-regulated ankyrin repeat-containing protein (NRARP) which interrupts Notch-mediated cell cycle arrest and activates mitogenic Wnt pathway, thus promoting stalk proliferation and elongation[291]. On the contrary, stalk cells express predominantly Jag1 which antagonizes Dll4-Notch signaling back to tip cells by competitively binding to Notch and suppressing its activity. The ability of Jag1 and Dll4 to generate antagonistic effects on Notch activity is dependent on Notch modification via glycosyltransferase Fringe which enhances Dll4 activation and represses Jag1 stimulation, consequently fine-tuning Notch-mediated vascular density[292]. Loss of function studies in mice underscored the indispensable role of Notch ligands in regulating tip-to-stalk cell ratio and in establishing proper vascular network. Blockade of Dll4 results in excessive tip cell formation and hypersprouting[293, 294], whereas perturbation of Jag1 reduces vascular branching and impairs angiogenesis[292].

At the vascular forefront, tip cells probe the environment and steer the growing sprout to non-vascularized areas in response to guidance signals released by proximal cells (Figure 4C). VEGF, a potent EC morphogen, chemoattractant and mitogen, triggers filopodia formation by

activating small GTPase Cdc42 in tip cells to direct cell migration[295]. CXCR4 receptor is also abundantly expressed on tip cells rendering them attractant to the chemokine stromal derived factor 1 (SDF-1)[296]. Axon guidance cues, which were initially described for axonal pathfinding, are implicated in vascular branching either by acting directly on ECs or by modulating EC response to VEGF[297]. Roundabouts (ROBOs) (1-4) are membrane-bound receptors known for their repulsive effects upon activation by their ligands Slits (1-3). ROBO4, an EC-specific receptor, is crucial for vessel integrity as its deficiency results in leakiness and hypervascularization[298]. Slit2 binding to ROBO4 inactivates GTPase Arf6 activity inhibiting EC protrusion[299]. ROBO4 can also interact with Unc5b, a vascular Netrin receptor, blocking VEGF-induced permeability and angiogenesis by disrupting Src kinase recruitment necessary for VEGF signal transduction[300]. Unc5b anti-angiogenic effects are further mediated via Netrin1 and 4 causing filopodia retraction[301, 302]; conversely, Netrins may induce vaso-attraction by binding to other receptors (DCC or neogenin)[303]. Semaphorins (Sema) are a large family of secreted or membrane-bound ligands that exert their effects by binding to Nrp/Plexin complexes or to Plexin alone[297]. Sema3E negatively regulates angiogenesis and functions as a repulsive cue by binding to PlexinD1 receptor triggering cytoskeletal rearrangements and filopodia retraction[13]. Sema3E-PlexinD1 signaling further modulates tip-stalk cell formation by inhibiting VEGF-upregulated Dll4 expression in tip cells[12]. Eph receptors and their ephrin ligands regulate vascular morphogenesis in a cell-contact dependent manner. They are known for their unique capacity to initiate bidirectional signaling driven either by the receptor (forward signaling) or by the ligand (reverse signaling)[304]. ephrinB2-mediated reverse signaling controls tip cell behaviour by modulating VEGFR2 internalization[305, 306]. Collectively, these disparate signals converge to orient the nascent vessel and ultimately determine whether to advance or collapse.

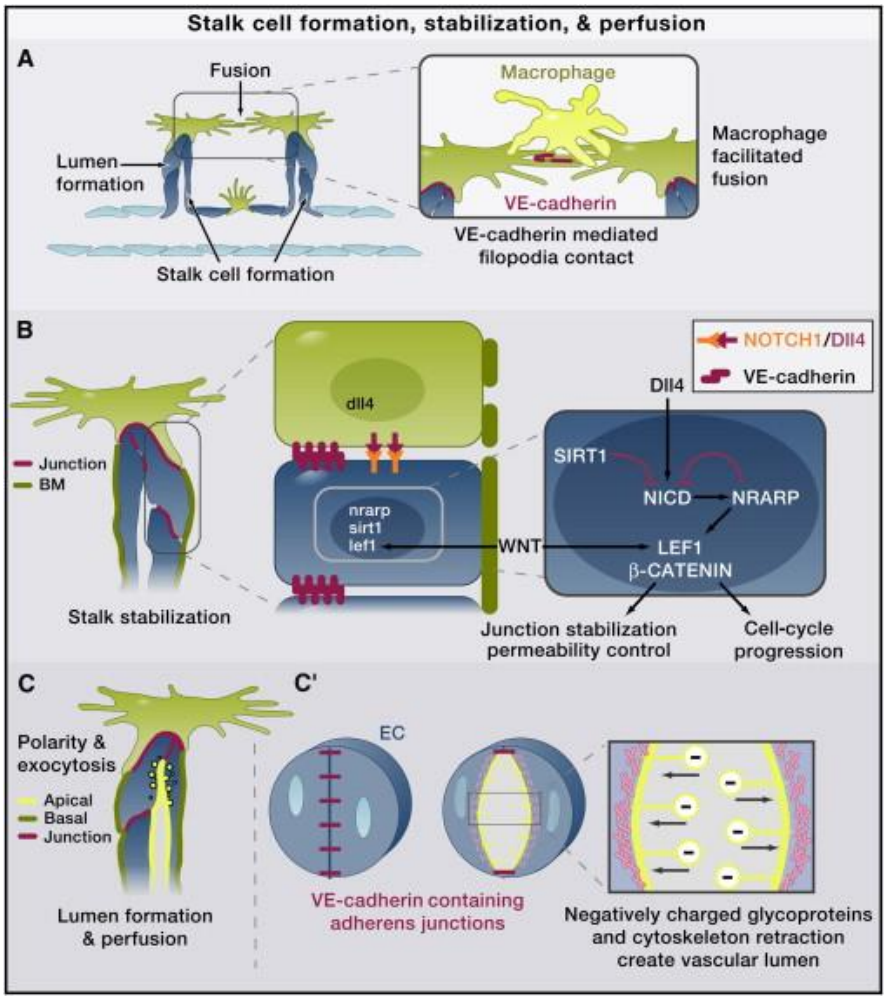

Figure 5. Stalk cell elongation, stabilization, and lumen formation. (A) Two tip cells meet and fuse together to form an interconnecting highly branched vascular circuit, facilitated by tissue resident macrophages. (B) As stalk cells continue to proliferate, Notch-regulated ankyrin repeat protein (NRARP) regulates Notch signaling to maintain intercellular tight junctions and stabilizing vessel extension (C) Endothelial cells establish an apical-basal polarity whereby negatively charged glycoproteins on the luminal side of ECs repel allowing lumen formation and expansion. (Figure adapted from Potente *et al.*[263])

Stalk cells play an important role in sprouting as they are responsible for stalk elongation and lumen formation. Regulation of Notch and canonical Wnt signaling (via Notch-regulated ankyrin repeat protein (NRARP)) in stalk cells promote cell proliferation while maintaining intercellular tight junctions, consequently preventing dispersed growth and stabilizing vessel extension[291] (Figure 4B). Stalk cells also deposit ECM proteins, such as EGFL7 (epidermal

Growth factor like-domain 7), which regulate spatial distribution of ECs. Deletion of EGFL7 halts stalk elongation as proliferating ECs accumulate at the base of the sprout[307]. As the vessel elongates, the process of vascular lumen formation is initiated to establish perfused neovessels. ECs first establish a defined apical-basal polarity by redistributing junctional proteins from the EC-EC apical interface to the periphery[308] (Figure 4C). Negatively charged glycoproteins aligned at the interface trigger an electrostatic repulsion creating an ECM-free space where lumen is formed. Separation of the luminal EC surfaces is further driven by VEGF, Rho-associated coiled-coil kinase (ROCK) and Ras-interacting protein 1 (RASIP1) which trigger cell shape adjustments enabling lumen expansion.

Sprouts continue to grow in a directional manner until two tip cells meet and fuse together by anastomosis to form an interconnected highly branched vascular circuit. Fusion of ECs is consolidated by establishing vascular endothelial cadherin (VE-cadherin) junctions and chaperoned by tissue macrophages[263, 309] (Figure 4A). Blood flow is then initiated in the new lumen and partakes in vascular remodeling. Fluid shear stress activates the expression of mechanosensitive transcription factor Kruppel-like factor 2 (KLF2) which promotes quiescence by upregulating artheroprotective and anti-coagulant factors (such as endothelial nitric oxide synthase and thrombomodulin), and by downregulating VEGFR2 to decrease EC responsiveness to VEGF and prevent sprouting[310, 311]. By contrast, non-perfused vessels undergo regression. Mural cells are subsequently recruited by ECs to the perfused vessel for coverage and stability. Pericytes line capillaries whereas smooth muscle cells (SMCs) encase arteries and veins. ECs secrete transforming growth factor β (TGF-β) and platelet-derived growth factor B (PDGF-B) to promote migration, proliferation and differentiation of mural cells which deposit ECM to reinforce vessel

stability[263, 284]. Secretion of bioactive lipid Sphingosine-1-phosphate (S1P) and fibroblast growth factors (FGFs) further maintain interactions between ECs and mural cells, which are tightly bound by tight junction proteins (occludins) and cell-adherent proteins (Neural-cadherin and Vascular Endothelial-cadherin)[312-315]. Pro-survival factors, VEGF and Ang1, are also involved in maintaining EC quiescence. VEGF promotes EC survival in an autocrine fashion by activating PI3K/Akt pathway[316, 317], while pericyte-derived Ang1 acts on ECs via Tie2 receptors solidifying EC-mural contact and preserving vascular integrity[284, 285]. Stabilized vessels enter a non-proliferative non-migratory quiescent state as ECs adopt a phalanx phenotype characterized by cobblestone-like structures[318]. Of note, vessel quiescence can be switched at any moment to vessel growth in response to pro-angiogenic signals released during development or injury.

Intussusceptive angiogenesis:

Non-sprouting or intussusceptive angiogenesis (IA) is a rapid expansion process of the vascular bed without requiring tip-stalk cell specification. It is defined as the longitudinal partitioning of a pre-existing vessel to generate two parallel daughter branches. A hallmark of IA is the formation of intravascular pillar initiated by the migration of opposing ECs towards the lumen creating a transluminal endothelial bridge[319, 320]. Cytoskeletal rearrangements ensue and pull the connective tissue along with the migrating ECs. As the process evolves, a cylindrical bridge wrapped by ECs is formed and extends down the lumen of the vessel. This process is concluded by pillar maturation characterized by the invasion of supporting perivascular cells and deposition of ECM[321]. The cellular and molecular basis of IA are poorly defined; however, several factors have been proposed to regulate this process. Sufficient evidence implicates hemodynamic alterations and increased blood flow as major biomechanical factors driving IA[320, 322]. Angiogenic

factor VEGF may function as molecular regulator of IA. While high VEGF levels modulate vessel sprouting and permeability, a drop in VEGF levels promotes vascular remodeling via intussusceptive branching[323]. This mechanism reshapes the vascular architecture and optimizes the number of vascular branches to meet local metabolic demands, either by relocating branching points or by pruning superfluous vessels[319]. Ang1-Tie2 axis may also play an important role in IA. Deletion of Tie2 impairs pillar formation whereas overexpression of Ang1 results in vessel enlargement with many small invaginations that are reminiscent of intraluminal pillars[324, 325]. Recently, EC-derived MT1-MMP was shown to partake in intussusceptive remodeling by upregulating nitric oxide (NO) production which induces vasodilation and initiates formation of transluminal pillars[326]. Other potential candidates include FGF2, PDGF-B, ephrins and Eph-B receptors, and monocyte chemotactic protein 1 (MCP-1)[319, 321, 327].

2.2.2.3 Arteriogenesis

Arteriogenesis is the formation of large vessels (arteries) from small vessels (arterioles). Increased blood flow in small vessels exert significant physical forces against the endothelium, known as fluid shear stress (FSS), which act as primary mechanical stimuli triggering vascular remodeling to accommodate flow alterations[264]. ECs sense these changes and transduce them into intracellular biochemical signals which are translated to structural modifications. While the molecular mechanism is not clearly elucidated, several factors have been shown to partake in this process[328].

In response to increased FSS, ECs acquire a proliferative state, release vasodilation factor nitric oxide (NO), and upregulate the expression of adhesion molecules (ICAM-1, VCAM-1, and PECAM-1)[329, 330]. They also produce tumor necrosis factor-α (TNF-α), granulocyte-macrophage

colony-stimulating factor (GM-CSF) and MCP-1 which recruit circulating monocytes to the expanding vessel[331-334]. Adhesion and activation of monocytes ensue and in turn, secrete additional TNF-α to attract more monocytes. Activated macrophages release MMPs to degrade the ECM allowing their intravascular invasion as well as liberating ECs and mural cells to create space for vessel expansion[335-337]. Macrophage-derived bFGF along with EC-derived PDGF-B promote subsequent migration and proliferation of SMCs which ensheath the enlarged vessel and reconstitute the ECM[338, 339]. In the final phases of arteriogenesis, vessel maturation takes places as ECs return to their quiescent state and SMCs adopt a contractile phenotype[340].

Growth of vessel size is proportional to FSS. Vessels continue to expand and adapt to increased blood flow until FSS is normalized. With increased vessel diameter, FSS proportionally drops, and vessel expansion is consequently arrested[341].

2.2.3 Formation of the retinal vascular network

The retinal vascular network is the last one to develop in the eye. Vascularization of the human retina begins at ~16 WG with the formation of the superficial plexus in the NFL originating at the optic nerve head and growing in a radial fashion, reaching the nasal side of the ora serata at ~36WG and the temporal retina at ~40WG. As the retina differentiates and matures during development, metabolic demands of neurons and astrocytes increase substantially instituting a wave of hypoxia which drives vascular growth to the peripheral edge of the retina[342]. In hypoxic conditions, the oxygen-sensitive α subunit of hypoxia-inducible factor-1 (HIF-1) evades degradation resulting in stabilization and activation of HIF-1, which in turn translocates to the nucleus to mediate transcription of a battery of target genes, notably VEGF[343-346]. Under the control of HIF-1, a gradient of VEGF, secreted by RGCs and astrocytes, is found in the developing

retina and governs the spatiotemporal growth of the vascular network. Moreover, the nascent vessels advance to non-perfused areas along an astrocytic template in an R-cadherin-dependent manner[347]. Retinal vessels extend their network by angiogenesis involving proliferation, migration and fusion, as discussed above, to form a highly branched dense vascular bed.

Once the superficial retina is vascularized, the primary plexus undergoes remodeling to establish 3 parallel layers of well-organized and interconnected vascular networks. New vessels branch from the primary plexus and dive down towards the photoreceptor layer to first form the deep plexus layer (also referred to as outer vascular plexus) followed by the intermediate plexus. Unlike the superficial plexus, the deep vascular network develops independent of an astrocytic template. The vascular sprouts migrate perpendicular to the primary plexus guided by cell surface expression of integrin αVβ8 on pan-retinal Müller cells[348]. Relative hypoxia in deep retinal layers presumably also results in VEGF gradient guiding vascular growth in those regions. Once the sprouting vessels reach the photoreceptor layer, they turn sideways parallel to the primary plexus and grow at the outer boundary of the INL towards the peripheral retina, via suppressive Wnt5a-Flt1 pathway by deep retinal myeloid cells[349] and expression of repulsive Sema3F by RPE[350]. Formation of the intermediate vascular plexus follows at the inner boundary of the INL, though the molecular mechanisms are not fully understood.

2.2.4 Guidance cues in retinal vascular development

In the 16th century, Andreas Vesalius proposed that neuronal and vascular networks are anatomically coupled throughout the body[351]. Later in the 19th century Ramon y Cajal discovered that axonal migration is steered by a highly motile structure, the growth cone[352]. It was then recognized that endothelial cells share a similar growth pattern to axons. Endothelial ‘tip’ cells,

equivalent to the neuronal growth cone, navigate vascular growth and migration by responding to guidance cues[351]. Only recently, we have begun to unravel the molecular mechanisms by which axonal guidance cues govern vascular patterning.

2.2.4.1 Semaphorins

Semaphorins (Sema) are a family of cell surface and soluble proteins involved in many developmental processes. There are 5 mammalian classes (Class 3 to 7) of Semaphorin: class 3 are secreted proteins; classes 4-6 are transmembrane proteins and class 7 are membrane-bound proteins. Class 3 Semaphorins are extensively studied in axonal pathfinding and vascular sprouting and are expressed in the retina[353, 354].

Semaphorins elicit their effects via Plexins, a group of 9 transmembrane receptors classified into 4 subfamilies A-D (Plexin A1-A4, Plexin B1-3, Plexin C to Plexin D). Semaphorins 4 -7 signal via direct binding to their Plexin receptors (A-D), while class 3 Semaphorins interact with Plexins in a Neuropilin-dependent manner with the exception of Sema3E[354]. Neuropilins are single-pass transmembrane with a large extracellular domain subdivided into 3 subdomains (a1a2, b1b2, and c). These distinct subdomains enable the binding of 2 structurally unrelated ligands (Sema3A and VEGF-165) which mediate antagonistic effects on angiogenesis[355, 356]. Nrp-1acts as a co-receptor of VEGFR2 when bound to VEGF-165 to promote EC chemotaxis and growth. In contrast, Sema3A binding to a1a2 and b1b2 Nrp-1 sites promotes recruitment of Plexins, which harbor a split GAP cytoplasmic domain. Sema3A-PlexinA1 interaction then recruit and sequester Rac-1small GTPase which activate GAP domain to recruit R-Ras, subsequently leading to cytoskeletal rearrangements and inactivation of β1 integrin, which inhibits cell adhesion and migration[357]. Moreover, Sema3A can induce caspase-3-dependent apoptosis of endothelial cells[358].

Of relevance, Sema3A was found to upregulated in hypoxic neurons in response to elevated IL-1β levels resulting in repulsion of neovessels away from the ischemic OIR mouse retina[14]. Conversely, inhibiting neuron-derived Sema3A disrupted neovascularization and promoted vascular regeneration. In non-proliferative DR, overexpression of RGC-derived Sema3A resulted in inner BRB disruption via Nrp1 and consequent macular edema formation, while neutralization of Sema3A diminished vascular leakage[359]. Sema3C is another potent antiangiogenic factor. Sema3C hinders VEGF-driven EC migration by signaling through Nrp1 and PlexinD to inactivate kinases AKT, FAK and p38MAPK as well as to promote internalization of VE-cadherin, which is otherwise essential for vessel integrity and EC survival[15]. Similarly, Sema3F elicits antiangiogenic effects by binding to Nrp2-PlexinA1 and mediating RhoA inactivation to induce cytoskeletal collapse in glioma[360]. Moreover, administration of Sema3F in mouse model of laser-induced choroidal neovascularization elicited protective effects[361]. Sema3E also plays an important role in retinal vascular development. In developing mouse retina, Sema3E-PlexinD1 modulates angiogenesis by inhibiting VEGF-induced Delta-like 4 expression on tip cells[12]. In ischemic retina, Sema3E acts on the abnormal tufts via PlexinD1 activating RhoJ which in turn hinders VEGF-induced filopodia extension and mediates EC contraction[13].

2.2.4.2 Netrins

Netrins, derived from the Sanskrit word “Netr” which means “guide”, are a family of laminin-like proteins consisting of 3 secreted (Netrins1, 3 and 4) and 2 membrane-anchored (Netrins G1 and G2) proteins[362]. Netrins were initially discovered as axonal guidance cues in the midline brain steering the outgrowth of commissural neurons by binding to the receptor deleted in

colorectal cancer (DCC). Netrins are also known to exert chemorepellent effects via Unc5 family (A-D).

Netrin-1 is considered and classical guidance cue which plays an important role in angiogenesis. Its receptor Unc5b is enriched on ECs unlike Unc5a and DCC. Upregulation of Unc5b coincides with active vascular growth whereby endothelial tip cells of sprouting retinal vessels express the receptor, but not quiescent adult vessels[363]. In the developing mouse retina and midbrain, activation of Unc5b by Netrin-1 induces retraction of EC filopodial projections and impairs angiogenesis. Furthermore, the role of Netrin1-Unc5b is limited to angiogenesis and not vasculogenesis since disruption of Unc5b does not impact arterio-venous specification[364].

In the context of ROP, the role of Netrin-1 remains not clear. Netrin-1 expression was reported to be elevated in ischemic retinas to induce neovascularization and vascular permeability[301, 365]. Un5b expression is also elevated in OIR mice while its deletion curtailed formation of extraretinal tufts[365]. In another study, however, ischemia-driven endoplasmic reticulum (ER) stress in RGCs degraded Netrin-1 via inositol-requiring enzyme-1α (IRE1α)-dependent mechanism which in turn aggravated neurovascular injury in retinas of OIR mice[366]. Inhibition of IRE1α or expression of Netrin-1 promoted reparative vascularization by activating ERK1/ERK2 kinase pathway in retinal macrophages to produce VEGF at the vascular front.

2.2.4.3 Slits

Slits are secreted guidance proteins known for their role in multiple biological functions including axonal patterning[351], kidney induction[367], leukocyte migration[368], and angiogenesis[351, 369]. Slits signal through single pass transmembrane receptors known as Roundabouts (Robos)[370].

In vertebrates, there are 3 Slit proteins and 4 Robo receptors, of which Robo4 is selectively localized on ECs[371, 372].

Robo4 is implicated in vascular development by maintaining vessel integrity and sprouting. Robo4 is expressed predominantly in stalk cells during development to counteract VEGF-induced vessel elongation and branching[298]. OIR retinas of Robo4 knockout mice exhibited hypervascularization and vascular permeability, though this phenotype can be rescued by administering Src kinase inhibitor to disrupt VEGF-driven Src activation[298, 300]. These results ascertain the role of Robo4 in mediating an anti-angiogenic effect by impairing VEGF-induced sprouting.

2.2.4.4 Ephrins

Ephrins and their Eph receptors regulate a broad range of biological processes including axon guidance and angiogenesis in a contact-dependent manner[373, 374]. Eph receptors represent the largest family of receptor tyrosine kinases subdivided into class A (EphA 1-8) and class B (EphB 1-4 and EphB6) based on similarities in their extracellular sequence and binding preference for Eph ligands[374]. Similarly, Ephrin ligands are subclassified into A and B based on their structural properties whereby Ephrin A ligands are anchored to the cell membrane via a glycosyl-phosphatidyl-inositol modification whereas EphrinB contain transmembrane passages and cytoplasmic regions. Eph A or B receptors typically bind Ephrins ligands of similar class though cross-reactivity can happen[374, 375]. A distinctive feature of Ephrin-Eph signaling is the capacity to propagate signals bidirectionally: ‘forward signaling’ from Eph receptors to the receiving cell and ‘reverse signaling’ from ephrin to expressing cell[376].

Class B Ephrins and Eph receptors play critical roles in vascular development including vasculogenesis, sprouting and mural cell recruitment. As discussed in section 2.2.1, EphrinB2-EphB4 signaling governs and maintains arteriovenous specification[270, 272]. In sprouting angiogenesis, the role of Ephrin-Eph signaling is less clear. Knockdown of Ephrin B2 or its receptor EphB2 resulted in significant impairment of sprouting and vascular remodeling, suggesting that Ephrins elicit a stimulatory role in mediating vessel growth[377, 378]. EphrinB2-EphB4 interaction mediated EC migration and proliferation in part via PI3K and AKT pathways[379, 380]. Other studies, however, reported that forward EphB4 signaling inhibited EC proliferation, migration, and adhesion by hindering VEGF-induced intracellular Ras/MAPK signaling[381-383]. Moreover, EphrinB2 facilitates proper adhesion of mural cells to the endothelium as selective deletion of mural cell-specific EphrinB2 prevented mural-cell coverage of the endothelium resulting in poorly organized and leaky vessels as well as perinatal lethality[384].

2.2.5 Treatment modalities for Ischemic Retinopathies

IRs are typically treated with laser photocoagulation which utilizes thermal energy to target and destroy the peripheral ischemic retina in order to arrest aberrant intravitreal angiogenesis. It remains to date the standard of care. Its use has been shown to slow down vision loss by decreasing risk of retinal detachment, promoting regression of neovascularization and attenuating VEGF[156, 385]. However, this could lead to significant vision loss [386-388].

A new era of anti-angiogenesis therapy emerged with the advent of anti-VEGF treatments in 2004[389]. One of the first anti-VEGF agents developed to treat ocular neovascularization was Bevacizumab (Avastin by Genetech Inc., San Francisco, CA), a monoclonal antibody that neutralizes all isoforms of VEGF-A[390, 391]. Bevacizumab was mistakenly believed to diffuse poorly in the retina to reach the choroid because of its full length, which prompted the development of a

truncated variant of the antibody, ranibizumab (Luncetis, Genetech Inc.)[392, 393]. An alternative strategy devised to block VEGF angiogenic effects is VEGF-trap[394] (Aflibercept, Regeneron Pharmaceuticals, NY). It is a soluble fusion protein comprising of extracellular VEGF-binding domains derived from VEGFR1 and VEGFR2 and acts as a decoy receptor to sequester VEGF blocking its effects. Aflibercept and ranibizumab received FDA approval for DR/DME while bevacizumab is used off-label to treat retinal neovascularization. Anti-VEGF therapy is less invasive and easily administered via intravitreal injection under topical anesthesia[395].

Addition of anti-VEGF therapy to the treatment of ROP neonates can change the natural course of the disease as demonstrated by clinical studies showcasing superior short-term outcomes compared to laser photocoagulation. In eyes with stage 3 ROP, bevacizumab induced regression of neovascularization, reduced recurrence rate and preserved peripheral vision in contrast to laser-treated eyes[396-400]. However, a critical concern of anti-VEGF is the potential long-term systemic effects on the development of neonates. Anti-VEGF injected intravitreally has been shown to leak into systemic circulation and suppress serum VEGF levels up to 8 weeks [401-403]. To date, the long-lasting sequela of circulating anti-VEGF remains uncertain. One study found no association between anti-VEGF therapy and neurodevelopment impairment[404], whereas another retrospective review noted severe neurodevelopmental disabilities in bevacizumab-treated neonates[405]. This indicates a dire need for robust safety data assessing the benefits of anti-VEGF therapy in neonates, particularly since VEGF is a neuroprotective factor and an essential angiogenic agent during development[267, 406, 407]. Moreover, there is substantial controversy in the literature on the optimal dosage of anti-VEGF treatments for neonates, complicated by individual variability and by changes in VEGF levels throughout the stages of ROP[398, 408-411].

In the case of diabetic retinopathy, clinical studies have also favored anti-VEGF therapy over laser photocoagulation. One-year treatment of DME retinas with bevacizumab or ranibizumab resulted in increased visual acuity and decreased macular thickness in contrast to laser-treated group[412, 413]. However, a serious complication of anti-VEGF therapy is the high risk of tractional retinal detachment which can adversely affect vision. Reduction in VEGF levels following anti-VEGF administration activates an 'angio-fibrotic switch' which can accelerate fibrosis creating tractional forces and eventually leading to retinal detachment[414, 415]. This complication seems to occur mostly in patients with poorly controlled diabetes[416, 417]. Moreover, anti-VEGF therapy raises concerns on possible systemic complications. Such concerns arise from the reported side effects of intravenous injection of anti-VEGF in cancer patients, which include arterial thromboembolism, stroke, hypertension and hemorrhage[418, 419]. Further safety studies on the intravitreal use of anti-VEGF in DR are required.

Given the limitations of anti-VEGF therapy for IRs, there is a need for novel therapies that go beyond merely preventing neovascular formation. The current therapies fail to arrest vascular degeneration or repopulate the avascular regions of the retina. Furthermore, IRs are complex multifactorial diseases that progress as a result of interdependent pathophysiological mechanisms. As such, use of single agent treatments may not be an optimal therapeutic approach to preserve and restore vision. IRs would greatly benefit from combination or multifaceted therapeutic avenues, such as stem cell therapy.

2.3 Mesenchymal Stromal Cells

2.3.1 Historical perspective

"Stammzelle", German for stem cell, was first introduced in the 19th century by the German biologist Ernest Haeckel to describe the fertilized egg as an ancestor of all cells of an organism[420]. At that time, ongoing research on hematopoiesis was questioning the existence of a common precursor capable of giving rise to all distinct cell types of the blood and sparked 2 schools of thought[421, 422]. The dualists believed that myeloid and lymphoid cells are derived from 2 distinct precursor cells residing in 2 separate hematopoietic tissues, the bone marrow and the lymphatic system, respectively. On the other hand, the unitarians supported the theory of the existence of a single precursor for all blood cell types irrespective of size, morphology, and granularity. This debate continued for several decades until the first definitive evidence of a common stem cell, the hematopoietic stem cell, was shown by James Till and Ernest McCulloch at the University of Toronto in 1960s[423, 424]. Their seminal work not only confirmed the unitarian theory of hematopoiesis but also provided evidence of the existence of adult stem cells capable of differentiating into multilineage descendants while retaining their capacity to self-renew. The latter knowledge of adult tissues as a source of stem cells triggered active investigation in the fields of regenerative medicine and tissue engineering to identify other somatic stem cells and harness their therapeutic potential.

The concept that non-hematopoietic stem cells exist in bone marrow dates back to 1867 when German experimental pathologist Julius Cohnheim injected a dye in circulation of animals and observed that labeled blood-borne inflammatory cells as well as fibroblastoid cells extravasated to injury sites to mediate tissue healing[425]. A year later, French physiologist Goujon

noticed that transplantation of bone marrow in heterotropic anatomical sites resulted in *de novo* generation of ectopic bone and marrow[426]. In the early twentieth century, Russian scientist Alexander Maximov surmised that, as part of the unitarian theory of hematopoiesis, bone marrow-resident fibroblastoid cells partake in hematopoiesis by signaling to hematopoietic stem cells[427]. But it was not until the 1970s that the soviet scientist Alexander Friedenstein discovered that the bone marrow harbors a distinct population of stem cells. In his pioneering work, Friedenstein demonstrated that these non-hematopoietic stem cells can be isolated by adhering to the plastic tissue-culture plate and can proliferate at clonogenic density *in vitro* by forming fibroblastoid colonies (colony-forming unit fibroblasts; CFU-F)[428-430]. He further showed that these cells possess osteogenic properties[431, 432]. Upon their transplantation under renal capsules of mice, the fibroblastoid cells formed a bone tissue, exclusively of donor origin, capable of sustaining hematopoiesis. Friedenstein initially referred to these cells as colony-forming unit-fibroblastic cells, but then used osteogenic stem cell, stromal stem cell and marrow stromal fibroblast[433, 434]. In 1991, developmental bone biologist Arnold Caplan coined the name 'mesenchymal stem cells' due to their differentiation potential to mesodermal lineages and putative developmental origin from the embryonic mesenchyme[435].

2.3.2 Identification of MSCs

Since their original description, MSCs have gained rapid interest in the field of regenerative medicine. Though commonly isolated from the bone marrow, MSCs were subsequently isolated from other tissue sources such as adipose tissue[436, 437], dental pulp[438], skeletal muscle[439], synovial membrane[440], umbilical cord[441], and placenta[442] among others, suggesting that nearly every vascularized tissue harbors putative MSCs[443, 444]. They expressed similar markers despite being

isolated from different tissue sources[445]. However, in the absence of a well-established characterization protocol, these plastic adherent MSCs seemed to exhibit incomparable self-renewal and *in vivo* differentiation capabilities rendering 'mesenchymal stem cells' nomenclature problematic[446]. Accordingly, in an effort to minimize confusion and standardize MSC identification, the International Society for Cellular Therapy (ISCT) recommended the term "multipotent mesenchymal stromal cells" while retaining the 'MSC' acronym[447] and put forth a set of minimal criteria to identify these fibroblastic-like cells[448]:

1. Adherence to plastic in standard culture conditions;
2. Expression of surface molecules CD73, CD90 and CD105;
3. Lack of expression of CD34, CD45, HLA-DR, CD14 or CD11b, CD79α or CD19;
4. Capacity to differentiate *in vitro* into adipocytes, osteocytes and chondrocytes.

Almost two decades since ISCT proposal, the criteria for MSC characterization remains unresolved mainly due to the lack of an exclusive molecular signature. MSCs are rare a population of the bone marrow (0.001% – 0.01% of nucleated cells)[449] and thus *ex vivo* expansion and enrichment are inevitable. Isolated plastic adherent MSCs are often enriched using a positive immunostaining of CD73, CD90 and CD105, coupled with negative staining for CD34 (hematopoietic stem cell surface antigen), CD45 (leukocyte surface antigen), or CD11b (monocyte surface antigen), as suggested by the ISCT. Because these markers are not specific to MSCs, numerous research studies have emerged in search of exclusive cell surface markers. Stro-1 was shown to prospectively isolate MSCs from human bone marrow and form enriched CFU-Fs (about 100-fold) when combined with negative staining of glycophorin A (an erythroid marker)[450]. However, recent studies suggested that *in vivo* Stro-1 is expressed by the endothelium rather than

MSCs[451]. Stro-1 expression is also limited to human bone marrow without equivalent markers in other species. CD146, also known as melanoma cell adhesion molecule (MCAM), is another putative MSC cell surface marker[452]. It was shown to label bone marrow adventitial reticular cells which gave rise to CFU-F colonies when cultured and supported hematopoiesis in immunocompromised mice. Of note, *in situ* MSCs expressed CD146 and are capable of self-renewal. Other markers identified were CD106[453], CD166[454], CD271[455], and SSEA-4[456]. A further complication in characterizing MSCs is the potential impact of cell culture conditions on surface marker expression. Some studies have shown that cultured MSCs may exhibit certain features that otherwise differ from native MSCs. For example, expression of CD271, also known as low-affinity nerve growth factor receptor (LNGFR), was found on freshly isolated MSCs but is lost when grown in bFGF-containing medium[457]. Similarly, CD44 and STRO-1 only label cultured stem cells and are believed to be artifacts of cell culture[451, 458]. Oxygen levels can further influence surface marker expression as in the case of CD146, which was found to be upregulated in bone marrow MSCs expanded in normoxic environment but downregulated in hypoxia[459]. Interestingly, this difference in CD146 expression correlated with MSCs *in situ* localization: high CD146+ MSCs resided in perivascular regions whereas low CD146+ MSCs lined the bone surface. Moreover, expansion of MSCs in low oxygen levels was shown to sustain MSC proliferation and trilineage differentiation, improve cell viability and reduce expression of p53 and ROS[460].

2.3.3 Mechanisms of tissue repair

Application of MSCs in tissue regeneration and repair involves 4 potential mechanisms: (1) homing to damaged sites in response to chemotactic signals, (2) differentiation and cell replacement by which engrafted MSCs differentiate into tissue-relevant cell type, (3) interacting

directly with immune cells to modulate the inflammatory response, and (4) secretion of bioactive factors that promote an environment favourable for regeneration (Figure 6).

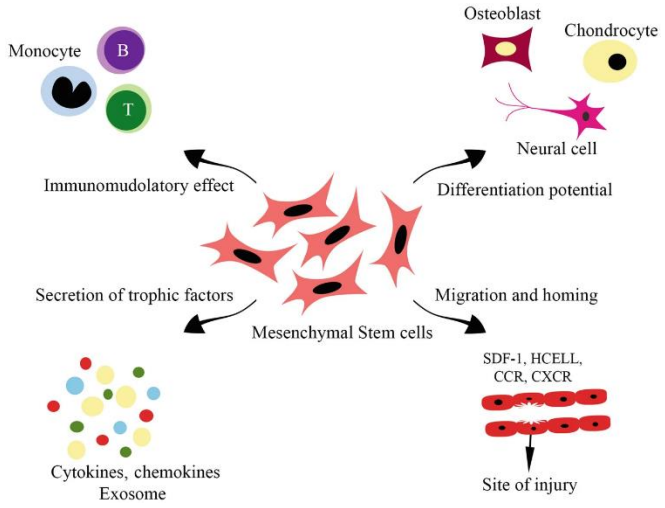

Figure 6. Mechanisms of MSC-driven tissue repair. MSCs mediate their therapeutic effect via multiple mechanisms including migration and homing, multi-lineage differentiation potential, secretion of trophic factors, and modulation of inflammation (Figure adapted from Hosseini *et al.*[461])

2.3.3.1 MSC Homing and Engraftment

MSCs express a wide range of integrins and adhesion molecules that facilitate their migration along the endothelium, similar to the principles of leukocyte migration[462 463]. Upon systemic administration, MSCs upregulate P-selectin expression on endothelial cells and bind to the endothelium in a P-selectin-dependent manner. However, MSCs don't express similar P-selectin ligands such as P-selectin glycoprotein ligand 1 and CD24 typically found on circulating leukocytes[462, 464]. Rather, glycoproteins and galectin-1 present on the surface of MSCs mediate binding to EC-specific-P-selectin but with low affinity which, with the speed of blood flow, results in a cycle of adherence and separation mimicking a rolling motion[465, 466]. In response to inflammatory signals released by ECs or damaged tissues, mobilization of MSCs ensues via the

activation of C-X-C-motif chemokine receptors such as CXCR4 and CXCR7[467, 468]. Damaged tissues upregulate secretion of stromal cell-derived factor-1 (SDF-1), a chemokine in the CXC chemokine family, which then binds to CXCR4 receptors expressed by MSCs prompting MSC homing to the injury site along a gradient of SDF-1[469, 470]. SDF-1 also activates expression of very late antigen 4 (VLA4, also known as α4β1) on MSCs promoting firm adhesion to ECs by binding to VCAM-1[462, 471, 472]. Subsequently, MSCs begin migrating transcellularly through the endothelium by secreting matrix metalloproteinases (MMPs)[473, 474] and urokinase-type plasminogen activator (uPA)[475] to breakdown the endothelial basement membrane. Once MSCs cross the endothelial barrier, they are steered to the injury site by tissue-specific chemotactic signals. However, very few systemically administered MSCs reach the injury sites and thus MSC homing is considered as a "bottleneck" to harnessing their full therapeutic potential[476]. Furthermore, ex vivo expansion and cell culture techniques can diminish MSC homing capacity[477]. Preconditioning MSCs to hypoxia elevates expression of chemokine receptors such as CXCR4 and CXCR7 thereby improving MSC homing and engraftment efficiency to ischemic tissues[478-480]. Similarly, priming MSCs with TNF-α or IL-8 has been shown to improve MSCs homing efficiency[481, 482]. Other strategies include magnetic guidance, cell surface engineering, direct injection and genetic modification[476].

In addition to their low homing capacity, MSCs exhibit poor engraftment efficiency as the majority of intravenously infused MSCs are cleared from circulation within 5 minutes of administration[483]. Less than 5% of viable MSCs are found in tissues, primarily in the lung where MSCs get trapped as emboli with a half-life of 24 hours[483-485]. MSCs do not persist in tissue following transplantation but rather die within 48 hours of systemic infusion[483, 486]. This was further confirmed in tissues analyzed from patients treated with allogeneic MSCs showing scarce

levels of donor MSC DNA[487]. MSC entrapment in the lung may be attributed to their large size (20µm in diameter) relative to the circulating immune cells and to the width of pulmonary microcirculation. Combining vasodilators and anticoagulants with MSC injections was shown to reduce their accumulation in the lung and improve homing to other sites[488, 489]. Alternative routes of administration could bypass lung entrapment and target tissues of interest. For example, MSCs injected via the portal vein localized in the liver[490]. Moreover, MSCs locally delivered to target tissues tend to persist and maintain their viability for weeks[491, 492], though very low proportion of injected MSCs engraft within the tissue[493, 494]. Nonetheless, several studies have reported positive functional outcomes following the systemic delivery of MSCs, suggesting that MSCs exert their beneficial effects from a distance in a paracrine fashion[485, 495, 496]. This mechanism is referred to as a 'hit and run' whereby MSCs' therapeutic benefit remains detectable long after their early disappearance and is thought to be mediated via the release of soluble trophic and immunomodulatory factors[487].

2.3.3.2 Differentiation and Cell replacement

Because of their multilineage differentiation potential, MSCs can potentially be used to replace tissue-specific damaged cells. A key application of MSCs is bone regeneration and cartilage repair. The osteoblastic and chondrogenic differentiation pathways are very well established. *In vitro* exposure of MSCs to dexamethasone or to TGF-β proteins induces their differentiation to osteoblasts[497, 498] or chondrocytes[499, 500], respectively. Differentiation of MSCs into functional osteoblasts was further demonstrated in animal models and in humans to treat osteogenesis imperfecta and bone loss[501, 502]. MSCs also have the potential to differentiate into other cell types than mesodermal lineages. Numerous studies have demonstrated that MSCs can

be differentiated into ECs by exposing them to VEGF-supplemented culture medium, resulting in expression of EC markers such as CD31, CD34, VEGF receptors 1 and 2, as well as formation of capillary-like structures[503-505]. However, this remains controversial as other studies have not been able to skew MSC differentiation to ECs using VEGF[506]. *In vivo*, bone marrow-derived MSCs expressed EC markers including von Willebrand Factor, CD34 and factor VIII few weeks following their transplantation in a rat model of hindlimb ischemia[507-509]. Similarly, DAPI-labeled MSCs injected in rat vein grafting model localized in contact with injured blood vessels and expressed CD31 and endothelial nitric oxide synthase[510]. As mentioned above, engraftment rate of MSCs is low and very few cells incorporate in the host vascular structures. The angiogenic effect of MSCs is predominantly mediated by paracrine mechanisms rather than by differentiating into ECs[509-511].

In the context of retinal repair, some studies have investigated MSC differentiation potential into various retinal cell types. In laser-induced retinal injury model, BM-MSCs injected intravenously engrafted in injured retina and differentiated into neurons, pigment epithelium (RPE), endothelium, pericytes and photoreceptors[512, 513]. Furthermore, neuroglial differentiation of BM-MSCs into retinal ganglion cells was demonstrated in an animal model of optic nerve crush[514, 515]. Intravenous injection of adipose-derived MSCs in streptozotocin rat model of diabetic retinopathy engrafted in injured retina and differentiated into astrocytes and photoreceptors by expressing glial fibrillary acidic protein (GFAP) and rhodopsin, respectively[516]. One week after MSC-administration, blood-retinal barrier integrity was shown to have improved and glucose levels declined substantially.

2.3.3.3 Contact-dependent tissue repair

MSCs can exert their immunomodulatory actions by interacting directly with immune cells such as macrophages and T cells. In response to an inflammatory environment, MSCs release chemokines that attract T cells to their vicinity and mediate direct cell-to-cell contact resulting in release of immunosuppressive factors and consequently T cell suppression. For example, interferon-γ (IFN-γ) induces production of MSC-derived indoleamine 2,3-dioxygenase (IDO) which depletes tryptophan, an essential amino acid for cell survival and protein synthesis, leading to the suppression of T cell proliferation and cell apoptosis[517-519]. Other studies have shown that in response to IFN-γ and other inflammatory signals, MSCs secrete nitric oxide (NO), an unstable molecule with limited range of action, which then suppresses Stat5 phosphorylation in T cells inhibiting their proliferation and in turn dampening the immune response[520, 521]. Interestingly, this effect is dependent on the expression of adhesion molecules VCAM-1 and ICAM-1 on the surface of MSCs[522]. Other contact-dependent mechanisms include FAS/FASL[523], Notch signaling[524, 525] and programmed death-1/programmed death ligand-1[526].

MSCs can also modulate macrophages to adapt an anti-inflammatory signature. Upon contact with LPS-stimulated macrophages, MSCs produce prostaglandin E-2 (PGE2) prompting a switch in the polarization state of M1 pro-inflammatory macrophages to M2 immunosuppressive phenotype[527, 528]. As a result, macrophages release significant levels of anti-inflammatory IL-10 inhibiting neutrophil migration and reducing inflammation. Moreover, cell-to-cell contact with pro-inflammatory macrophages trigger MSCs to produce tumor necrosis factor-stimulated gene-6 (TSG-6) which acts in a paracrine fashion to inhibit T cell proliferation and reprogram macrophages to M2 phenotype[529], suggesting that direct cell-contact can further potentiate MSC immunomodulatory capacity. Alternatively, some studies proposed that clearance of lung-

entrapped MSCs, following intravenous infusion, by macrophages (via phagocytosis) can promote tissue repair by modulating macrophage polarization to M2 anti-inflammatory phenotype and eliciting an IL-10 response[530-532]. This theory may explain the persistent therapeutic effect observed after the early disappearance of infused MSCs.

2.3.3.4 Paracrine Action of MSCs

MSC-driven tissue repair is considered to be largely mediated via paracrine mechanisms. MSCs secrete a plethora of factors, referred to as 'secretome', which includes growth factors, cytokines and chemokines, and extracellular vesicles (EVs) carrying RNA, miRNA, proteins, and lipids. In response to tissue stressors, MSCs are activated and release a wide range of cytoprotective bioactive factors which regulate biological processes including wound healing, angiogenesis, immunity and cell motility[533, 534], and thereby transforming the local microenvironment from insult to pro-regenerative milieu.

Angiogenic mechanisms:

Accumulating evidence point to the angiogenic potential of MSCs in promoting vascular regeneration via the secretion of a myriad of factors, notably VEGF, fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), monocyte chemoattractant protein (MCP-1), interleukin (IL)-6 and SDF-1α. VEGF and FGF are critical inducers of angiogenesis whereby they regulate various functions of endothelial cells ranging from proliferation, survival, migration to capillary tube formation. As such, MSC-derived conditioned media significantly promoted EC proliferation. This mitogenic effect was elicited by VEGF and FGF secreted by MSCs, while simultaneously blocking both factors partially attenuated EC proliferation thus confirming their

role in MSC-mediated angiogenesis in a paracrine manner[535, 536]. Furthermore, MSCs cultivated in hypoxic conditions were shown to produce IL-6, VEGF and MCP-1 which inhibited hypoxia-induced endothelial apoptosis and stimulated sprouting of new vessels via the activation of PI3K/AKT pathway[537]. These angiogenic effects were found to be abrogated by pre-treating MSCs with neutralizing antibodies against IL-6, VEGF and MCP-1[538]. IL-6 is a potent pro-survival factor while MCP-1 is an essential chemoattractant for angiogenesis[539, 540]. In addition, MCP-1 upregulates expression of HIF-1α which subsequently increases VEGF transcription[541]. In mouse hindlimb ischemia model, MSCs promoted vascular regeneration by secreting VEGF, IL-8, MCP-1 and angiogenin[542]. Moreover, intramuscular injection of conditioned media from TNF-α-primed MSCs accelerated tissue angiogenesis and repair via IL-6 and IL-8 dependent mechanisms [543, 544]. Another soluble factor shown to promote angiogenesis is MSC-derived hepatocyte growth factor (HGF) which exerts its angiogenic effect via tyrosine phosphorylation of its receptor c-met localized on ECs and mural cells[545].

Use of MSCs was also documented in retinal vasculopathies. In an OIR mouse model, human placental amniotic membrane-derived MSCs injected intraperitoneally (to avoid getting trapped in lungs) migrated to damaged areas of the retina and significantly decreased neovascularization in a paracrine manner via the secretion of TGF-β1[546]. Ezquer *et al.* showed that intravitreal administration of bone marrow-derived MSCs in retinas of diabetic mice prevented loss of retinal ganglion cells by upregulating neuroprotective factors and attenuated aberrant neovascularization by augmenting expression levels of the anti-angiogenic factor thrombospondin-1 (TSP1) while VEGF and PEDF levels remained unchanged[547]. In another study, intravenous injection of MSCs into laser-induced choroidal neovascularization mouse model migrated to damaged lesions and promoted vascular regression by producing antiangiogenic pigment epithelial-derived factor[548].

Immunomodulatory mechanisms:

MSCs are known for their strong immunomodulatory properties on both innate and adaptive immune cells. Functional regulation of immune cells is mediated via direct cell-contact as discussed above and by the secretion of anti-inflammatory factors[549]. For example, MSCs can suppress proliferation and cytotoxic activity of CD8+ T cells by producing PGE2, IDO and TGF-β[550, 551]. MSc-conditioned media suppressed expression of TNF-α and IL-6 in macrophages by inhibiting MAPK and NF-κB pathways[552]. Inflammation is a major mechanism in the pathogenesis of ischemic retinopathies. An inflammatory microenvironment is established in ischemic retinas due to elevated expression of pro-inflammatory mediators and persistent infiltration of immune cells, which was shown to be significantly reduced following intravitreal injection of MSCs[553, 554]. In mouse model of retinal inflammation, intravitreal injection of MSCs dampened the inflammatory response by reducing levels of IL-1β, IL-6, iNOS, TNF-α and VEGF as well as macrophage infiltration[555, 556]. Moreover, MSCs curtailed neuroinflammation in glaucoma retinas by downregulating TLR4 signaling pathways[557]. MSC-derived exosomes were reported to be as effective as injected MSCs in reducing retinal damage and suppressing inflammation in laser injury-induced mouse model by, partially, downregulating MCP-1[558].

Another mechanism by which MSCs regulate inflammation is by modulating the polarization state of macrophages. Macrophages can be classified as pro-inflammatory M1 or anti-inflammatory M2 based on their function and cytokine profile[559]. Treatment with MSCs skews macrophage polarization towards M2 phenotype by releasing immunomodulatory factors notably PGE2[527, 560], IL-1 receptor antagonist[561], IDO[562], TGF-β[563] and TSG6[564]. In addition to M2 polarization, anti-inflammatory cytokine IL-10 and Argina-1 (Arg-1) are upregulated concomitant

with diminished expression of pro-inflammatory cytokines IL-1β, TNF-α and IL-12[565, 566]. The shift towards M2 phenotype further potentiates MSC-mediated immune suppression by inhibiting T cell response and stimulating regulatory T cell via the production of TGF-β[567, 568]. Furthermore, intracerebroventricular injection of MSCs in post-brain injury promoted brain repair by reprogramming microglial polarization to immunoprotective M2 phenotype. This is accompanied by an upregulation of M2 markers Ym1 and Arg-1 as well as downregulation of co-stimulatory molecule CD86 (M1 marker)[569]. MSC-derived TSG-6 is proposed to mediate microglial M2 polarization[570]. Exosomes also play a role in modulating macrophage polarization. MSC-derived EVs elicit an anti-inflammatory effect upon internalization by macrophages driving their switch from M1 to M2 polarization state[571].

Preconditioning of MSCs:

Hypoxia has been shown to enhance MSCs' therapeutic properties. Numerous studies demonstrated the importance of *in vitro* MSC expansion in low oxygen tension environment as it mimics the stem cell native niche[572, 573]. Hypoxic environments help maintain stem cell characteristics and functions[574, 575], enhance their proliferative and differentiation potential[576-578], and reduce senescence and oxidative stress[460, 555, 579]. Cultivation of MSCs under hypoxic conditions stabilizes HIF-1α which drives a metabolic switch from oxidative phosphorylation to glycolysis curtailing ROS generation.[580-582] Furthermore, stabilization of HIF-1α potentiates MSCs beneficial properties by activating the transcription of downstream targets involved in angiogenesis, metabolism, immunity and stemness [583, 584]. Treatment of ECs with conditioned media from hypoxic MSCs reduced hypoxia-induced endothelial apoptosis substantially and promoted tube formation compared to that from normoxic MSCs. In a rat model of myocardial

infarction, increased vascularization was observed following the administration of hypoxic-preconditioned MSCs not those cultivated in normoxic conditions[585]. Similarly, EV-derived from hypoxic MSCs increased vascular tube formation[586] and promoted macrophage polarization to M2 type[571]. in comparison to normoxic EVs.

Pre-treatment of MSCs with toll-like receptors (TLR)-agonists or pro-inflammatory factors is an alternate approach to enhancing MSCs' immunosuppressive properties. Expression of TLRs 1-9 except for TLR8 has been documented in human and murine MSCs from different tissue sources including bone marrow, adipose tissue, umbilical cord, and others [587-589]. For example, activation of TLR3 with polyinosinic:polycytidylic acid triggers release of VEGF, SDF-1, IL-6 and IL-10[590]. Co-treatment of MSCs with LPS and TNF-α prompted PGE2 secretion and induced macrophage polarization to M2 state expressing high levels of CD206 and Arg1[591]. Similarly, co-exposure to IFN-γ and TNF-α augmented IDO activity driving monocyte differentiation into M2 type macrophages by expressing CD14 and CD206 and secreting IL-10 which in turn suppresses T cell proliferation[562]. In addition, analysis of IL-1β MSCs revealed upregulation of NF-κB-driven factors involved in cell survival, angiogenesis, and immune responses[592]. EVs derived from TGF-β and IFN-γ primed MSCs were more effective in promoting generation of regulatory T cells than EVs from unprimed MSCs[593].

2.3.4 Clinical applications of MSCs

In 2004, a landmark case study on severe graft-versus-host disease (GvHD) was the first to clinically report an immunosuppressive response to allogeneic MSCs administered intravenously[594], which prompted Osiris Therapeutics to launch large-scale clinical trials on treating GvHD with allogeneic stem cells. By 2012, Health Canada approved its first MSC product

Prochymal (now Mesoblast) by Osiris Therapeutics for GvHD treatment[595]. Since then and with the publication of numerous proof-of-concept preclinical studies, more and more clinical trials are devised to harness the therapeutic potential of MSCs and treat a multitude of medical disorders. As of December2022, there are 1,470 clinical trials registered on the database of the US National Institutes of Health (https://clinicaltrials.gov/) to treat various diseases including ocular (such as diabetic retinopathy, glaucoma, retinitis pigmentosa, age related macular degeneration), cardiovascular (such as ischemic heart disease, angina, myocardial infarction), respiratory (such as chronic obstructive pulmonary disease, COVID-19, pulmonary fibrosis), and neurodegenerative diseases (such as Parkinson's, Alzheimer's, multiple sclerosis).

The hype around MSCs stems from their ease of isolation, their low immunogenicity averting the need for immunosuppressants upon administration, and their exceptional tissue regenerative capacity across various medical conditions. Yet, translating basic research findings into clinical practice remains a challenge particularly given the heterogeneity of the MSC population. To date, multiple sources of MSCs are documented demonstrating 'stemness' as per the minimal criteria put forth by ISCT in 2006[448]. However, variation in surface markers, differentiation capacity and proteomic signatures of MSCs from different sources[596-599] underscore the need to update the ISCT criteria with the aim of standardizing MSC characterization to facilitate their clinical use. Moreover, *in vitro* expansion protocols and cell culture conditions may introduce variations in the expression of surface antigens resulting in inconsistent reports[600-602]. While the number of clinical trials investigating the therapeutic use of MSCs continues to rise, there is a dire need to implement optimization and standardization measures to ensure reliable production of high grade MSCs.

MSCs are also known for their poor engraftment efficiency when administered systemically. The majority of intravenous infused MSCs were shown to get trapped in the lungs resulting in increased risk of thrombosis, whereas less than 1% migrate to the damaged site[483]. Yet, numerous studies from different animal models have showed that MSCs mainly exert their therapeutic effect in a paracrine fashion through the release of bioactive products mediating tissue reparation and regeneration without the risk of cell loss or entrapment[495, 603-606]. Interestingly, administration of MSC-derived bioactive molecules demonstrated similar therapeutic efficacy in promoting tissue regeneration to MSCs themselves[607, 608]. Such results shifted the interest in MSC-based therapies from stem cells to their secreted factors where about 4% of the 1470 registered clinical trials are investigating MSC secretome, exosomes or conditioned medium. The use of MSCs trophic properties as an alternate therapeutic avenue over its cellular counterpart offers several advantages: they tend to have better safety profile and lower immunogenicity than infused MSCs due to their low expression of surface proteins, eliminate the risk of pulmonary entrapment and consequent emboli formation, can be used as a delivery system to modulate specific molecular processes, and are more economical and time-effective for mass-production[609]. Nonetheless, our understanding of the molecular biology and function of MSCs secretome is quite limited given its complex composition, which includes a broad range of soluble proteins, lipids, microRNAs and extracellular vesicles[610]. This is further complicated by the intrinsic heterogeneity of MSCs which could impact the identity and concentrations of the secreted bioactive molecules. Exploiting MSCs secretome as a stem cell free approach is an attractive and promising therapeutic avenue, intended to overcome the challenges with transplanting MSCs themselves; however more studies are warranted to study the quality, efficacy and safety of the secretome prior to their implementation in clinical practice.

Chapter 3. Hypothesis

Evidence discussed above in the literature review (Chapter 2) highlights the complexity of Ischemic Retinopathies pathogenesis which involves an intricate interplay between endothelial cells, neurons, and myeloid cells. While VEGF has been depicted to be the main culprit of Ischemic Retinopathies, anti-VEGF treatments merely target neovascular tuft formation without repopulating the vaso-obliterated regions of the retina. Moreover, numerous studies have demonstrated that the imbalance in the expression of other factors including inflammatory mediators and guidance cues play a major role in facilitating vascular damage and hindering vascular regeneration. As such, there is a dire need for new therapeutic avenues which would foster proper retinal revascularization. Mesenchymal Stem/Stromal Cells have emerged as a promising treatment modality because of their remarkable angiogenic and anti-inflammatory properties. We thus surmised that MSCs can promote vascular regeneration by modulating the angiogenic and inflammatory microenvironment in ischemic retinas.

The objective of this thesis is to understand the mechanisms by which MSCs elicit their therapeutic benefit in ischemic retinas. Using oxygen-induced retinopathy mouse model, we first compared the efficacy of intravitreal injection of MSCs to MSC secretome in promoting revascularization. Then, we explored the effects of MSC secretome on the expression of growth factors, inflammatory mediators, and guidance cues. To dissect the mechanisms of tissue repair observed in treated ischemic retinas, we investigated the role of MSC secretome (1) in restoring Sema3E levels and the resulting effect on the expression of pro-inflammatory cytokines, particularly IL-17A (Chapter 4), and (2) in reprogramming pro-inflammatory myeloid cells to an an-inflammatory phenotype (Chapter 5). Collectively, this thesis examines the multifaceted

potential of MSCs in establishing a favorable microenvironment permissive for healthy vascular regeneration.

Chapter 4. Mesenchymal stromal cells promote retinal vascular repair by modulating Sema3E and IL-17A in a model of ischemic retinopathy

Running Title: Sema3E negatively regulates IL-17A in ischemic retina

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Keywords: mesenchymal stem cells, vascular regeneration, ischemic retinopathies, semaphorin 3E, interleukin-17A.

Abstract

Ischemic retinopathies (IRs), such as retinopathy of prematurity and diabetic retinopathy, are characterized by an initial phase of microvascular degeneration that results in retinal ischemia, followed by exaggerated pathologic neovascularization (NV). Mesenchymal stromal cells (MSCs) have potent pro-angiogenic and anti-inflammatory properties associated with tissue repair and regeneration, and in this regard exert protection to neurons in ischemic and degenerative conditions; however, the exact mechanisms underlying these functions remain largely unknown. Class III Semaphorins (A-G) are particularly implicated in regulating neural blood supply (as well as neurogenesis) by suppressing angiogenesis and affecting myeloid cell function; this is the case for distinct neuropilin-activating Sema3A as well as PlexinD1-activating Sema3E; but during IR the former Sema3A increases while Sema3E decreases. We investigated whether retinal vascular repair actions of MSCs are exerted by normalizing Semaphorin and downstream cytokines in IR. Intravitreal administration of MSCs or their secretome (MSCs-conditioned media [MSCs-CM]) significantly curtailed vaso-obliteration as well as aberrant preretinal NV in a model of oxygen-induced retinopathy (OIR). The vascular repair effects of MSCs-CM in the ischemic retina were associated with restored levels of Sema3E. Vascular benefits of MSCs-CM were reversed by anti-Sema3E; while intravitreal injection of anti-angiogenic recombinant Sema3E (rSema3E) in OIR-subjected mice reproduced effects of MSCs-CM by inhibiting as expected preretinal NV but also by decreasing vaso-obliteration. To explain these opposing vascular effects of Sema3E we found in OIR high retinal levels respectively of the pro- and anti-angiogenic IL-17A and Sema3A-regulating IL-1β; IL-17A positively affected expression of IL-1β. rSema3E decreased concentrations of these myeloid cell-derived pro-inflammatory cytokines *in vitro* and *in vivo*. Importantly, IL-17A suppression by MSCs-CM was abrogated by anti-Sema3E neutralizing antibody. Collectively, our findings provide novel evidence by which MSCs inhibit aberrant

neovascularization and diminish vaso-obliteration (promoting revascularization) in retinopathy by restoring (at least in part) neuronal Sema3E levels that reduce pathological levels of IL-17A (and in turn other proinflammatory factors) in myeloid cells. The ability of MSCs to generate a microenvironment permissive for vascular regeneration by controlling the production of neuronal factors involved in immunomodulatory activities is a promising opportunity for stem cell therapy in ocular degenerative diseases.

Contribution to the field

Ischemic retinopathies are the leading causes of visual impairment and blindness worldwide. Anti-VEGF therapy is currently used as the major treatment in some neovascular eye diseases; however, it has shown limited benefits and a number of undesirable side effects. The development of innovative therapeutic strategies to prevent pathological retinal angiogenesis is desirable. Mesenchymal stem cells (MSCs) are multipotent cells that play important roles in the regulation of angiogenesis and tissue repair; however, the exact mechanisms underlying these functions remain largely not well understood. In this study, we showed that MSCs promote revascularization and inhibit aberrant neovascularization in ischemic retinas, at least in part, by restoring neuronal levels of Sema3E that reduces pathological levels of IL-17A in myeloid cells. We uncovered a novel mechanism by which MSCs-secretome regulates the interplay between neurons and immune cells, facilitating healthy revascularization and preventing the development of ischemic retinopathies.

Introduction

Ischemic retinopathies (IRs), such as retinopathy of prematurity (ROP) and diabetic retinopathy (DR), are leading causes of severe visual impairment and blindness in children and the working population respectively[2, 3]. These ocular diseases are characterized by retinal vasculature impairment that originates from local hypoxia, which triggers an exaggerated and uncontrollable pathological neovascularization (NV). Several molecules have been described to play an important role in pathological ocular NV, of which vascular endothelial growth factor (VEGF) is most reported. Anti-VEGF therapy has proven effective in reducing NV areas and improving the vision of some patients with retinopathy. However, anti-VEGF agents do not facilitate revascularization of the retina, resulting in undesired outcome[611-613]. Lately, adult stem-cell based therapies have emerged as alternative therapeutic avenues to treat ischemic retinopathies[614-616]. In particular, Mesenchymal Stem/Stromal Cells (MSCs) are a most promising modality for the treatment of ocular diseases[617, 618] because of their remarkable immunomodulatory and angiogenic capacities[10, 11, 619, 620]; correspondingly, MSCs exert protective functions on brain and retinal neurons[621, 622]. These adult multipotent stromal cells exert their reparative effects either by cell differentiation to replace damaged cells or through paracrine fashion via their secretome[623-626]; however, since MSCs have low engraftment efficiency and poor differentiation at the site of injury, an action involving secretion of bioactive molecules seems to be favored in their modulation of tissue microenvironment[622, 627]. But the exact mechanism of MSC functions on vascular repair is likely diverse and remains largely unclear.

We previously highlighted the importance of neuron-derived signaling molecules on retinal endothelial cell function[14, 628], particularly as it applies to guidance molecules of the class III semaphorins family (Sema3A-Sema3G), which are derived from retinal ganglion cells (RGCs),

and are key regulators of developmental and pathological angiogenesis in the retina[12-15, 350, 629]. Semaphorins can in turn affect the activity of myeloid cells[16, 17]. Most Sema3s (as clearly described for Sema3A-E) exert anti-angiogenic functions[18]. Interestingly, Sema3E (a key axon repulsive protein) is implicated in immune cell regulation and vascular growth and remodeling[20, 21, 630-633], as it selectively inhibits disoriented outgrowth of extraretinal vessels and restores the normal vasculature in ischemic retina[13]. Sema3E exerts its actions on endothelial cells via a single-pass transmembrane receptor, PlexinD1[19]. During retinal development as well as in ischemic retinopathy, PlexinD1 is located on endothelial cells exclusively at the front of actively sprouting blood vessels, while its ligand Sema3E is generated by retinal ganglion cells (RGCs)[12, 634]. Sema3E-PlexinD1 complex trigger an antiangiogenic signaling pathway that suppresses endothelial cell motility by inducing cytoskeletal rearrangements that cause filopodial retraction[13, 635], and inhibits endothelial cell growth and tube formation[12]. Semaphorins can also modulate angiogenesis (and tumor progression) by regulating immune cell function, as documented for Sema3A and Sema3E[636, 637]. Conversely, decreased expression of Sema3E aggravates inflammation and exacerbates disease severity by upregulating the release of pro-inflammatory cytokines, notably of interleukin-17A (IL-17A)[20, 21], which in turn can affect NV[638, 639]; concordantly, IL-17A plasma levels are high in DR and ROP[640, 641]. Hence, Semaphorins seem to represent an important class of factors that exert important vascular, immuno-modulatory and neuronal functions. However, the role of Sema3E and its interaction with IL-17A as it applies to MSC actions in ischemic retinopathies is not known.

Using an oxygen-induced retinopathy (OIR) mouse model, we showed that MSCs modulate the microenvironment of ischemic retinas enabling vascular regeneration and thus diminishing vaso-obliteration and inhibiting aberrant pre-retinal NV. MSCs secretome suppresses the

expression of Sema3A and intriguingly markedly stimulates that of Sema3E, both of which arise from the ganglion cells in retina of animals subjected to OIR. Upon exploring the role of Sema3E it has been found that this Semaphorin not only inhibits neovascularization by acting directly on vascular endothelium[18] but also acts on myeloid cells by repressing pro-angiogenic IL-17A expression[638], that negatively impacts production of primary pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in these cells; these cytokines are known to upregulate expression of the anti-angiogenic Sema3A and conversely its suppression enhances retinal revascularization of vaso-obliterated areas[14, 628]. Thus, in presence of MSCs, we hereby find that Sema3E mediates to a significant extent the vascular benefits incurred by MSCs secretome, and assists Sema3A to performing opposing functions, specifically accelerating revascularization and diminishing aberrant NV, by controlling inflammation. Collectively, we highlight some evidence by which MSCs secretome regulates the interplay between neurons and immune cells to facilitate healthy revascularization and diminish abnormal NV in ischemic retina.

Materials and methods

4.1.1.1 Animals

Adult C57BL6/J mice were purchased from Jackson Laboratories. Adoptive lactating CD-1 females were purchased from Charles River to tend to C57BL6/J pups. All experiments adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement regarding use of animals in ophthalmic and vision research and were approved by the Animal Care Committee of Maisonneuve-Rosemont Hospital in accordance with guidelines established by the Canadian Council on Animal Care.

4.1.1.2 Isolation and characterization of mesenchymal stem/stromal cells (MSCs) from compact bone

MSCs characterized by their potential therapeutic properties, easy isolation, wide expansion in vitro, and their abundance in compact bone of many species[642, 643] were isolated from the compact bone of mice as previously outlined[644]. Long bones of adult C57BL6/J mice (6-8 weeks) were removed and thoroughly cleaned from any connective tissue. Using a 23G needle, the bone marrow was flushed out with PBS and saved for subsequent isolation of bone-marrow-derived macrophages (BMDM). Bones were gently crushed and digested with (2.5mg/ml) collagenase (Sigma) and 5% TrypLE Express (Gibco) for 1 hour at 37ºC. Cell suspension was discarded, and bone fragments were cultivated in αMEM (Gibco) supplemented with 20% Mesenchymal-tested FBS (Wisent) and 1% penicillin/streptomycin (Corning) at hypoxia (5%O_2) allowing the migration of MSCs out of the compact bone. MSCs were enriched at P2 by negative selection using EasySep Mouse Mesenchymal Progenitor Enrichment Kit (Stem Cell Technologies) to deplete non-mesenchymal lineages. At 80% confluency, the media was changed to basal αMEM. The supernatant, referred herein as conditioned media (MSCs-CM), was collected 24 hours later, centrifuged, filtered through 0.22μm filter (Millipore), and concentrated 10 times using a 10K molecular weight cut off centrifugal filter (Millipore). MSCs-CM was collected between passages 3 and 5. MSCs were characterized based on the minimal criteria put forth by the International Society of Cellular Therapy[448]. Immunophenotyping of MSCs was performed by flow cytometry analysis using monoclonal conjugated antibodies against putative MSC surface markers FITC anti-mouse CD90.2 (eBioscience), PE/Cy7 anti-mouse CD105 (BioLegend), PE anti-mouse CD73 (BioLegend), and against cell lineage markers FITC anti-mouse CD11b (eBioscience) and PE anti-mouse CD34 (BioLegend). FACS was performed on BD LSRFortessa X-20 and data was analyzed using FlowJo software. Compact bone derived-MSCs highly expressed the classical stem cells surface makers CD90.2 (≥97%), CD73 (≥94%) and CD105 (≥95%) previously characterized in

C57BL6/J mice[645] and were negative for hematopoietic markers including CD11b (≤1%) and CD34 (≤0.3%) (**Suppl. Fig. 1A and B**). MSCs were also differentiated in adipocytes or osteocytes following the instructions described in the mouse Mesenchymal Stem Cell Functional Identification Kit (RnD systems). MSCs' adipogenic and osteogenic potential was confirmed by immunostaining against fatty acid binding protein 4 (FABP4) and osteopontin respectively (**Suppl. Fig. 1C**).

4.1.1.3 Isolation and stimulation of bone-marrow derived macrophages (BMDM)

Bone marrow from tibia and femur of adult mice were flushed with PBS and gently dissociated with 23G needle. The cell suspension was filtered through 70μm strainer and centrifuge at 4^{o}C, 500xg for 10 minutes. Cell pellet was resuspended in DMEM containing 10% FBS and 20 ng/ml monocyte colony stimulating factor (M-CSF; PeproTech) to differentiate monocytes into macrophages and seeded in 6-well plates. On days 3 and 5, half of the differentiation media was changed. By day 7, mature macrophages have formed and attached to the plate. BMDM then were pre-exposed to hypoxia for 24 hours to assess IL-17A expression levels. Subsequently, BMDM were treated with vehicle, MSCs-CM, HypRGC-CM, HypRGC-MSCs-CM, HypRGC-MSCs in presence of a monoclonal Sema3E antibody (15μg/ml; R&D Systems), recombinant mouse Sema3E (5ng/ml), or recombinant mouse IL-17A (100ng/ml) proteins for another 24 hours. RNA was isolated using RNA isolation kit (Qiagen), whereas protein was collected in commercial RIPA buffer (Cell Signaling Technology).

4.1.1.4 Oxygen-Induced Retinopathy (OIR) model

The OIR model has been well established and standardized to mimic the vascular dysfunction observed in ROP[103, 244]. Postnatal day 7 (P7) C57BL6/J pups were placed with an adoptive lactating CD-1 female in a hyperoxic environment set to 75% O_2 (OxyCycler A820CV; BioSpherix, Ltd., Redfield, NY, USA) until P12 to trigger VO. Then, the animals were returned to room air whereby hypoxia-driven neovascularization develops at P14 and reaches its peak at P17. Pups at P17 were anesthetized in 3% isoflurane with oxygen and sacrificed by decapitation. Eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour at room temperature. Retinas were dissected and stained overnight at 4^{o}C with fluorescein labeled GSL I, Isolectin B4 (Vector Labs, 1:100) in PBS containing 1mM $CaCl_2$. Lectin-stained retinas were whole-mounted onto Superfrost/Plus microscope slides (Fisher Scientific) with the photoreceptor side down and embedded in Fluoro-gel (Electron Microscopy Sciences). Multiple 10x images of each whole-mounted retina were taken using the MosiaX option built in Zeiss AxioObserver.Z1 and then merged into a single file using the in the AxioVision 4.6.5 software (Zeiss). Quantification of VO and NV was determined at P17 using SWIFT_NV as described previously[249].

4.1.1.5 Stimulation of RGC-5

RGC-5 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Cell Applications) and 1% penicillin/streptomycin (Cell Applications) either in normoxia or hypoxia (5% O_2) and treated with MSCs-CM for 48 hours to determine Sema3E mRNA levels. Normoxic and hypoxic RGC-5 cells were stimulated with recombinant IL-17A in a dose-dependent manner for 24 hours. RNA was collected in Ribozol (Amersco), whereas protein was collected in commercial RIPA buffer (Cell Signaling Technology).

4.1.1.6 Immunohistochemistry

Eyes of P17 C57BL6/J pups exposed to normoxia or OIR were enucleated, fixed in 4% paraformaldehyde for 1 hour at room temperature, and saturated overnight at 4°C in a 30% sucrose solution prior to embedding in OCT compound (TissueTek®). Sagittal cross-sections of 10 μm was sectioned using a Cryostat (Leica) and permeabilized for 1 hour at room temperature. Immunostaining against Sema3E (R&D Systems; 1:100), NeuN (EMD Millipore; 1:100), F4/80 (Abcam; 1:100) or IL-17A (Abcam; 1:200) overnight at 4°C, followed by fluorochrome-conjugated secondary antibody (goat anti-mouse IgG Alexa Fluor 488 and goat anti-rabbit IgG Alexa Fluor 594; Invitrogen) for localization studies according to manufacturers' recommendations. Nuclei were stained with DAPI (Invitrogen; 1:5000). Cross-sections were visualized using 30x objectives with an IX81 confocal microscope (Olympus), and images were obtained with Fluoview 3.1 software (Olympus).

4.1.1.7 Retinal Whole-Mounts

Mice eyes were collected and processed for retinal flat-mounts as previous published studies[253, 646]. Briefly, eyes were fixed in 4% paraformaldehyde for 1 hour and then, retinas were isolated and incubated overnight at 4°C in a solution containing 1% Triton X-100-1 mM $CaCl_2$/phosphate-buffered saline (PBS) and TRITC-conjugated lectin endothelial cell marker Bandeiraea simplicifolia (1:100; Sigma-Aldrich, St. Louis, MO). Retinas were mounted and photographed under a Zeiss AxiObserver Z1 motorized inverted microscope (Zeiss, Canada) at 10x. Vaso-obliterated areas were assessed as the retinal area devoid of vasculature over the total retinal area by using Adode Photoshop CS5[247]. Neovascularization was analyzed using the SWIFT-NV method[249] which was developed to quantify all the pixels represented by neovascular tufts and clusters, but not normal vessels in lectin-stained retinal whole mounts.

4.1.1.8 Intravitreal injections

P12 mice pups were anesthetized in 3% isoflurane with oxygen and injected intravitreally with 2µl of CM, whereas basal αMEM (vehicle) was injected in the contralateral eye as control using a Hamilton syringe equipped with 50-gauge glass capillary. 2µl of mouse recombinant Sema3E Fc Chimera (20ng/µl; R&D systems) or PBS were administered into vitreous cavity of P17 OIR pups. To assess the effect of blocking IL-17A on the retinal vascularization, 2µl of 5µg/µl neutralizing monoclonal IL-17A antibody (Clone 17F3, BioXCell) or of 5µg/µl mouse IgG1 isotype control monoclonal antibody (Clone MOPC-21, BioXCell). To reverse the benefits of Sema3E induced by MSCs-CM administration during OIR, the eyes were injected at P12 and P14 with 2µl of blocking Sema3E (5µg/µl) antibody (AF3239, R&D Systems). To assess the dose-response of MSCs on vaso-obliteration, 50,000, 100,000 and 200, 000 cells on passage 3-5 were intravitreally injected in a volume of 2µl. Retinal vasculature was analyzed in whole-mounts at P17.

4.1.1.9 Reverse-transcription PCR and quantitative real-time PCR

Freshly dissected retinas (2 retinas pooled per n) were placed immediately in RiboZol (Amresco) and homogenized using Precellys 24 homogenizer. Cultured cells were scrapped using RiboZol (Amresco). RNA was extracted following manufacturer's instructions and then treated with DNase I (Sigma) to remove genomic DNA. 1µg of RNA was reverse transcribed into cDNA using iScript RT Supermix (Biorad) as described by manufacturer's instructions. cDNA was analyzed by Quantitative real-time PCR using iTaq™ Universal SYBR® Green Supermix (BioRad) with primers targeting mouse Sema3E (Fwd 5'-TCTGCAACCCATCCATTCTGAG-3'

and Rev 5’-ACCACAAGAGGGAAGCACAGAC-3’), mouse IL-1β (Fwd 5’-CTGGTACATCAGCACCTCACA-3’ and Rev 5’-GAGCTCCTTAACATGCCCTG-3’), mouse IL6 (Fwd 5’-ACAGAAGGAGTGGCTAAGGA-3’ and Rev 5’-AGGCATAACGCACTAGGTTT-3’), mouse TNF-α (Fwd 5’-GCCTCTTCTCATTCCTGCTTG-3’ and Rev 5’-CTGATGAGAGGGAGGCCATT-3’), mouse IL-17A (Fwd 5’-CACCGCAATGAAGACCCTGA-3’ and Rev 5’-TTCCCTCCGCATTGACACAG-3’), mouse Rorγ (Fwd 5’-CCACTACGGGGTTATCACCTG-3’ and Rev 5’-TGCAGGAGTAGGCCACATTAC-3’), mouse PlexinD1 (Fwd 5’-TTCCATTTGGTGCTACCTACG-3’ and Rev 5’-CAATACTTTCTTGCGGTGGC-3’), mouse Sema3A (Fwd 5’-GGGGAACCAGATGACAGAGA-3’ and Rev 5’-GCTCCTGCTCCGTAGCCTGC-3’), mouse VEGF (Fwd 5’-TCGGCGTTGCTTTCGGTCCC-3’ and Rev 5’-GCCCTGAGTCAAGAGGACAG-3’), mouse IL-4 (Fwd 5’-CCATATCCACGGATGCGACA-3’ and Rev 5’-CGTTGCTGTGAGGACGTTTG-3’), mouse IL-10 (Fwd 5’-GCTCTTACTGACTGGCATGA-3’ and Rev 5’-AGAAAGTCTTCACCTGGCTG-3’). Primers were designed by NCBI Primer-BLAST software and synthesized by Alpha DNA (Montreal). Quantitative gene expression analysis was evaluated using ABI 7500 Real-Time PCR system (Applied Biosystems) and normalized to QuantumRNA^{TM} 18S universal primer (Invitrogen) using ΔΔCT method.

4.1.1.10 Western blot

Cultured cells were washed with ice-cold PBS, collected in RIPA lysis buffer (Cell Signaling Technology) and centrifuged to eliminate cell debris. 50μg of cell lysate was loaded on SDS-PAGE gel. After blocking, membranes were blotted against mouse IL-17A (Abcam), IL-β

(Abcam), IL-6 (Abcam), TNF-α (Abcam), Sema3E (Abcam) and β-actin (Santa Cruz Biotechnology). After washing, membranes were incubated with horseradish peroxidase-conjugated (HRP) anti-mouse or HRP anti-goat or -rabbit secondary antibodies (Millipore). Membranes were imaged with LAS-3000 imager. Protein band intensities were assessed using densitometry plugins in ImageJ and expressed as a ratio between corresponding protein and β-actin.

4.1.1.11 Choroidal Sprouting Assay

Choroidal explants were prepared from adult C57BL6/J mice as previously described[647]. Briefly, eyes were rapidly enucleated and dissected to remove the cornea, lens and retina. The choroid/RPE complex was cut into approximately 1x1 mm sections and embedded in growth-factor reduced Matrigel in 24-well plates. The choroidal explants are cultured in EBM-2 medium (Lonza, Cat. CC-3156) supplemented with endothelium growth medium (EGM) kit (Lonza, Cat. CC-4147) at 37^{o}C in 5% CO_2 for 3 days. On day 4, the media was changed to vehicle or recombinant mouse IL-17A (200ng/ml) in absence or presence of recombinant mouse Sema3E (5ng/ml), for another 24 hours. Phase-contrast photos of individual explants were taken with ZEISS AxioOberver microscope before and 24-hours after treatment. The microvascular sprouting area was measured using Image J software version 1.50i (National Institutes of Health, USA).

4.1.1.12 Flow cytometry and Cell Sorting

Freshly dissected retinas from treated-OIR pups were pooled (2 retinas per treatment per n) and digested in HBSS solution containing 750U/ml DNase I (Sigma-Aldrich) and 1mg/ml collagenase D (Roche) for 20 minutes at 37^{o}C with gentle shaking. Homogenized retinas were then

filtered through 70μm cell strainer and supplemented with fluorescent activated cell sorting (FACS) buffer (PBS with 3% FBS) to quench enzyme activity. Cell suspensions were incubated with LEAF purified anti-mouse CD16/32 (Biolegend) for 10 minutes at 4^{o}C to block Fc receptors, followed by 30 minutes incubation at 4^{o}C of the following antibodies: FITC anti-mouse CD11b (eBioscience) and APC anti-mouse F4/80 (BioLegend). Dead cells were excluded using 7-AAD viability staining solution (BioLegend). FACS was performed on BD LSRFortessa X-20 and data was analyzed using FlowJo software. Myeloid cells ($CD11b^{+}$ $F4/80^{+}$) were sorted by BD FACSARIA III.

4.1.1.13 Statistical analysis

Results are presented as mean ± S.E.M. for all studies. One-way or two-way analysis of variance with significance $\alpha=0.05$ or higher were used for processing data. Bonferroni post-hoc analysis was used for calculating significance between groups. Two-tailed student t-tests were used to test for significance between two means.

Results

4.1.1.14 MSCs promote normalization of ischemic retinal vasculature in a paracrine fashion

MSCs were intravitreally injected in OIR animals at the beginning of neovascularization phase on postnatal day 12 (P12). At P17, retinas were isolated to evaluate the degree of vaso-obliteration (VO) and consequential pathological neovascularization (NV) (**Fig. 1A-C**). Intravitreally administered MSCs (50, 000 cells; similar regenerative efficacy to higher cell concentrations [**Suppl. Fig. 2A**]) significantly reduced the extent of VO, thus enhancing revascularization, and consequently diminished pathological NV, compared to naïve media-treated retinas (**Fig. 1A-C**). Although MSCs were effective, occasionally some cells remained clustered

at the injection site, while conditioned medium derived from the same number of MSCs (MSCs-CM) reproduced effects of MSCs on retinal angiogenesis and no drawbacks were detected (**Fig. 2A-C**).

4.1.1.15 MSCs-secretome modulates retinal expression of factors that affect retinal vascular architectural network in OIR

A gene expression array of growth (angiogenic), inflammatory and guidance/apoptotic factors (**Fig. 3A**) performed at P17 after OIR revealed in retinas of MSCs-CM-treated animals (at P12) a marked increase in VEGF, a decrease in pro-inflammatory cytokines IL-1β and IL-17A, as well as an increase in anti-inflammatory IL-10 in comparison to vehicle-treated. These MSCs-CM-induced changes in OIR were associated with a considerable suppression of Sema3A and a notable (3-fold) augmentation of Sema3E which is nearly undetected in untreated OIR retinas at P17 (**Fig. 3B**), consistent with previous reports [13]. The MSCs-CM-induced increase in Sema3E in OIR at P17 was confirmed by RT-PCR (**Suppl Fig. 2B**), as well as by immunohistochemistry where, as expected[13, 648, 649], Sema3E was detected in the ganglion cell layer (**Fig. 3C**).

In an attempt to mimic *in vitro* the *in vivo* conditions related to OIR, we confirmed that MSCs-CM triggered induction of Sema3E gene expression and protein in hypoxic (5% O_2) retinal ganglion cells (RGC) (**Fig. 4A**), but not in normoxic (21% O_2) RGCs (**Suppl Fig. 2C**). Concordantly, anti-Sema3E prevented the improved retinal vascularization in MSCs-CM-treated mice subjected to OIR (**Fig. 4B-C**). In addition, treatment of mice subjected to OIR with recombinant Sema3E (rSema3E, at P12) markedly curtailed aberrant neovascularization (at P17), in line with a gradual OIR-associated increase in the expression of the Sema3E receptor PlexinD1

(**Suppl Fig. 3A**); yet surprisingly anti-angiogenic Sema3E also diminished the degree of retinal vaso-obliteration (**Fig. 5A-C**).

4.1.1.16 Effects of MSCs-CM and Sema3E in OIR are associated with downregulation of angio-active IL-17A and other proinflammatory cytokines in myeloid cells

As is the case for MSCs[650], Sema3E does not only act on endothelial cells[651], by inhibiting directly proangiogenic effects of IL-17A (**Suppl Fig. 3B**), but also exerts effects on myeloid cells[652]. In an attempt to explain opposing actions of Sema3E on vasculature we explored if Sema3E can regulate inflammatory mediators (myeloid cell-derived) which exhibit distinct functions. A lack of Sema3E has been shown to aggravate inflammation[21, 653] and exacerbate neovascular retinopathy by increasing the release of pro-inflammatory cytokines, particularly the pro-angiogenic IL-17A[639]. As expected, IL-17A mRNA and protein (immunoreactivity) increased during the neovascular phase starting at P14 ($p<0.05$) and furthermore by P17 ($P<0.001$) (**Fig. 6A,B**); likewise, mRNA expression of the retinoic acid receptor-related orphan nuclear receptor γ (RORγ), a key regulator in the production of IL-17A[639], also increased at P17 in OIR (**Fig. 6C**). Intravitreal injection of MSCs-CM or rSema3E reduced significantly mRNA and protein levels of IL-17A, and mRNA expression of RORγ (**Fig. 6D,E**) as well as that of other downstream pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (**Suppl Fig. 4A**), in OIR retinas evaluated at P17; importantly, contrary to IL-17A, IL-1β elicits retinal vascular degeneration by upregulating Sema3A[628].

IL-17A co-localized with F4/80 myeloid cells in the retina, as these are main producers of IL-17A[639]. Attenuation of the pro-inflammatory cytokine profile of myeloid cells by MSCs-CM and rSema3E, was not related to their density (flow cytometry analysis) (**Suppl. Fig. 4B**),

suggesting that MSCs-CM and rSema3E modulate activation state of immune cells rather than infiltration rate of these cells. Correspondingly, we found in PlexinD1-expressing bone-marrow derived macrophages (BMDM)[633] that hypoxia-driven IL-17A mRNA expression (**Suppl Fig. 4C**) was dose-dependently reduced by rSema3E (**Fig. 7A**). Concordant protein expression of IL-17A and of IL-1β, IL-6 and TNF-α was also decreased *in vitro* by rSema3E as well as MSCs-CM (**Fig. 7B,C**), as observed *in vivo* (**Suppl Fig. 4A**); relevantly, IL-17A induced expression of IL-1β, IL-6 and TNF-α in BMDM (**Fig. 7D**). Correspondingly *in vivo* in retina of mice subjected to OIR, expression of IL-1β and IL-1β-dependent Sema3A[14, 628] were attenuated by anti-IL-17A antibody (**Suppl Fig. 5A**), consistent with induction of IL-1β by IL-17A (**Fig. 7D**).

Finally, we ascertained a prominent role for MSCs-CM-triggered Sema3E derived from RGC, in suppressing IL-17A mRNA expression in myeloid cells; this effect was abrogated by anti-Sema3E (**Fig. 7E**). Vice versa, the expression of the Sema3E and its receptor was unaffected by IL-17A or anti-IL-17A (**Suppl Fig. 5A,B**).

Discussion

Progression of ischemic retinopathies is driven by a complex interplay of factors that activate various signaling pathways involved in dysregulation of vascular, inflammatory, neuronal and metabolic processes[646, 654]. The ability to target critical factors involved in initial vascular damage and subsequent disoriented vessel formation remains a major challenge for preventing such oculo-vascular diseases. Emerging therapies against ischemic retinopathies often focus on targeting VEGF to ameliorate pathological neovascularization and prevent vision loss; however, anti-VEGF therapy has limited benefits and can exert potential adverse outcomes[611, 655]. Alternatively, stem-cell based therapies offer a multifaceted therapeutic approach by targeting multiple underlying pathologic pathways and providing an environment favorable for vascular regeneration. In this

study, we harnessed the reparative, regenerative and immunomodulatory potential of MSCs-secretome to promote proper vascular growth in ischemic retinas and prevent pathological neovascular formation. Several studies have shown that the therapeutic effects of MSCs can be associated with soluble paracrine factors and show even better tissue repair than the cells themselves[656-660]. MSCs-secretome has shown a low risk of toxicity, and immune rejection[661] making it a promising therapeutic alternative for the treatment of ischemic retinopathies. We investigated the modulatory response of the MSCs-secretome on the expression of key retinal factors involved in vascular repair, notably in enhancing revascularization and thus reducing vaso-obliteration. We found that MSCs-CM preventing the development of pathological neovascularization and induced a healthy revascularization in OIR retinas. This beneficial effect of MSCs-CM was associated with a modulation of gene expression of angiogenic, guidance cue and inflammatory factors involved in the progression of retinopathy. Such restoration of growth as well as pro/anti-inflammatory factors, such as VEGF, IL-1 and IL-10, is reported to decrease the occurrence of retinopathy[662, 663]. Moreover, MSCs-CM controlled in opposite directions the expression of two neuron projection repulsive signals, Sema3A and Sema3E, known to exert anti-angiogenic properties, such that MSCs-CM induces Sema3E which in turn suppresses pro- and anti-angiogenic inflammatory factors, which respectively curtail pre-retinal neovascularization and facilitate revascularization.

A variety of factors affected by the inherent environment have been identified to partake in reparative and immuno-modulatory properties of MSCs. These include peptide growth factors, lipid mediators, nucleic acids as well as organelles and exosomes[664]. A key factor presented in this work that partakes in benefits incurred by MSCs applies to Sema3E. Neuronal-derived Sema3E is markedly upregulated by MSCs-CM, and in turn exerts potent anti-inflammatory effects on

myeloid cells which are the main producers of potentially detrimental cytokines[224, 628, 639, 665] that contribute to vascular injury and pathological neovascularization[666]. The effects of Sema3E on macrophages are mostly controversial[632, 633]. In this paper MSCs-CM restores Sema3E, which tends to normalize IL-17A concentrations and in turn those of other proinflammatory cytokines, some of which upregulate expression of other Semaphorins, notably Sema3A[14, 628]. The mechanism by which Sema3E down-regulates IL-17A is possibly through suppression of RORγ, an important regulator of IL-17A[639]. IL-17A is known to play an important role in aggravating and sustaining local tissue inflammation by enhancing the inflammatory signaling pathways of pro-inflammatory cytokines that impede normal revascularization and enhances pathological neovascularization[667, 668]. In contrast, IL-1β causes retinal vascular degeneration by inducing Sema3A expression[628]. The shift in the inflammatory profile of OIR retinas treated with MSCs-CM or rSema3E resulted in a beneficial advantage by favoring an environment suitable for vascular regeneration and dampening preretinal neovascularization in the ischemic tissue.

Although we attribute a significant role for inflammatory mediators IL-17A, IL-1β and possibly IL-6 and TNF-α in the actions of MSCs-CM and Sema3E we cannot rule out the possible contribution of anti-inflammatory mediators, such as angiopoietin-like 4 (ANGPTL4) or IL-10 highly secreted by MSCs[669, 670]. Such factors could indirectly modulate Sema3E production through suppressing the secretion of proinflammatory cytokines[670-672] which induce ROR-α upregulation and subsequently inhibition of Sema3E expression in the retina during pathological conditions. ROR-α is up-regulated by pro-inflammatory cytokines such as IL-1β and TNF-α[670, 673]and suppresses Sema3E expression in pathological retinal neovascularization[12, 13]. We can also not exclude an effect of this Semaphorin directly on its receptor PlexinD1 on endothelium[12, 13, 674]. PlexinD1 activation promotes filopodial retraction in endothelial tip cells by disrupting integrin-

mediated adhesive structures resulting in inhibition of angiogenesis[635]. Its expression is particularly augmented in pathological endothelium as found herein. Altogether, these claims favor PlexinD1 as a viable target in abnormal conditions, relative to VEGF essential in vascular proliferation.

In this study, we unveiled an unprecedented interplay between MSCs, neurons and myeloid cells through which MSCs inhibit aberrant neovascularization and promote revascularization in retinopathy, at least in part, by restoring neuronal Sema3E levels and reducing pathological levels of IL-17A in myeloid cells. A limitation in this study is to uncover the identity of MSCs-derived factors that trigger Sema3E production in RGCs. In this regard, MSCs-derived exosomes and microvesicles[675, 676] that contain a complex set of multiple soluble biologically active molecules could be ideal candidates to regulate Semaphorins in RGCs. The therapeutic efficacy of extracellular vesicles from MSCs has been widely demonstrated in many retinal disease models[558, 625, 677-680]. Due to their nano dimension, extracellular vesicles may rapidly reach and exert their therapeutic effects on RGCs[623] inducing axonal regeneration and improving survival and the maintenance of these cells[681, 682]. Notably, microRNAs (miRNAs) as a component of MSCs-derived extracellular microvesicles[683, 684] seem to be attractive candidates for semaphorins regulation on RGCs. miRNAs have been shown to modulate semaphorin expression, for instance, miR-497-5p is a critical target of Sema3A[685] while miRNA-4282 has been shown to be a key regulator of Sema3E expression[686]. Determining the presence of these miRNAs as part of MSCs-secretome could be of great relevance for evaluating its action on RGCs. Exosome-derived lipids could be other candidates for regulating semaphorins in RGCs. Exosomes contain large amounts of cholesterols[687, 688]. Cholesterol derivatives such as 7α-hydroxycholesterol, 7β-hydroxy-cholesterol and 7-keto-cholesterol are natural ligands and negative regulators of the nuclear

receptor ROR-alpha[689], responsible for inhibiting the expression of Sema3E in retinal ganglion cells during OIR[634]. Although we do not rule out a possible action of MSCs-derived cytokines/growth factors on RGCs, future work will certainly be aimed at evaluating the MSCs-CM-derived extracellular vesicle elements involved in the regulation of semaphorins in RGCs. So far, our study shows the ability of MSCs-CM to promote the production of neuronal factors involved in immunomodulatory activities which would generate a microenvironment permissive for vascular regeneration, and thus a promising opportunity for the treatment of ischemic retinopathies using stem cell therapy. Collectively, we show evidences by which MSC secretome regulates the interplay between neurons and immune cells establishing a healthy environment permissive for vascular regeneration in ischemic retina; a schematic diagram depicting the described mode of action of MSCs secretome in ischemic retinopathy is presented in **Fig. 8**.

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Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interest.

Authorship contributions

B.N., S.C and J.C.R. conceived and designed the study; B.N., J.C.R., R.D., A.P., S.O., and I.L. performed the experiments; B.N. and J.C.R. prepared the figures; J.C.R. designed the scheme. B.N., S.C. and J.C.R. wrote the manuscript.

Figures

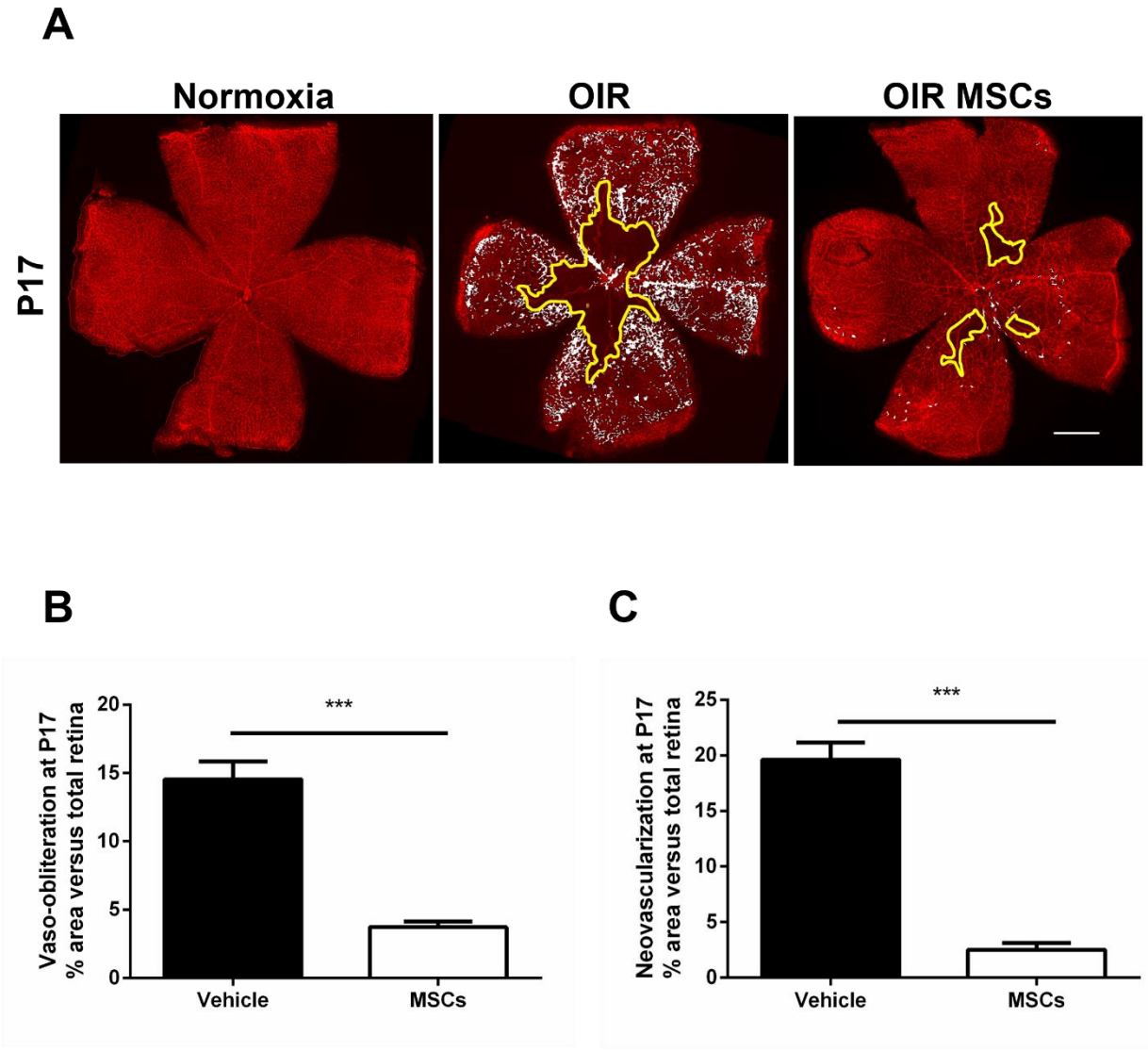


Figure 1

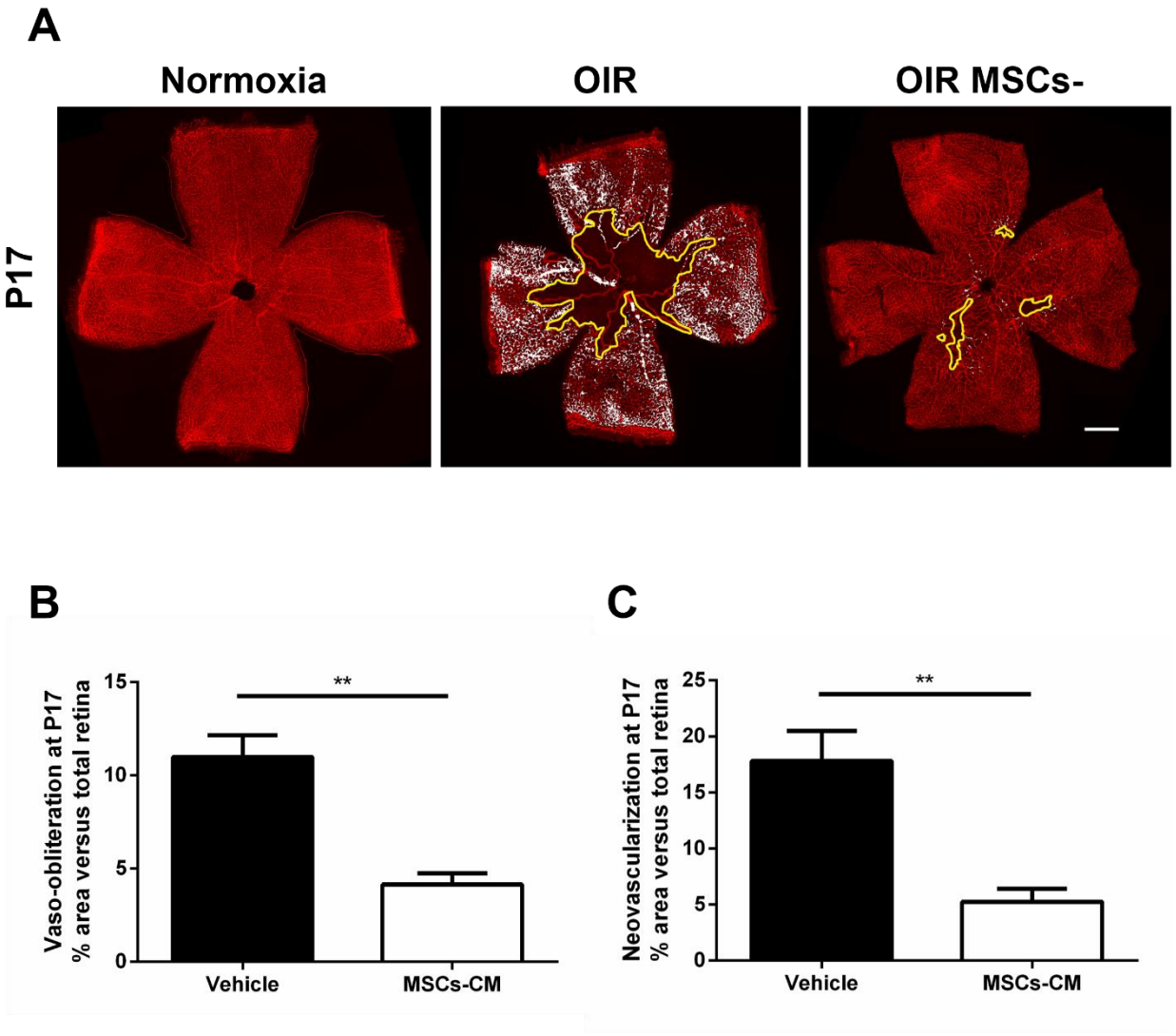

A
Normoxia
OIR
OIR MSCs-
P17
B
Vaso-obliteration at P17
% area versus total retina
15
10
5
0
**
Vehicle
MSCs-CM
C
Neovascularization at P17
% area versus total retina
25
20
15
10
5
0
**
Vehicle
MSCs-CM

Figure 2

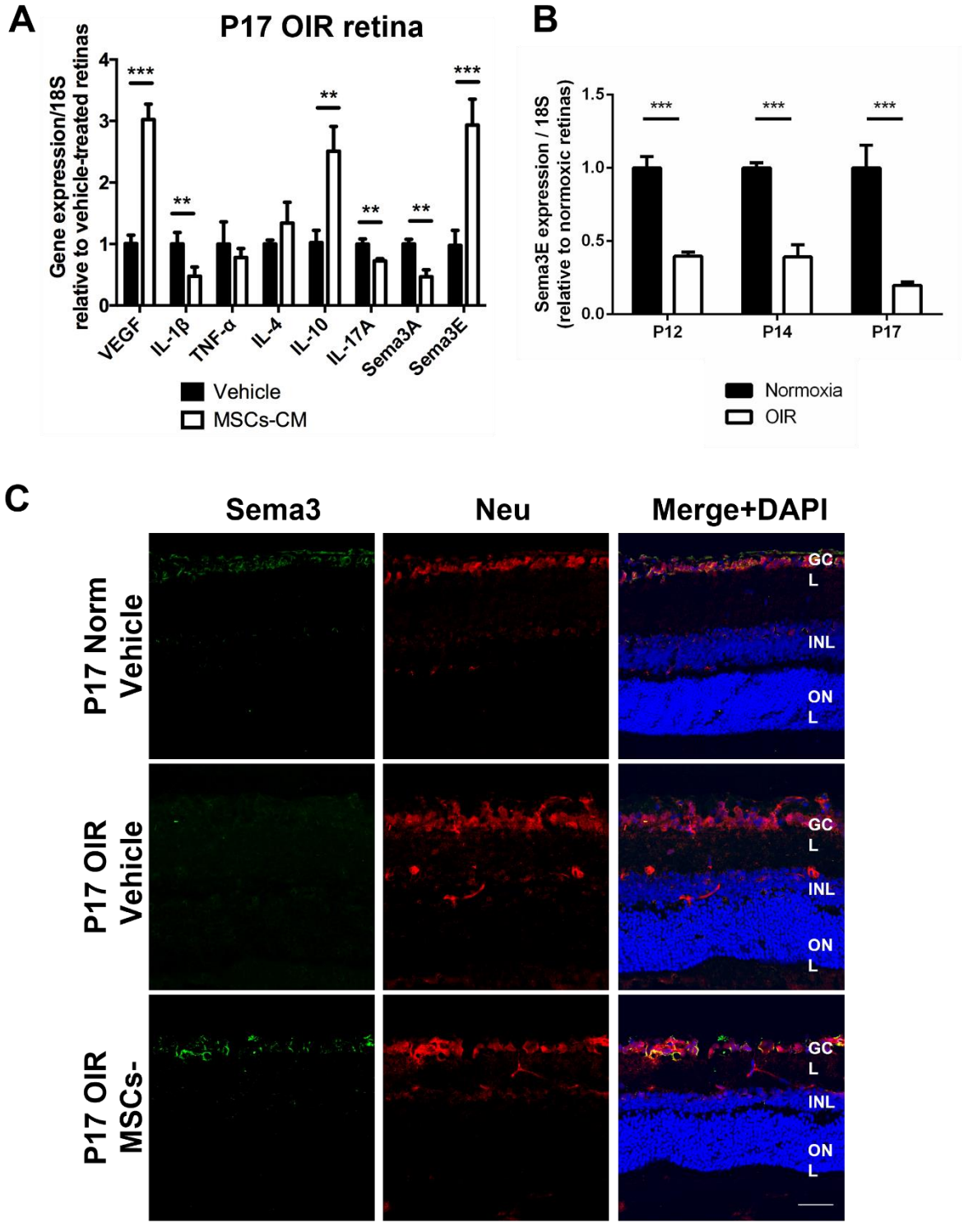

A
P17 OIR retina
Gene expression/18S relative to vehicle-treated retinas
VEGF
IL-1β
TNF-α
IL-4
IL-10
IL-17A
Sema3A
Sema3E
Vehicle
MSCs-CM
B
Sema3E expression / 18S (relative to normoxic retinas)
P12
P14
P17
Normoxia
OIR
C
Sema3
Neu
Merge+DAPI
P17 Norm Vehicle
P17 OIR Vehicle
P17 OIR MSCs-
GC L
INL
ON L

Figure 3

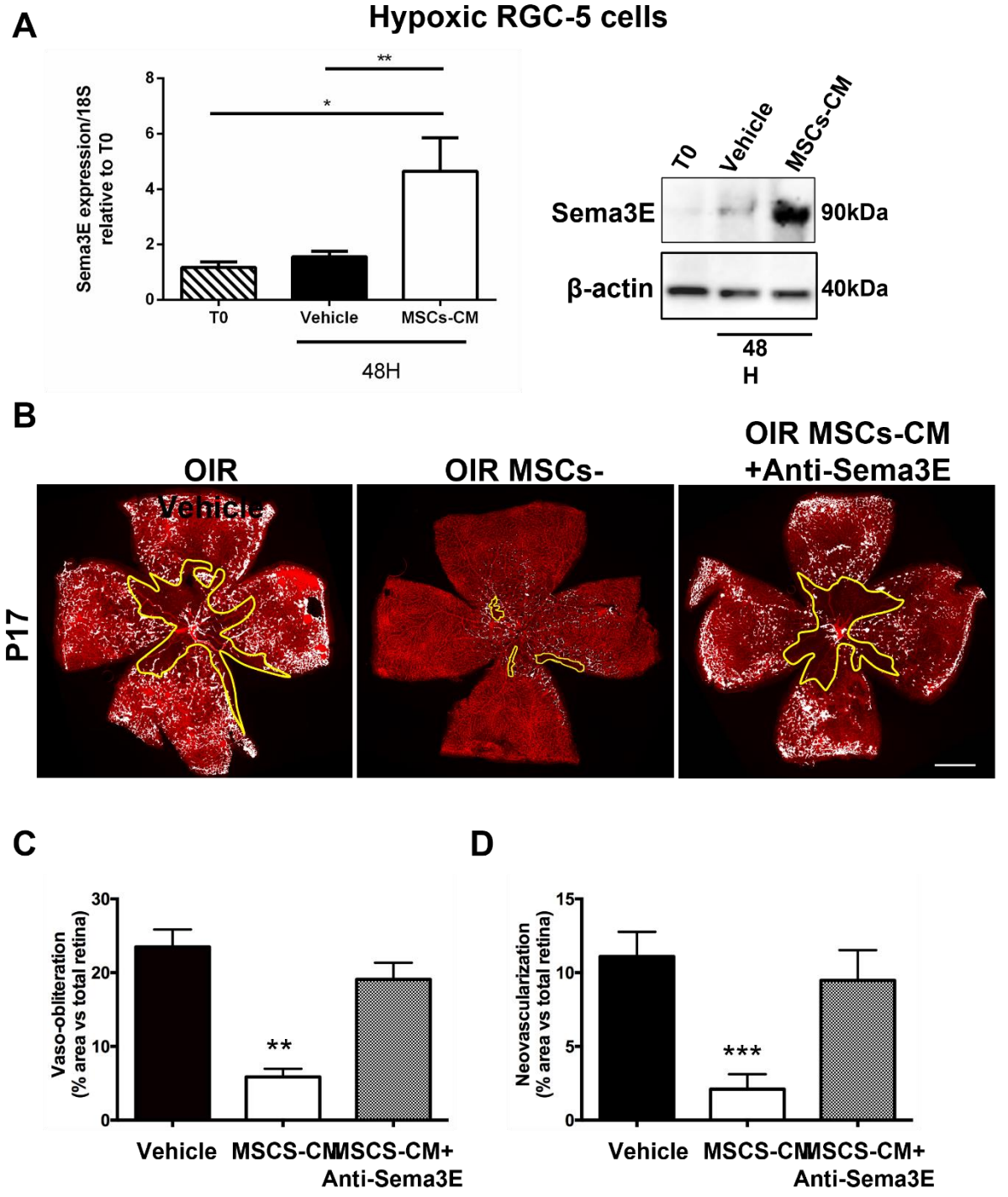

A
Hypoxic RGC-5 cells
Sema3E expression/18S relative to T0
T0
Vehicle
MSCs-CM
48H
Sema3E
β-actin
90kDa
40kDa
48 H
B
OIR
Vehicle
OIR MSCs-
OIR MSCs-CM +Anti-Sema3E
P17
C
Vaso-obliteration (% area vs total retina)
Vehicle
MSCS-CM
MSCS-CM+ Anti-Sema3E
D
Neovascularization (% area vs total retina)
Vehicle
MSCS-CM
MSCS-CM+ Anti-Sema3E

Figure 4

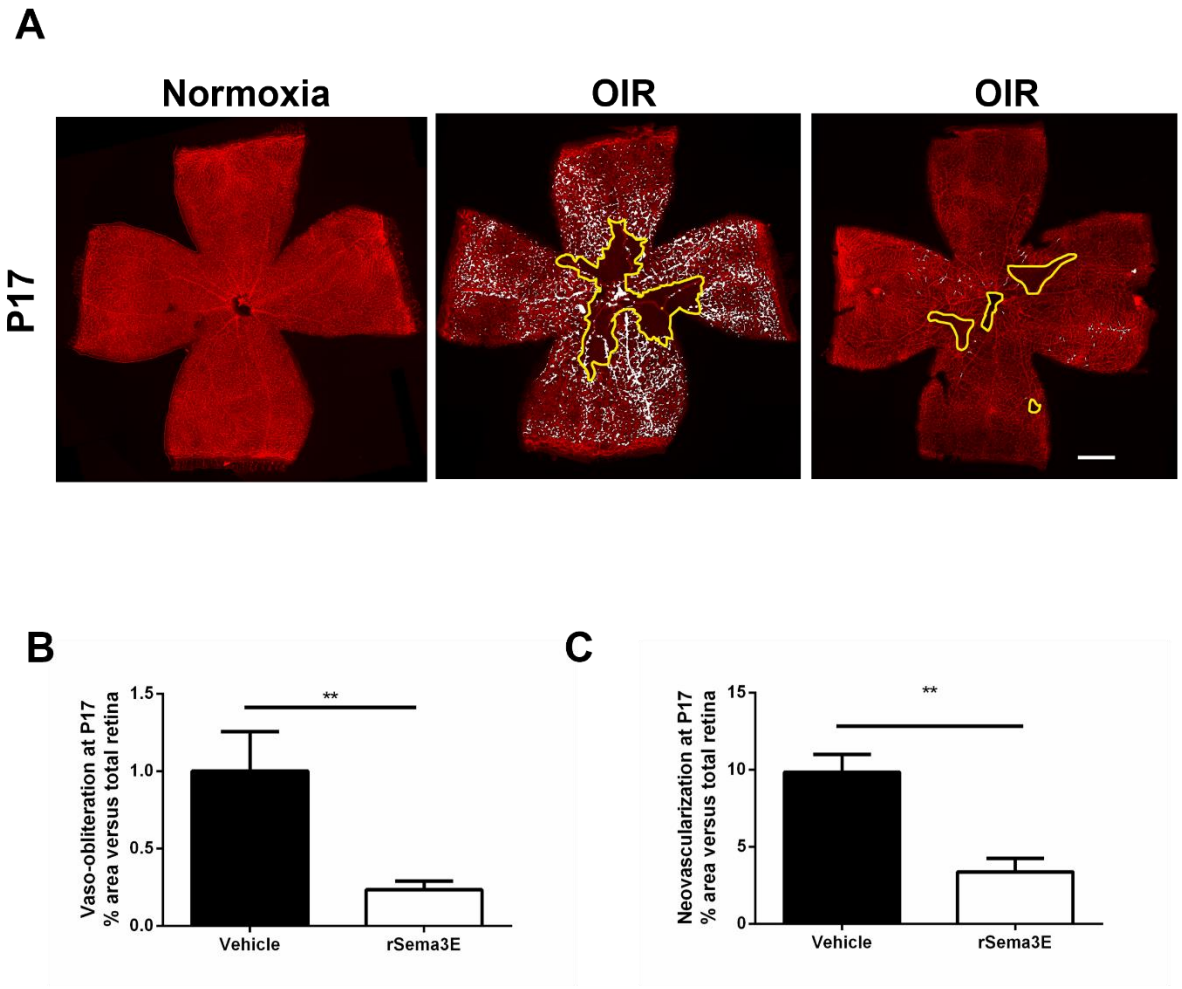

A
Normoxia
OIR
OIR
P17
B
Vaso-obliteration at P17
% area versus total retina
1.5
1.0
0.5
0.0
**
Vehicle
rSema3E
C
Neovascularization at P17
% area versus total retina
15
10
5
0
**
Vehicle
rSema3E

Figure 5

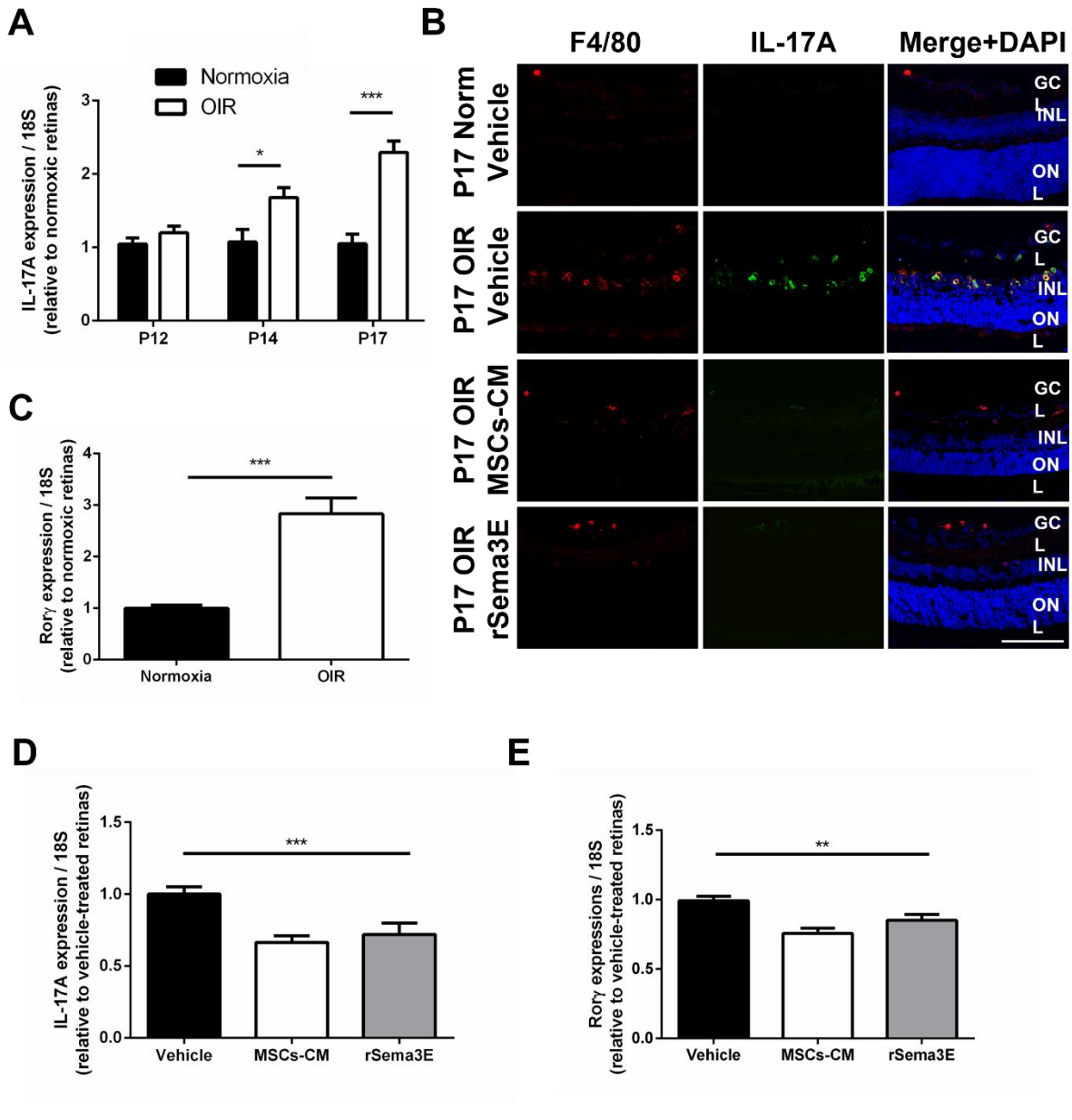

A
Normoxia
OIR
IL-17A expression / 18S
(relative to normoxic retinas)
P12
P14
P17
B
F4/80
IL-17A
Merge+DAPI
P17 Norm Vehicle
P17 OIR Vehicle
P17 OIR MSCs-CM
P17 OIR rSema3E
GC L
INL
ON L
C
Rorγ expression / 18S
(relative to normoxic retinas)
Normoxia
OIR
D
IL-17A expression / 18S
(relative to vehicle-treated retinas)
Vehicle
MSCs-CM
rSema3E
E
Rorγ expressions / 18S
(relative to vehicle-treated retinas)
Vehicle
MSCs-CM
rSema3E

Figure 6

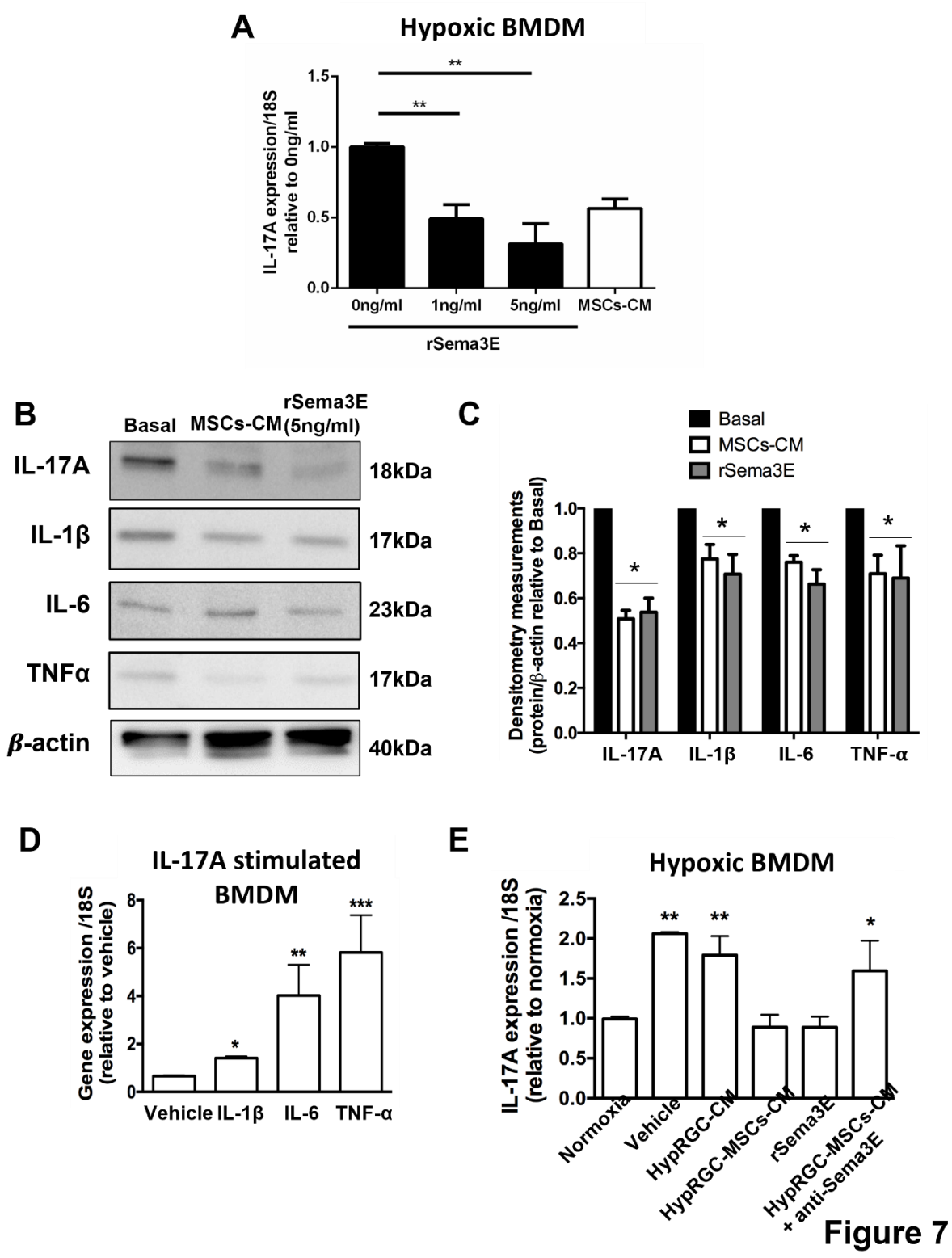

A
Hypoxic BMDM
IL-17A expression/18S relative to 0ng/ml
1.5
1.0
0.5
0.0
**
**
0ng/ml
1ng/ml
5ng/ml
MSCs-CM
rSema3E
B
Basal
MSCs-CM
rSema3E (5ng/ml)
IL-17A
IL-1β
IL-6
TNFα
β-actin
18kDa
17kDa
23kDa
17kDa
40kDa
C
Basal
MSCs-CM
rSema3E
Densitometry measurements (protein/β-actin relative to Basal)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
IL-17A
IL-1β
IL-6
TNF-α
D
IL-17A stimulated BMDM
Gene expression /18S (relative to vehicle)
8
6
4
2
0
*
**
***
Vehicle
IL-1β
IL-6
TNF-α
E
Hypoxic BMDM
IL-17A expression /18S (relative to normoxia)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
Normoxia
Vehicle
HypRGC-CM
HypRGC-MSCs-CM
rSema3E
HypRGC-MSCs-CM + anti-Sema3E

Figure 7

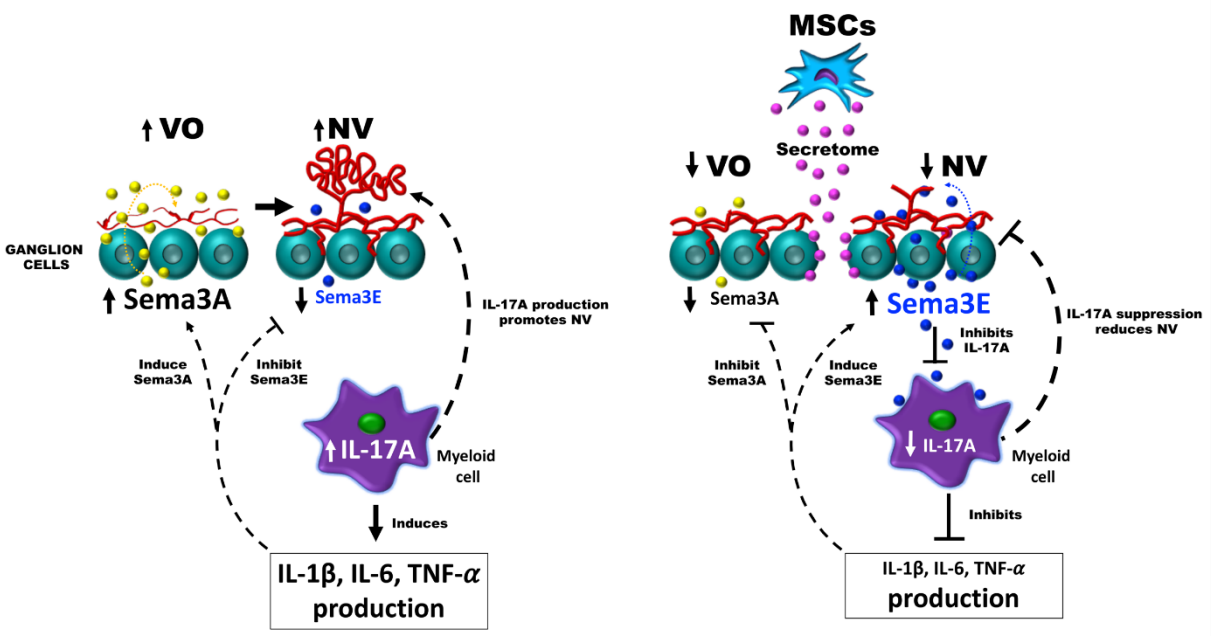

↑VO
↑NV
GANGLION CELLS
↑Sema3A
↓Sema3E
IL-17A production promotes NV
Induce Sema3A
Inhibit Sema3E
↑IL-17A
Myeloid cell
Induces
IL-1β, IL-6, TNF-α production
MSCs
Secretome
↓VO
↓NV
↓Sema3A
↑Sema3E
IL-17A suppression reduces NV
Inhibits IL-17A
Inhibit Sema3A
Induce Sema3E
↓IL-17A
Myeloid cell
Inhibits
IL-1β, IL-6, TNF-α production

Figure 8

Figure Legends

Figure 1. Intravitreal injection of MSCs induced revascularization in OIR retinas. (A) Representative photomicrographs from isolectin B4-stained retinal flatmounts from normoxic or OIR mice at P17 intravitreally treated with vehicle or MSCs. Scale bar 500μm. **(B)** Retinas intravitreally injected with MSCs demonstrate decreased vaso-obliterated (VO) areas (outlined with solid yellow) with respect to OIR vehicle at P17. Quantification of VO areas is shown in graph (***p<0.001 vs vehicle, values are mean ± SEM, n=6-10 retinas). **(C)** MSCs-treated OIR retinas further show less neovascularization (**NV**) areas (highlighted in white) compared to vehicle. Quantification of NV areas by *SWIFT_NV* are shown in graphs (***p<0.001 vs vehicle, values are mean ± SEM, n=6-10 retinas).

Figure 2. Conditioned media (CM) of hypoxic MSC (MSCs-CM) promoted vascular growth in OIR retinas. (A) Representative photomicrographs from isolectin B4-stained retinal flatmounts from normoxic or OIR mice at P17 intravitreally treated with vehicle or MSCs-CM. Scale bar 500μm. Retinas intravitreally injected with MSCs-CM demonstrate decreased VO **(B)** and NV **(C)** areas highlighted in yellow and white, respectively. Quantification of VO and NV is represented in the graphs (**p<0.01 vs vehicle, values are mean ± SEM, n=6-10 retinas).

Figure 3. MSCs-CM regulate gene expression and restored Sema3E levels in OIR retinas. (A) Real-time quantitative PCR (qPCR) analysis of whole retinas from P17 OIR mice treated with MSCs-CM show, in comparison with vehicle-treated retinas, regulated mRNA levels of growth factor (VEGF), inflammatory cytokines (IL-1β, TNF-α, IL-4, IL-10, IL-17A), and guidance cues (Sema3A, Sema3E), (**p<0.01, ***p<0.001 vs vehicle, values are mean ± SEM, n=4-5, pool of 2

retinas per n). **(B)** Real-time quantitative PCR (qPCR) analysis of whole retinas from mice pups exposed to normoxia or OIR. Sema3E levels consistently decreased following exposure to 75% O_2 in P12, P14 and P17 pups. (***=p<0.001 vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(C)** Representative images showing immunohistochemical analysis of P17 mice exposed to OIR (middle panel, vehicle) displayed absence of Sema3E (green) levels in NeuN-expressing retinal ganglion cells (red) in comparison to normoxic retina (top panel, normoxia). Treatment of OIR retinas with MSCs-CM restored neuronal Sema3E levels (bottom panel). Nuclei were counterstained with DAPI (blue). GCL=ganglion cell layer; INL=inner nuclear layer; ONL=outer nuclear layer. Scale bar 100μm.

Figure 4. Sema3E is promoted by MSCs-CM in retinal ganglion cells. (A) Sema3E levels were evaluated in lysates from RGC-5 cells subjected to MSCs-CM or vehicle for 48 hours under hypoxic conditions. No changes were observed on RGC-5 cells treated with vehicle, whereas MSCs-CM treatment upregulated mRNA levels evaluated by qPCR (**=p<0.01, *=p<0.05 vs Vehicle, values are mean ± SEM, n=4-5, pool of 2 retinas per n) and protein levels of Sema3E evaluated by Western blot (n=4, pool of 2 retinas per n). **(B)** Representative photomicrographs from isolectin B4-stained retinal flatmounts from P17 OIR mice treated intravitreally with vehicle (PBS) or MSCs-CM in absence or presence of an anti-Sema3E antibody. Scale bar 500μm. Histograms representing VO **(C)** and NV areas **(D)** that were significantly reduced in MSCs-CM respect to vehicle-treated but prevented in the presence of the anti-Sema3E (**p<0.01, ***p<0.001 vs vehicle, values are mean ± SEM, n=3-4 retinas).

Figure 5. Sema3E promoted vascular regeneration. (A) Representative photomicrographs from isolectin B4-stained retinal flatmounts from normoxic or P17 OIR mice treated intravitreally with vehicle (PBS) or recombinant Sema3E (rSema3E). Scale bar 500μm. Histograms representing VO **(B)** and NV areas **(C)** that were significantly reduced in rSema3E-treated retinas in contrast to vehicle-treated (**p<0.01 vs vehicle, values are mean ± SEM, n=4-5 retinas).

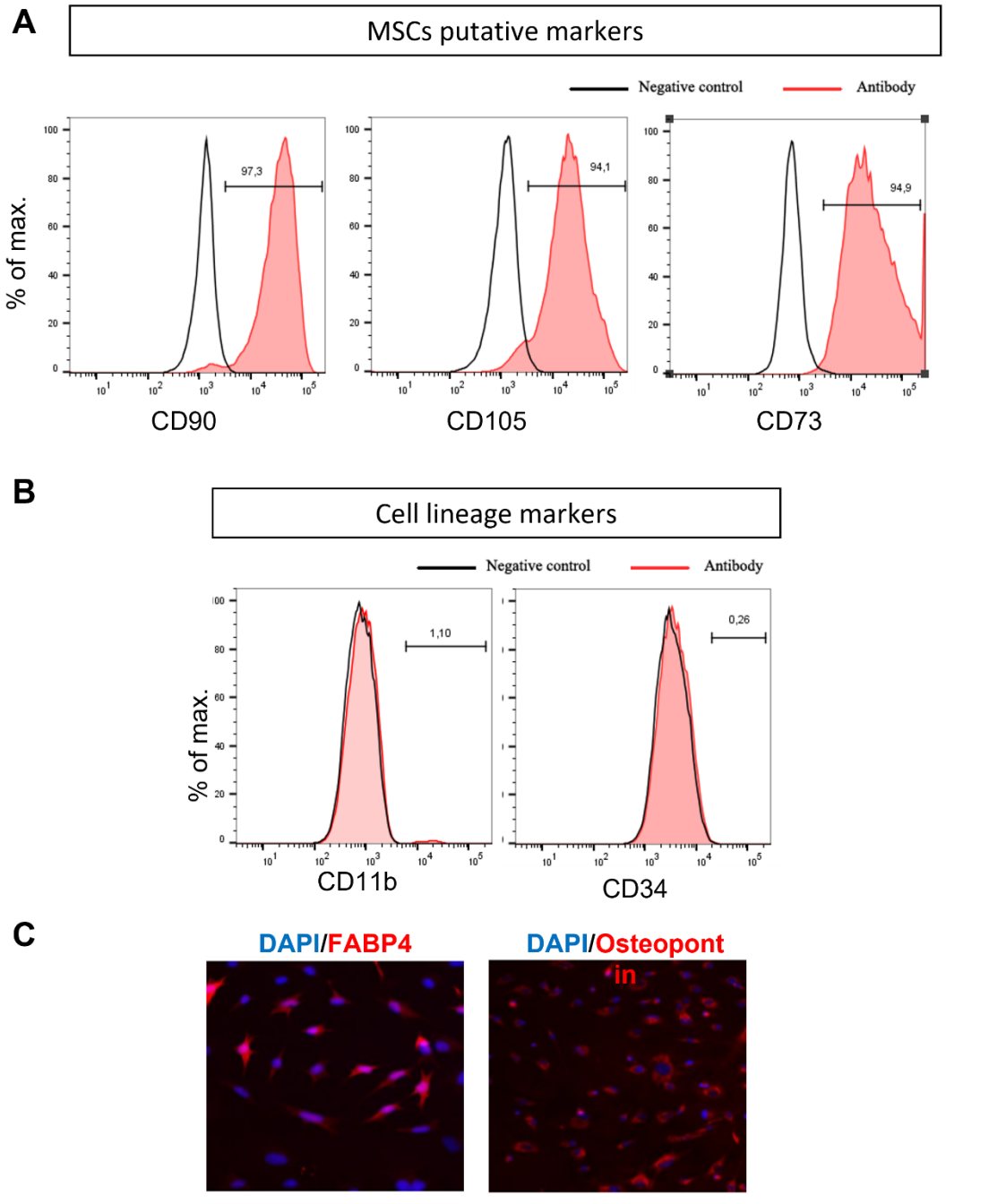
Figure 6. MSCs-CM downregulated IL-17A levels in retinal myeloid cells. (A) qPCR analysis of IL-17A in whole retina from mice pups at different time points of OIR demonstrating increased levels of IL-17A mRNA by P14 and P17. (*p<0.05, ***p<0.001 vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(B)** Representative cryosections of normoxic and OIR retinas treated with Vehicle, MSCs-CM or rSema3E demonstrating co-localization of IL-17A with myeloid cell F4/80 marker. Nuclei were counterstained with DAPI (blue). NFL, nerve fiber layer; GCL=ganglion cell layer; INL=inner nuclear layer; ONL=outer nuclear layer. Scale bar 100μm. **(C)** Real-time quantification (qPCR analysis) of P17 OIR retinas demonstrated increased expression of the nuclear receptor RORγ which regulates IL-17A transcription (***p<0.001, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(D)** Intravitreal injection of OIR retinas with MSCs-CM and rSema3E demonstrated lower levels of IL-17A at P17 versus vehicle-injected OIR retinas. (***p<0.001, n=3-4, pool of 2 retinas per n). **(E)** Intravitreal injection of MSCs-CM and rSema3E exhibited significant decreased RORγ expression in OIR retinas via qPCR analysis at P17 in comparison to vehicle-treated counterpart (**p<0.01, values are mean ± SEM, n=4-5, pool of 2 retinas per n).

Figure 7. rSema3E regulated myeloid cells-derived IL-17A in a dose-dependent manner. (A) Stimulation of hypoxic bone marrow-derived macrophages (BMDM) with rSema3E diminished IL-17A mRNA expression in a dose-dependent manner, while MSCs-CM have a partial effect (**p<0.01 vs 0 ng/ Sema3E, values are mean ± SEM, n=6). **(B)** Representative images from Western blot analysis of pro-inflammatory cytokines (IL-17A, IL-1β, IL-6, TNF-α) in hypoxic BMDM treated with MSCs-CM and rSema3E (5ng/ml) showing reduced protein levels of the cytokines with rSema3E and MSCs-CM treatments in comparison to vehicle. β-actin was used as internal control. **(C)** Densitometry quantification at right (*<0.05 vs Basal, values are mean ± SEM n = 3 independent experiments, pool of 2 wells per group). **(D)** Stimulation of hypoxic BMDM with rIL-17A (100 ng/ml) increases IL-1β, IL-6, TNF-α mRNA expression (*p<0.05, **p<0.01, ***p<0.001 vs vehicle, values are mean ± SEM, n=3). (**E**) Real-time quantitative PCR (qPCR) analysis of bone marrow-derived macrophages (BMDM) pre-exposed to hypoxia (5% O_2) showing the augmented BMDMs-derived IL-17A expression in vehicle (DMEM alone) and hypoxic RGC conditioned media (HypRGC-CM) treatments after 24 hours of incubation. BMDMs treated with the conditioned medium derived from RGCs previously stimulated with MSCs-CM (HypRGC-MSCs-CM) strongly supressed (p<0.01) the mRNA levels of IL-17A in a similar way to the supplementation with the rSema3E (5ng/ml). The anti-inflammatory effect of HypRGC-MSCs-CM was abrogated in the presence of a neutralizing antibody against Sema3E. (*p<0.05, **p<0.01, vs normoxia, values are mean ± SEM, n=3).

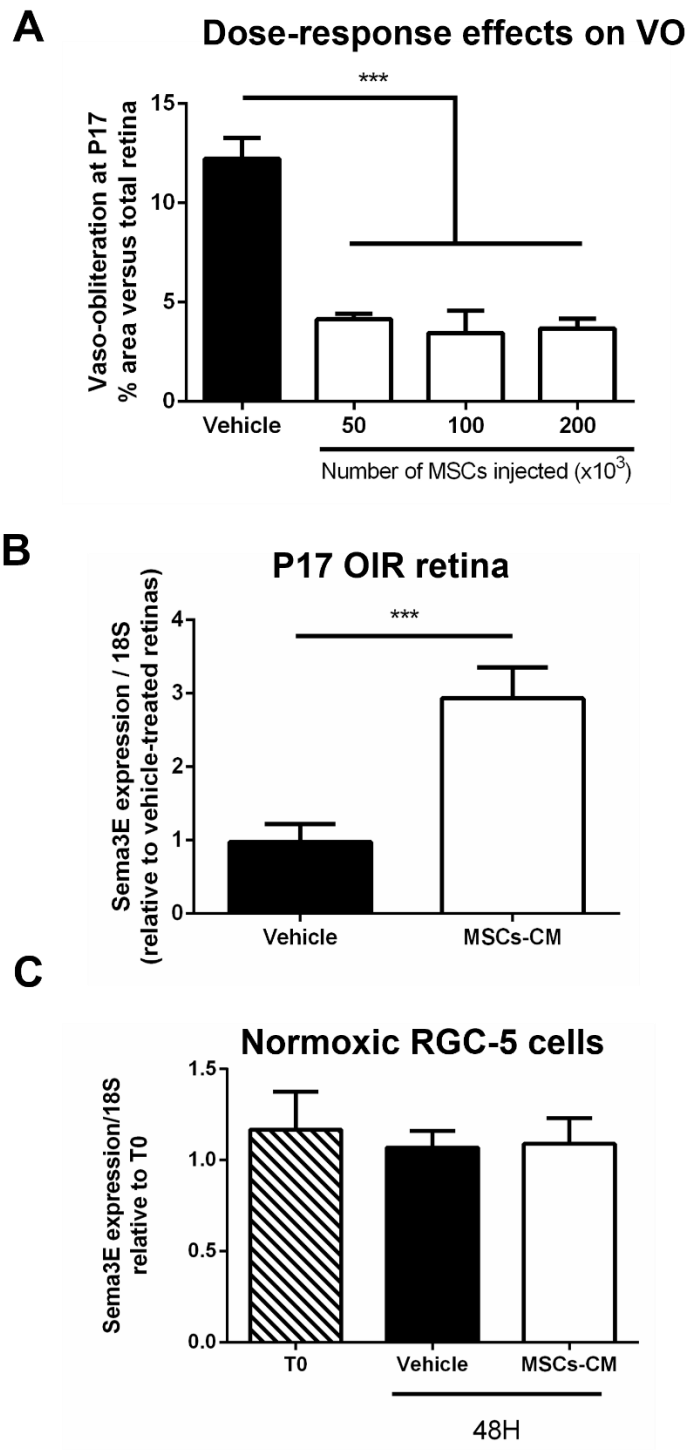
Figure 8. Diagram showing the interplay between neurons and immune cells to facilitate healthy revascularization and diminish abnormal NV in the ischemic retina. During oxygen-induced retinopathy (OIR), the increased production of IL-17A in myeloid cells allows the

development of pathological neovascularization (NV) and triggers an increase in the levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) that stimulate the expression of the anti-angiogenic factor Sema3A and conversely suppress the production of the neuronal factor Sema3E. In the presence of the MSCs secretome, RGCs restore their Sema3E levels that directly inhibit pathological neovascularization through their PlexinD1 receptor located in pathological vessels, or indirectly through supressing the production of angiogenic IL-17A on myeloid cells, which in turn results in a down-regulation of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), and of anti-angiogenic factor Sema3A.

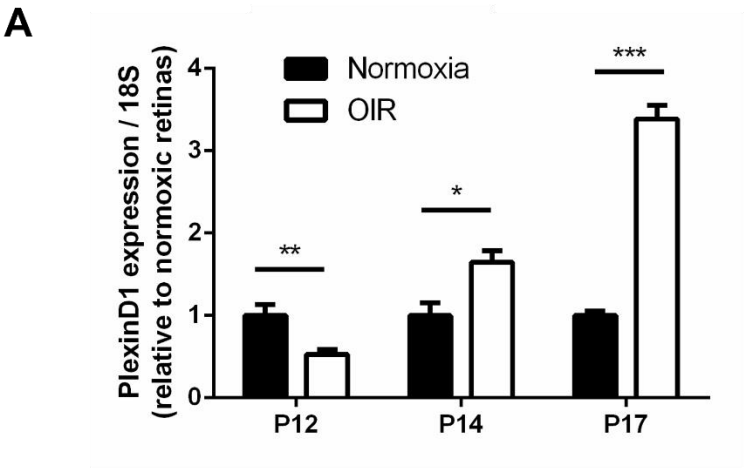
Supplemental Figures



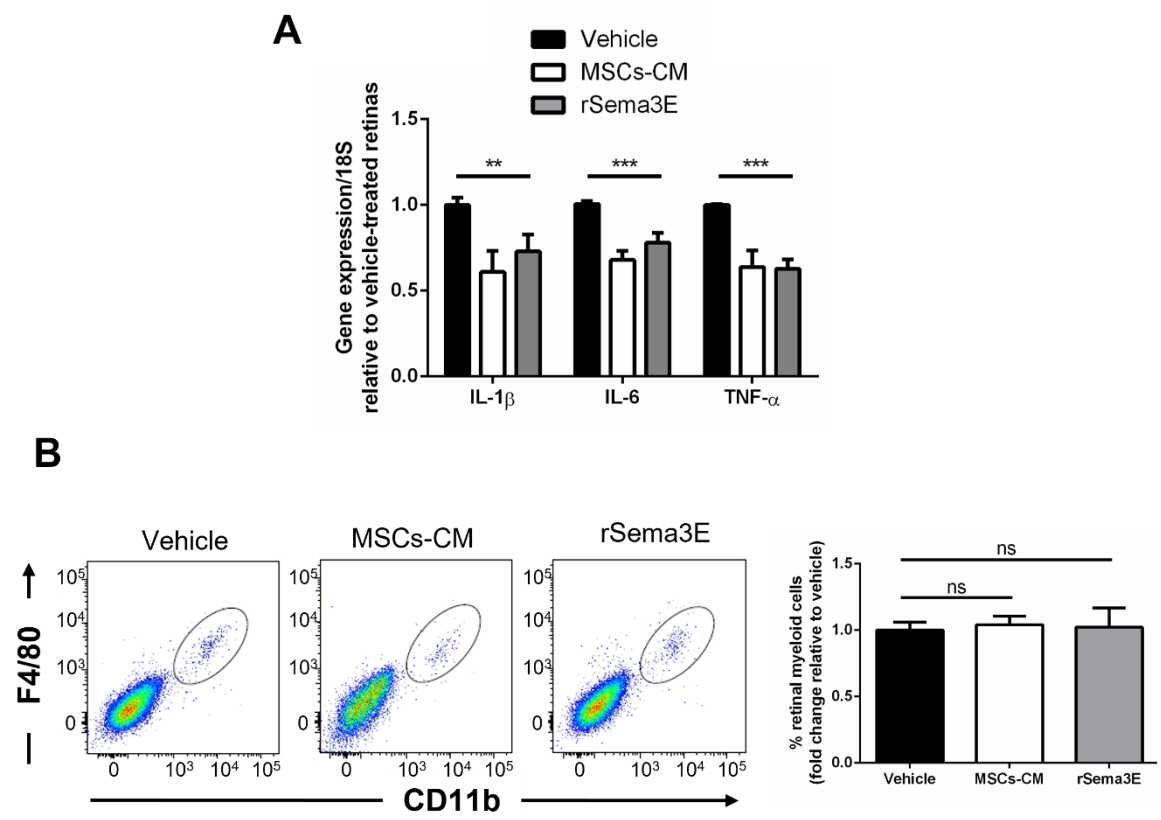
Supplementary Figure 1



Supplementary Figure 2

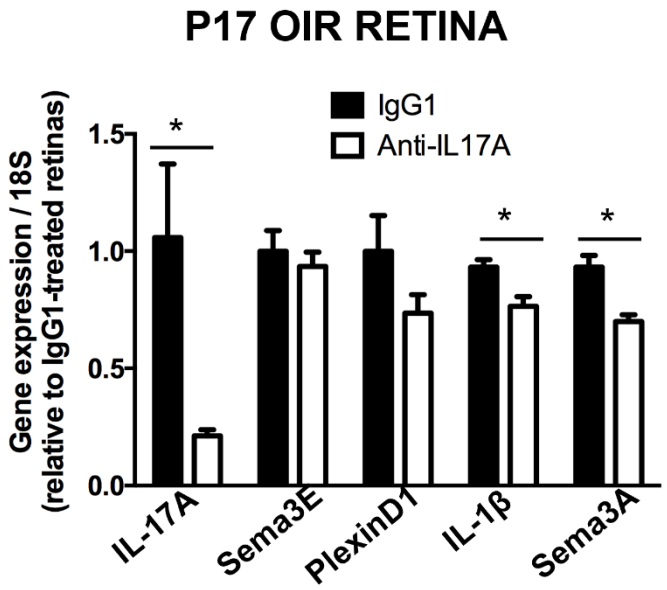


Supplementary Figure 3



Supplementary Figure 4

A



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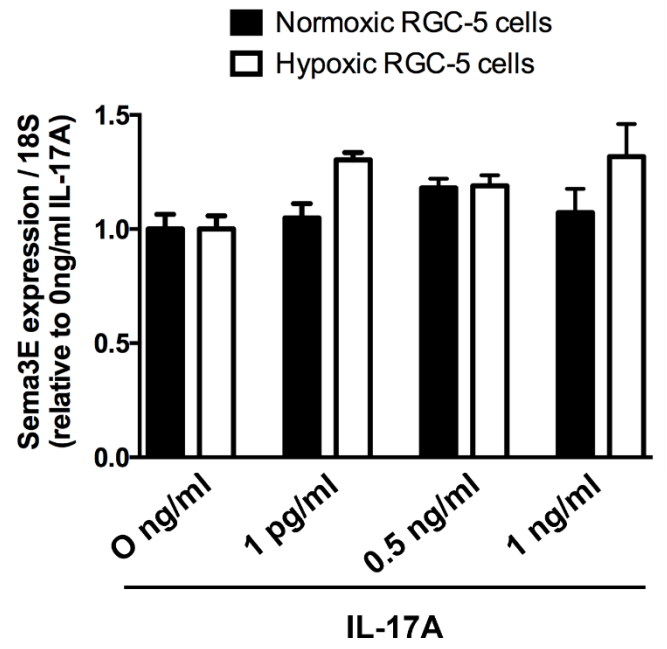

Supplementary Figure 5

Supplemental Figure Legends

Supplementary Figure 1. Characterization of MSC from mouse compact bone. (A) Representative images from flow cytometry quantification of MSCs demonstrated abundant expression of the positive markers CD90, CD105 and CD73. **(B)** Representative images showing flow cytometry quantification of MSCs exhibited negligible expression of the myeloid marker CD11b and hematopoietic stem cell marker CD34. **(C)** Representative microscopy images of differentiated MSCs into adipocytes by FABP4 staining (red, left panel) and osteocytes by oseteopontin staining (red, right panel). FABP4=Fatty Acid-Binding protein 4.

Supplementary Figure 2. Intravitreal administration of hypoxic MSCs cells reduces vasoobliteration, while MSCs-CM increases production of Sema3E in OIR retinas at P17. (A). Intravitreal administration of 50, 000 MSCs significantly reduced VO areas in OIR at P17 ($p<0.001$ vs Vehicle, values are mean ± SEM, n=4-5) to similar extent as higher cell doses (100, 000 and 200,00). **(B)** MSCs-CM-treated OIR retinas show increased Sema3E expression at P17. (***$p<0.001$ vs vehicle, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(C)** Real-time quantitative PCR (qPCR) analysis on Sema3E levels evaluated in RGC-5 cells subjected to vehicle or MSCs-CM for 48 hours under normoxic conditions. No changes on Sema3E mRNA levels were observed on RGC-5 cells treated with vehicle, or MSCs-CM (Values are mean ± SEM, n=4-5).

Supplementary Figure 3. PlexinD1 expression levels in the retina during neovascularization phase. (A) Real-time quantitative PCR (qPCR) analysis of OIR versus normoxic retinas indicated a steady increase of PlexinD1 levels, which is selectively expressed by neovascular tufts. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(B)** Representative

images from choroidal explants exposed to basal media or rIL-17A (200 ng/ml) in presence or absence of rSema3E (5ng/ml) for 24 hours. rSema3E significantly suppresses vascular sprouting induced by rIL-17A. Scale bar 200μm. The graphs represent the quantification of sprouting area (**p<0.01 vs Vehicle * p<0.05 vs rIL-17A, values are mean ± SEM, n=6-8).

Supplementary Figure 4. The production of proinflammatory cytokines rather than that the density of myeloid cell is affected by treatment with MSCs-CM or rSema3E. (A) Real-time quantification of different pro-inflammatory cytokine (IL-1β, IL-6, TNF-α) from retinal myeloid cells (CD11b+ F4/80+) sorted from vehicle, MSCs-CM, and Sema3E-treated retinas. MSCs-CM and rSema3E-injected retinas significantly downregulated the cytokines' expression (**p<0.01, ***p<0.001 vs vehicle, values are mean ± SEM, n=3-4). **(B)** Representative images from flow cytometry analysis of CD11b+ F4/80+ myeloid cells from P17 OIR retinas. Intravitreal injection of MSCs-CM or rSema3E did not change the myeloid cell density compared to vehicle treatment. Quantification at bottom. (ns=non-significant, n=6-10). **(A)** Real-time quantitative PCR (qPCR) analysis of BMDM exposed to normoxia (21% O_2) or hypoxia (5% O_2). Following 24-hour exposure to hypoxia, BMDM overexpressed the pro-inflammatory cytokine IL-17A. (**p<0.01 vs Normoxia, values are mean ± SEM, n=3).

Supplementary Figure 5. IL-17A antibody suppresses retinal expression of pro-inflammatory mediators in vivo, while IL-17A does not regulate Sema3E expression in RGC-5 cells. (A) Real-time (qPCR analysis) of P17 OIR retinas treated with Neutralizing IL-17A antibody showed decreased expression of IL-17A, IL-1β and Sema3A expression without affecting that of Sema3E and its receptor PlexinD1 (*p<0.05 vs IgG, values are mean ± SEM, n=3,

pool of 2 retinas per n). **(B)** Exposure of RGC-cells to IL-17A in a dose-dependent manner does not promote Sema3E secretion in normoxic or hypoxic conditions (values are mean ± SEM, n=3-4).

Bridging text

The following chapter explores the potential role of MSCs secretome (MSCs-CM) to modify the polarization phenotype of retinal myeloid cells, which can be classified as M1 pro-inflammatory or M2 anti-inflammatory based on the profile of secreted factors. In the previous study presented in chapter 4, we demonstrated that MSCs-CM can promote vascular regeneration in ischemic retinas. The vasoreparative effect was mediated, in part, by regulating the expression of neuronal repulsive cues and in turn, albeit indirectly, the expression of anti-inflammatory cytokines. Interestingly, the microglial cell counts in ischemic retinas were not impacted following MSCs-CM administration. Therefore, in this study, we investigated the impact of MSCs-CM on the reprogramming polarized bone-marrow derived macrophages M1 pro-inflammatory to M2 anti-inflammatory phenotype. We then determined which myeloid phenotype is dominant in ischemic retinas and whether MSCs-CM can skew their polarization state mediating vascular damage. This manuscript is in preparation.

Chapter 5. Immunomodulation of myeloid cell dynamics by Mesenchymal Stromal Cells in oxygen-induced retinopathy model

Running Title: MSCs skew microglial polarization towards M2-like immunosuppressive phenotype in ischemic retina

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Keywords: mesenchymal stem cells, vascular regeneration, ischemic retinopathies, immunomodulation, microglia, polarization, inflammation

Abstract

Inflammation plays an integral role in the pathogenesis and progression of Ischemic Retinopathies (IRs) including such as retinopathy of prematurity and diabetic retinopathy. In response to ischemia, tissue resident microglia cells secrete a wide range of pro-inflammatory cytokines which are implicated in vascular damage. Microglial activation was thus proposed as a major mechanism in mediating vascular degeneration than microglial cell counts. A new paradigm has recently emerged reporting that myeloid cells can be classified as M1 pro-inflammatory or M2 anti-inflammatory based on the profile of secreted factors. Mesenchymal Stem/Stromal cells (MSCs) are adult stem cells well known for their reparative properties, particularly immunomodulation. We have recently demonstrated that treatment of ischemic retinas with the secretome of hypoxic MSCs (MSCs-CM) promoted vascular regeneration by modulating cytokine expression without altering myeloid cell density. Hence, we investigated whether MSCs can reprogram the polarization state of myeloid cells. Using bone-marrow derived macrophages (BMDM), we first confirmed their capacity to polarize to M1 pro-inflammatory and M2 anti-inflammatory following their treatment with interferon gamma (IFNγ) and interleukin-4 (IL-4), respectively. Treatment of M1-polarized BMDM with MSCs-CM significantly reduced the expression of M1 markers iNOS, IL-β, TNF-α and IL-6 and concomitantly upregulated M2 markers arginase-1 (Arg-1), CD206 and IL-10. Moreover, MSCs-CM treatment of M2-polarized BMDM precipitated M2 phenotype and promoted expression of M2 markers. Using oxygen-induced retinopathy (OIR) mouse model, we demonstrated that M1 myeloid cells outnumber M2 phenotype in ischemic retinas. Intravitreal injection of OIR retinas reduced expression of M1 markers and upregulated Arg-1 and IL10 as M2 markers. To determine whether MSCs-CM skewed the polarization state of retinal myeloid cells, the ratio of iNOS to Arg-1 was assessed based on their competitive activity to metabolize the same

substrate L-arginine. MSCs-CM significantly curtailed iNOS/Arg-1 ratio in treated OIR retinas. Collectively, our results demonstrated that MSCs-CM modulates the pro-inflammatory microenvironment of ischemic retinas by shifting the polarization state of retinal myeloid cells to anti-inflammatory phenotype, which in turn promotes vascular regeneration.

In preparation for submission

Introduction

Ischemic Retinopathies, namely Retinopathy of Prematurity and Diabetic Retinopathy, are ocular vascular diseases characterized by an initial microvascular degeneration followed by aberrant intravitreal neovascularization. Inflammation has been shown to contribute considerably to the development of IRs in humans and animal models, mediated primarily by microglia[149, 228, 690]. These resident immune cells are essential for homeostasis and immune surveillance[22, 691]; however, under ischemic conditions, they become overactivated and produce a myriad of pro-inflammatory factors which propagate a hostile inflammatory milieu leading to vascular damage. Consistently, high concentrations of IL-1β, TNF-α and IL6 have been detected in patients[224, 665] and animal models of ocular vasculopathies[628, 692, 693].

It is well established that myeloid cells exhibit functional plasticity as they sense and actively respond to changes in their microenvironment[694]. Recently, a new paradigm emerged reporting that macrophages, and similarly microglia, exist on a spectrum of two polarization states M1 and M2[23, 695]. Classically activated M1 are considered pro-inflammatory, are involved in escalating inflammatory mechanisms and possess high inducible nitric oxide synthase (iNOS) activity; whereas M2 are anti-inflammatory, are associated with debris scavenging and wound healing, and have high activity of arginase-1 (Arg-1), which competes for the same substrate L-arginine as iNOS[696-698]. Both M1 and M2 phenotypes have been documented in ischemic retinas, albeit with conflicting data on their role in vascular damage. Some studies reported that M1 phenotype dominate M2 and contribute to pathological angiogenesis[699, 700], while others noted the involvement of M2 macrophage in promoting neovascularization[701, 702].

Mesenchymal Stem/Stromal cells (MSCs) have gained significant interest in the field of regenerative medicine due to their immunoregulatory properties. These multipotent stromal cells

exert their immunosuppressive effect on both innate and adaptive immune responses via multiple mechanisms involving direct cell-to-cell interaction or release of soluble factors[703]. In particular, it has been demonstrated that MSCs regulate the activity of macrophages and microglial cells by suppressing M1 pro-inflammatory cytokine profile and upregulating M2-specific markers, hence favoring M2 polarization[704-707].

We previously demonstrated that injection of MSCs secretome promoted vascular regeneration in ischemic retinas, partly by decreasing pro-inflammatory cytokine expression in retinal myeloid cells[708]. Interestingly, MSCs did not alter myeloid cell density in treated retinas. In the present study, we investigated the immunomodulatory effect of MSCs on retinal myeloid cells of oxygen-induced retinopathy (OIR) mouse model. Our findings indicate that MSCs secretome promoted M2-like phenotype by downregulating production of M1-associated cytokines and increasing expression of M2 markers. Moreover, we found that in OIR retinas M1 outnumber M2, while treatment with MSCs secretome shifted M1/M2 toward the immunosuppressive M2 phenotype. These results suggest that MSCs secretome resolved the pro-inflammatory microenvironment within ischemic retina by preferentially promoting M2 polarization and, hence enabling vascular regeneration.

Materials and Methods

5.1.1.1 Animal Care:

Adult C57BL6/J mice were purchased from Jackson Laboratories and adoptive lactating CD-1 females were purchased from Charles River to tend to C57BL6/J pups. All experiments were performed with strict adherence to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and were approved by

the Animal Care Committee of Maisonneuve-Rosemont Hospital in accordance with guidelines established by the Canadian Council on Animal Care.

5.1.1.2 Isolation mesenchymal stem/stromal cells (MSCs) from compact bone

MSCs were isolated from the compact bone of mice as previously outlined[644]. Long bones of adult C57BL6/J mice (6-8 weeks) were removed and thoroughly cleaned from any connective tissue. Using a 23G needle, the bone marrow was flushed out with PBS and saved for subsequent isolation of bone-marrow-derived macrophages (BMDM). Bones were gently crushed and digested with (2.5mg/ml) collagenase (Sigma) and 5% TrypLE Express (Gibco) for 1 hour at 37ºC. Bone fragments were then cultivated in αMEM (Gibco) supplemented with 20% Mesenchymal-tested FBS (Wisent) and 1% penicillin/streptomycin (Corning) at hypoxia (5%O_2) allowing the migration of MSCs out of the compact bone. MSCs were enriched at P2 by negative selection using EasySep Mouse Mesenchymal Progenitor Enrichment Kit (Stem Cell Technologies) to deplete non-mesenchymal lineages. At 80% confluency, the media was changed to basal αMEM. The supernatant, referred herein as conditioned media (MSCs-CM), was collected 24 hours later, centrifuged, filtered through 0.22μm filter (Millipore), and concentrated 10 times using a 10K molecular weight cut off centrifugal filter (Millipore). MSCs-CM was collected between passages 3 and 5.

5.1.1.3 Oxygen-Induced Retinopathy (OIR) mouse model

Mouse model of OIR is a well-established and standardized model reproducing the vascular abnormalities observed in ROP[103, 244]. Postnatal day 7 (P7) C57BL6/J pups were placed with an adoptive lactating CD-1 female in a hyperoxic environment set to 75% O_2 (OxyCycler A820CV;

BioSpherix, Ltd., Redfield, NY, USA) until P12 to trigger VO. Then, the animals were returned to room air whereby hypoxia-driven neovascularization develops at P14 and reaches its peak at P17. Pups at P17 were anesthetized in 3% isoflurane with oxygen and sacrificed by decapitation for gene expression and flow cytometry analysis.

5.1.1.4 Macrophage Isolation and In Vitro Polarization

Bone marrow was collected from tibia and femur of adult C57BL6/J mice (6-8 weeks) and gently dissociated with 23G needle. The cell suspension was filtered through 70μm strainer and centrifuge at 4^{o}C, 500xg for 10 minutes. Collected cells were cultured in DMEM containing 10% FBS and 20 ng/ml monocyte colony stimulating factor (M-CSF; PeproTech) for 7 days to differentiate monocytes into macrophages. Naïve BMDM (M0) were then stimulated for 24 hours with Interferon gamma (IFNγ; PeproTech) (20 ng/mL) for M1 differentiation or with interleukin-4 (IL-4; PeproTech) (20 ng/mL) for M2 differentiation[709, 710]. Polarized cells were then collected for validation using flow cytometry and RNA isolation (Qiagen). M1 BMDM expressed significantly iNOS, IL-1β, TNF-α, and IL-6 but not M2 markers (CD206 and Arg-1) **(Suppl. Fig. 1A and C).** M2-polarized BMDM expressed high levels of Arg-1, CD206, and IL-10 and not M1-specific pro-inflammatory cytokines IL-1β and TNF-α **(Suppl. Fig. 1B and C).** Polarized BMDM were treated with basal media (unsupplemented αMEM) or MSCs-CM for 24 hours. Conditioned media from treated-M1 and treated-M2 were collected 24 hours post-treatment for choroid angiogenesis assay.

Choroidal Sprouting Assay

Choroidal explants were prepared from adult C57BL6/J mice as previously described[647]. Briefly, the choroid/RPE complex was cut into approximately 1x1 mm sections and embedded in growth-factor reduced Matrigel in 24-well plates. The choroidal explants are cultured in EBM-2 medium (Lonza, Cat. CC-3156) supplemented with endothelium growth medium (EGM) kit (Lonza, Cat. CC-4147) at 37ºC in 5% CO_2 for 3 days. On day 4, the culture media was replaced with M1-CM, M1-MSCs-CM, M2-CM, or M2-MSCs-CM for 24 hours. Phase-contrast photos of individual explants were taken with ZEISS AxioOberver microscope before and 24-hours after treatment. The microvascular sprouting area was measured using Image J software version 1.50i (National Institutes of Health, USA).

5.1.1.5 Reverse-transcription PCR and quantitative real-time PCR

Retinas (2 retinas pooled per n) were rapidly dissected from enucleated eyes and placed immediately in RiboZol (Amresco) and homogenized using Precellys 24 homogenizer. Cultured cells were scrapped using RiboZol (Amresco). RNA was extracted following manufacturer's instructions and then treated with DNase I (Sigma) to remove genomic DNA. 1μg of RNA was reverse transcribed into cDNA using iScript RT Supermix (Biorad) as described by manufacturer's instructions. cDNA was analyzed by Quantitative real-time PCR using iTaq™ Universal SYBR® Green Supermix (BioRad) with primers targeting mouse IL-1β (Fwd 5'-CTGGTACATCAGCACCTCACA-3' and Rev 5'-GAGCTCCTTAACATGCCCTG-3'), mouse IL6 (Fwd 5'-ACAGAAGGAGTGGCTAAGGA-3' and Rev 5'-AGGCATAACGCACTAGGTTT-3'), mouse TNF-α (Fwd 5'-GCCTCTTCTCATTCCTGCTTG-3' and Rev 5'-CTGATGAGAGGGAGGCCATT-3'), mouse iNOS (Fwd

5’CTCCTCACGCTTGGGTCTTG-3’ and Rev 5’-CCAACGTTCTCCGTTCTCTTG-3’), mouse Arg1 (Fwd 5’CTGACATCAACACTCCCCTG-3’ and Rev 5’GGTACATCTGGGAACTTTCCTT-3’), mouse CD206 (Fwd 5’-TCTGTTCAGCTATTGGACGC-3’ and Rev 5’-GTTGCCGTCTGAACTGAGAT-3’), mouse IL-10 (Fwd 5’-GCTCTTACTGACTGGCATGA-3’ and Rev 5’-AGAAAGTCTTCACCTGGCTG-3’). Primers were designed by NCBI Primer-BLAST software and synthesized by Alpha DNA (Montreal). Quantitative gene expression analysis was evaluated using ABI 7500 Real-Time PCR system (Applied Biosystems) and normalized to QuantumRNA^{TM} 18S universal primer (Invitrogen) using ΔΔCT method.

5.1.1.6 Flow cytometry

Freshly dissected retinas from treated-OIR pups were pooled (2 retinas per treatment per n) and digested in HBSS solution containing 750U/ml DNase I (Sigma-Aldrich) and 1mg/ml collagenase D (Roche). Enzyme activity was quenched by resuspending digested tissue with fluorescent activated cell sorting (FACS) buffer (PBS with 3% FBS). Intracellular staining was done using Cytofix/Cytoperm^{TM} plus fixation/permeabilization kit with BD GolgiPlug^{TM} protein transport inhibitor containing brefeldin A (BD Biosciences). Cell suspensions are first incubated with BD GolgiPlug^{TM} to inhibit protein transport and then permeabilized with BD permeabilization/wash solution provided with the kit. To block Fc receptors, cell suspensions were incubated with LEAF purified anti-mouse CD16/32 (Biolegend) for 10 minutes at 4^{o}C, followed by 30 minutes incubation of the following antibodies for intracellular staining: Pacific Blue anti-mouse TNF-α (BioLegend), BV605 anti-mouse CD206 (BioLegend), PE anti-mouse Arg-1 (RnD Systems), PE-eFluor 610 anti-mouse iNOS (eBioscience), and Alexa Fluor700 anti-mouse CD86

(BioLegend). Cells are then washed and incubated with an antibody solution containing FITC anti-mouse CD11b (eBioscience) and APC anti-mouse F4/80 (BioLegend) for 30 minutes at 4^{o}C to stain cell surface markers. Cells are finally fixed with permeabilization/fixation solution provided by the manufacturer and evaluated on BD LSRFortessa X-20. Dead cells were excluded using Zombie Aqua^{TM} Fixable viability kit (BioLegend). Data was analyzed using FlowJo software.

5.1.1.7 Statistical analysis

All experiments were performed in triplicate and repeated independently at least three times. Results are presented as mean ± S.E.M. for all studies. One-way or two-way analysis of variance with significance α=0.05 or higher were used for processing data. Bonferroni post-hoc analysis was used for calculating significance between groups. Two-tailed student t-tests were used to test for significance between two means. Statistical significance was set on the basis of *p* value (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

5.1.1.8 MSCs-CM suppressed M1 markers in M1-polarized BMDM and shifted their polarization state to M2-like phenotype.

Undifferentiated bone-marrow derived macrophages (BMDM) were exposed to IFN-γ to promote their polarization to M1 phenotype. As expected[711], mRNA expression levels of M1 markers, particularly iNOS, IL-β, TNF-α, and IL-6 were found to be significantly elevated in polarized BMDM (M1) compared to naïve BMDM (M0) (**Suppl. Fig. 1A**). Flow cytometry analysis revealed positive staining of iNOS and TNF-α in M1 but not M0 (**Suppl. Fig. 1C**). Staining of M2 markers CD206 and Arg-1 was not detected in either M0 or M1 BMDM (**Suppl. Fig. 1C**). To determine the effect of MSCs on macrophage polarization, M1-polarized BMDM

were subjected to MSCs-CM which significantly reduced expression levels of iNOS, IL-β, TNF-α, and IL-6 compared to vehicle-treated M1 (**Fig. 1A**). Interestingly, M1-exposure to MSCs-CM upregulated M2 markers Arg-1, CD206 and IL-10 (**Fig. 1B**), suggesting that MSCs-CMs reprogrammed the polarization state of M1 to M2 anti-inflammatory profile. This result was corroborated via flow cytometry showing positive expression of CD206 and Arg-1, while iNOS and TNF-α only stained vehicle-treated BMDM (**Fig. 1C**). Because M2 macrophages are known for their angiogenic properties[712], choroidal explants exposed to the supernatant of MSCs-CM-treated M1 BMDM demonstrated a significantly larger sprouting area than vehicle-treated M1 macrophages (**Fig. 1D**), therefore confirming that MSCs-CM shifted M1 phenotype to M2.

5.1.1.9 MSCs-CM precipitates M2 phenotype

Exposure of undifferentiated BMDM to IL-5 for 24 hours induced their polarization to M2 phenotype, confirmed by the upregulation specific M2 markers Arg-1, CD206 and IL-10 (**Suppl. Fig. 1B**) in line with literature[711]. Moreover, M2 BMDM demonstrated positive staining of CD206 and Arg-1 via flow cytometry, without expressing M1 markers iNOS and TNF-α (**Suppl. Fig. 1C**). Treatment of M2-polarized macrophages with MSCs-CM potentiated the expression of M2 markers (**Fig. 2A-B**) but had no effect of iNOS and TNF-α expression (**Fig. 2B**). Moreover, the supernatant of MSCs-CM-treated M2 significantly promoted vascular sprouting then vehicle-treated M2 BMDM, thus indicating that MSCs precipitate M2 profile.

5.1.1.10 MSCs-CM reprogrammed retinal myeloid cells from M1 to M2 phenotype

To understand the myeloid profile in retina, genetic analysis revealed that P17 OIR retinas expressed high levels of pro-inflammatory cytokines compared to normoxic retinas (**Fig. 3A and**

Suppl. Fig 2A). Arg-1 and IL-10 expressions were low in P17 OIR retinas (**Fig. 3A and Suppl. Fig 2B**), whereas CD206 was significantly elevated in OIR retinas (**Fig. 3A**). Treatment of OIR retinas with MSCs-CM diminished expression levels of pro-inflammatory mediators, particularly iNOS (**Fig 3B and 3E**), and augmented expressions of Arg-1 and IL-10 (**Fig 3C and 3E**).

Interestingly, CD206 expression significantly elevated in OIR retinas (**Fig. 3A**) and was found to increase during neovascularization phase (**Suppl. Fig 2C**) when neovascular tuft formation peaks in ischemic retinas. This observation could be explained by the expression of CD206 found on endothelial cells[713]. Accordingly, intravitreal injection of MSCs-CM into OIR retinas significantly reduced CD206 levels (**Fig 3C and 3E**).

To determine the predominant macrophage phenotype in treated OIR retinas, the ratio of iNOS to Arg-1 was quantified based on their competitive activity to metabolize L-arginine[696-698]. Consistent with other studies[699, 700], M1 polarized macrophages were more abundant than M2 in vehicle-treated retinas, evident by a high iNOS/ARG-1 ratio, than in MSCs-CM treated OIR retinas, thereby suggesting that MSCs-CMs skewed M1 pro-inflammatory phenotype towards M2.

Discussion

Inflammation has been associated with the development and progression of IRs. Microglia are reported to partake in ocular pathological angiogenesis by mounting an inflammatory response to ensuing hypoxia. As a result, microglia become overactivated and act as a major source of pro-inflammatory mediators which further contribute to vascular abnormalities. IL-1β, TNF-α, and IL-6 have been shown to be rapidly released by microglia secondary to hypoxia and facilitate neurovascular injury in ischemic retinas[628, 714, 715]. Blocking the effects of these cytokines reduce hypoxia-induced neurovascular injury and mediate revascularization. Some studies have

highlighted the increased counts of myeloid cell population in ischemic retinas as evidence of hypoxia-driven inflammation[716, 717]. However, microglial activation rather than its density is implicated with vascular degeneration and is considered a key modulator of disease progression[228, 628, 690]. As such, inhibition of microglial activation reduced cytokine release and attenuated progression of IRs[149]. Consistently, we previously demonstrated that administration of MSCs secretome mediated vascular repair in part by downregulating expression of pro-inflammatory cytokines without altering myeloid cell density[708]. In this study, we investigated whether MSCs directly modulate microglial activity in ischemic retinas.

Microglia are sentinel cells of the retina and exhibit profound functional plasticity relative to changes in their microenvironment. Recent studies suggest that myeloid cells display two main activation status known as classically activated M1 and alternatively activated M2[23]. Both M1 and M2-like phenotypes were detected in OIR retinas though their roles in pathological neovascularization remain controversial[699, 700 701, 702]. In this study, we showed that OIR retinas have M1 macrophages predominantly, while intravitreal treatment with MSCs-CM skewed M1 polarization state to M2. Furthermore, MSCs secretome diminished expression of M1 markers in M1-polarized BMDM while concomitantly upregulating M2 markers, suggesting that MSCs prompted change in the activation status of macrophages towards an anti-inflammatory phenotype.

iNOS partakes in retina vascular damage. Blocking iNOS expression in ischemic retinas was shown to inhibit pathological neovascularization and improve retinal vascularization[692, 718]. In addition, OIR retinas from TNF-α-deficient mice exhibited reduction in iNOS activity and an increase in arginase activity[719]. These results confirm the role of macrophage polarization in ischemic retinopathies. Herein, we show that MSCs-CMs can modulate cytokine expression of macrophages without affecting their cell density. MSCs-CM-mediated suppression of pro-

inflammatory cytokines concomitant with elevated expression of IL-10 and Arg-1 reprogrammed macrophage polarization to M2 phenotype and promoted vascular regeneration in ischemic retinas.

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Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interest.

Authorship contributions

B.N., S.C, J.C.R. and S.O conceived and designed the study; B.N. and J.C.R., performed the experiments; B.N. prepared the figures and drafted the manuscript.

Figures

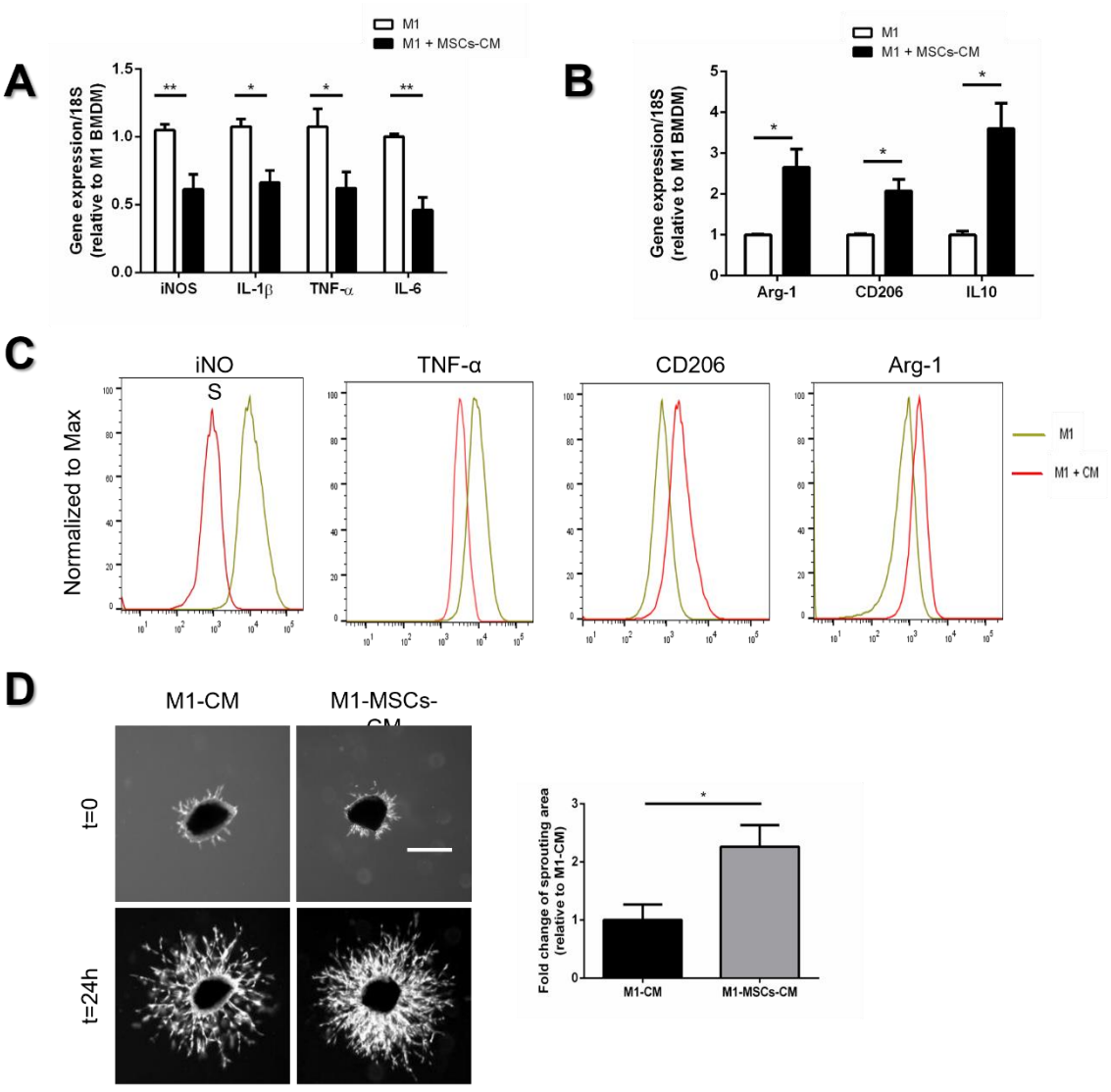


Figure 1

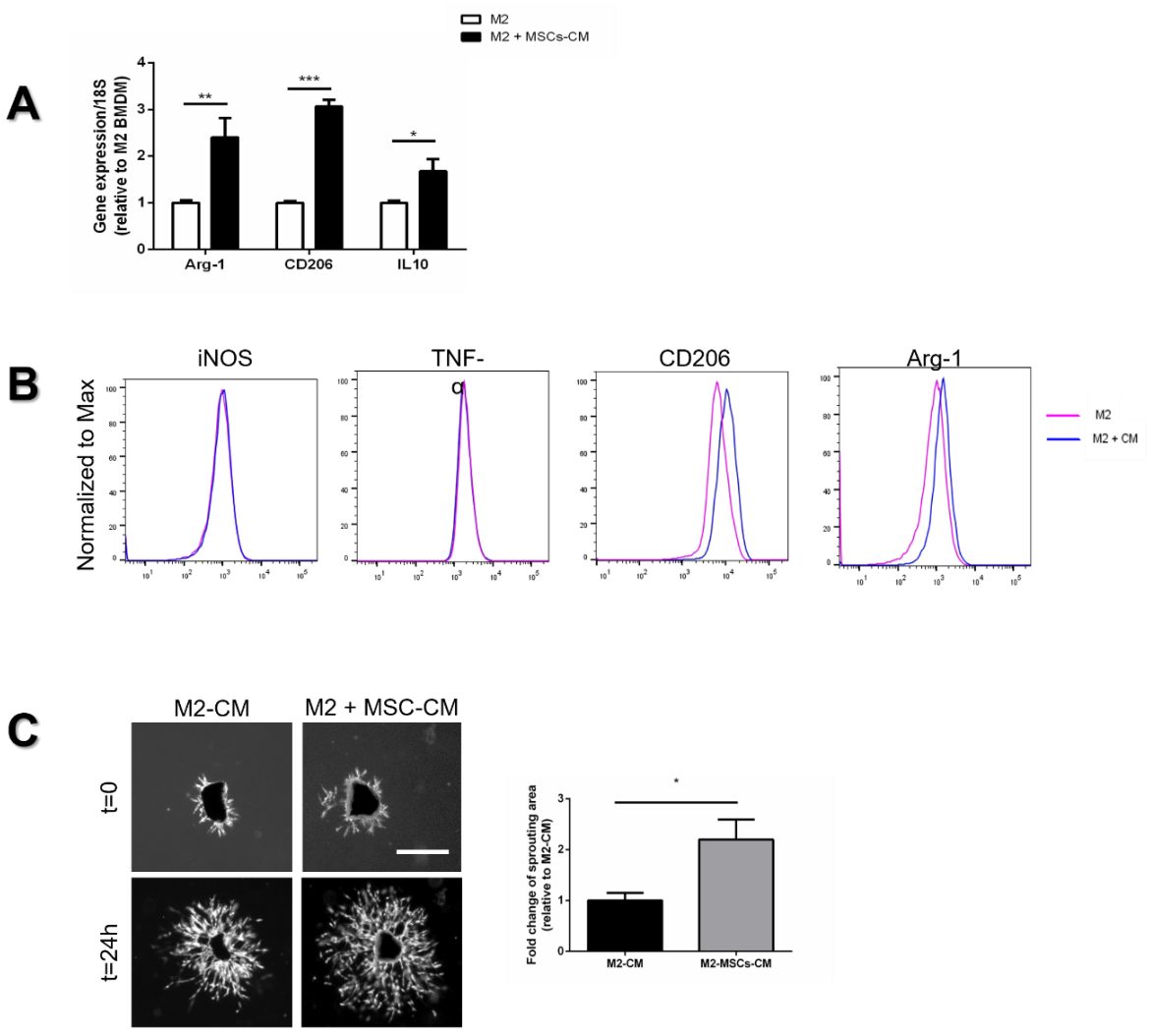

A
M2
M2 + MSCs-CM
Gene expression/18S (relative to M2 BMDM)
Arg-1
CD206
IL10
B
iNOS
TNF-α
CD206
Arg-1
Normalized to Max
M2
M2 + CM
C
M2-CM
M2 + MSC-CM
t=0
t=24h
Fold change of sprouting area (relative to M2-CM)
M2-CM
M2-MSCs-CM

Figure 2

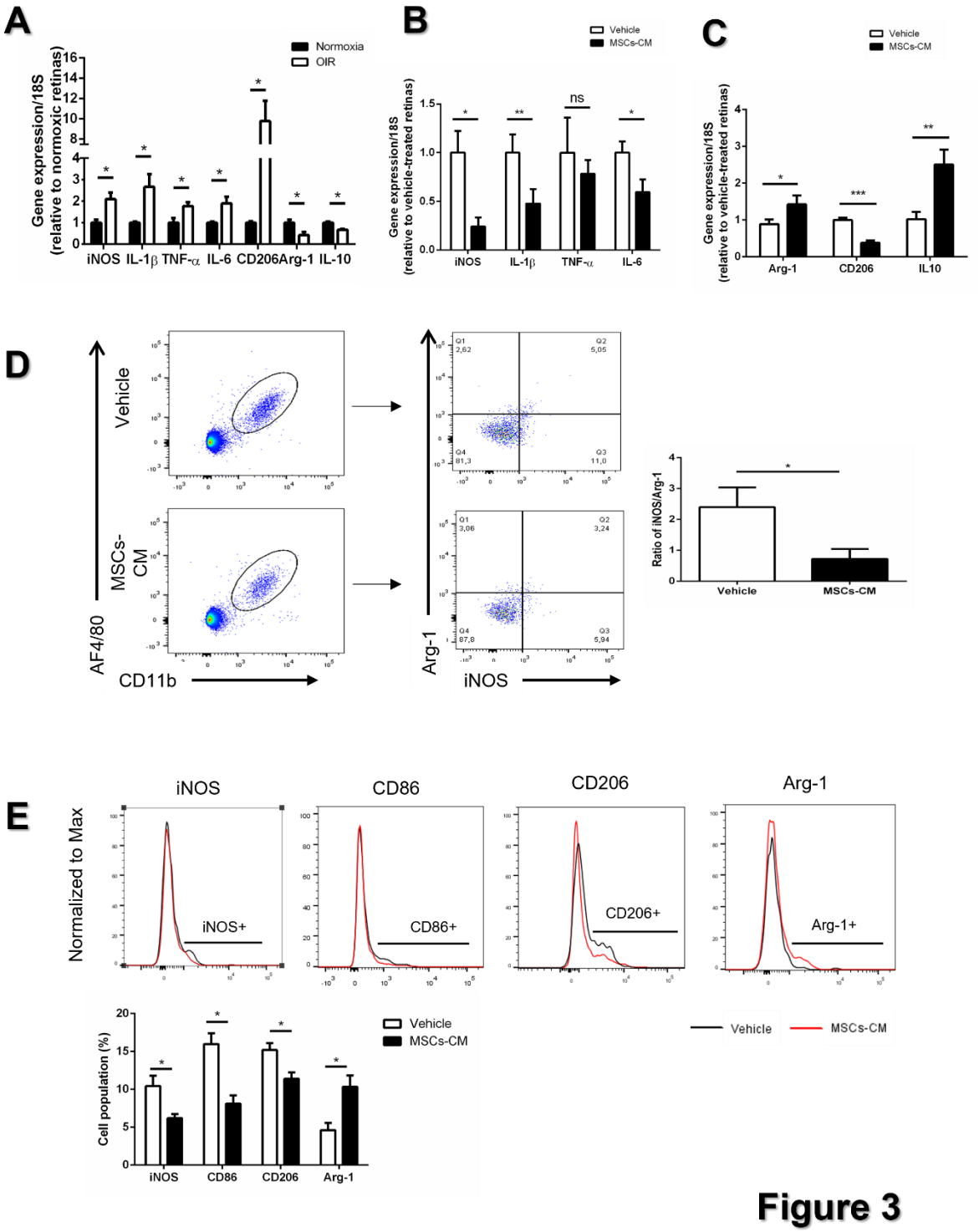

A
Normoxia
OIR
Gene expression/18S (relative to normoxic retinas)
iNOS IL-1β TNF-α IL-6 CD206 Arg-1 IL-10
B
Vehicle
MSCs-CM
Gene expression/18S (relative to vehicle-treated retinas)
ns
iNOS IL-1β TNF-α IL-6
C
Vehicle
MSCs-CM
Gene expression/18S (relative to vehicle-treated retinas)
Arg-1 CD206 IL10
D
Vehicle
MSCs-CM
AF4/80
CD11b
Q1 2,62
Q2 5,05
Q4 81,3
Q3 11,0
Q1 3,06
Q2 3,24
Q4 87,8
Q3 5,94
Arg-1
iNOS
Ratio of iNOS/Arg-1
Vehicle
MSCs-CM
E
iNOS
CD86
CD206
Arg-1
Normalized to Max
iNOS+
CD86+
CD206+
Arg-1+
Vehicle
MSCs-CM
Cell population (%)
iNOS CD86 CD206 Arg-1

Figure 3

Figure Legends

Figure 1. MSCs-CM suppressed M1 phenotype and promoted their shift toward M2-like phenotype. (A) Real-time quantitative PCR (qPCR) analysis of M1-polarized BMDM treated with MSCs-CM showed significant reduction of expression of iNOS, IL-1β, TNF-α, and IL-6.) compared to M1 exposed to basal media. (**p<0.01, *p<0.05 vs M1 BMDM, values are mean ± SEM, n=4-6). **(B)** qPCR analysis of MSCs-CM-treated M1 further demonstrated upregulation of M2-specific markers (Arg-1, CD026, ad IL-10) (**p<0.01, *p<0.05 vs M1 BMDM, values are mean ± SEM, n=4-8). **(C)** Representative flow cytometry histograms of iNOS, TNF-α, CD206 and Arg-1 intracellular expression in treated M1-differentiated BMDM. Positive staining for cytokines iNOS and TNF-α was detected in M1 but not in MSCs-CM-treated M1 cells, whereas positive staining for Arg-1 and DC206 was detected specifically in MSCs-CM-treated M1. **(D)** Representative images from choroidal explants exposed to conditioned media derived from M1 (M1-CM) or from M1 previously stimulated with MSCs-CM (M1-MSCs-CM). M1-MSCs-CM promoted vascular growth compared to M1-CM. The graphs represent the quantification of sprouting area (* p<0.05, values are mean ± SEM, n=3-5). Scale bar 200μm.

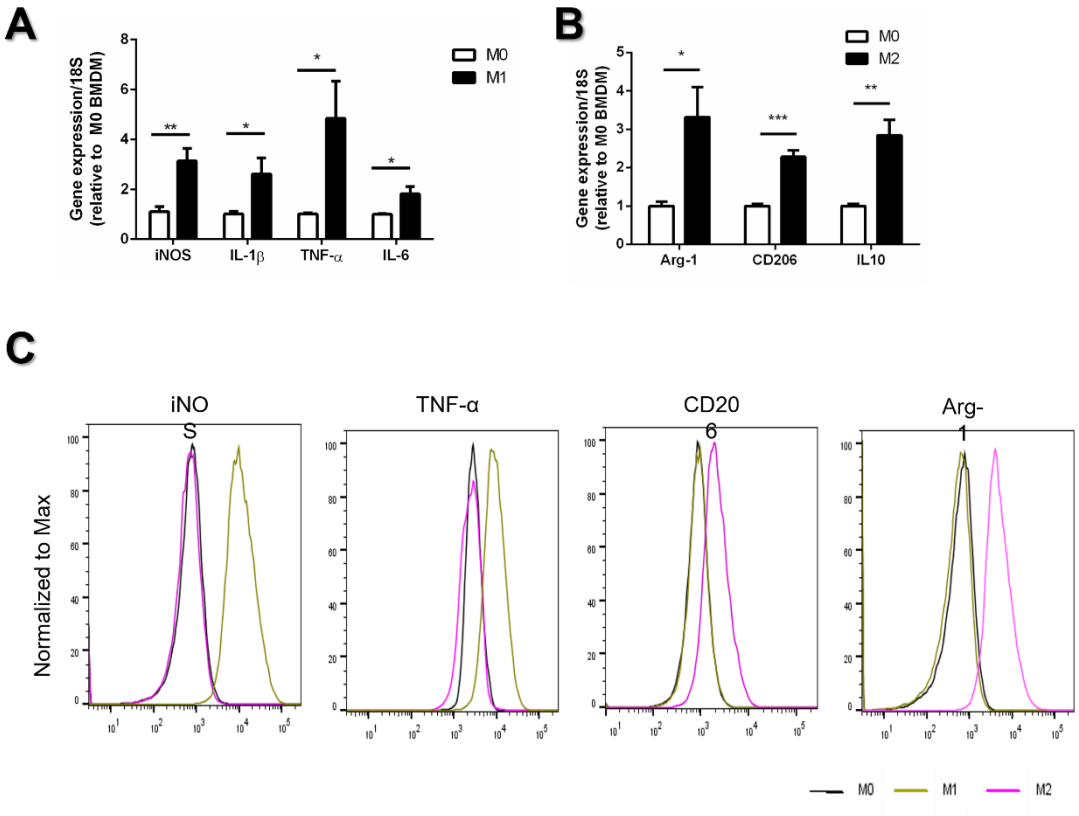
Figure 2. MSCs-CM sustained M2 phenotype. (A) qPCR analysis of M2-polarized BMDM treated with MSCs-CM showed upregulation of M2 markers Arg1, CD206 and anti-inflammatory cytokine IL-10. (*p<0.05, **p<0.01, ***p<0.001vs M2 BMDM, values are mean ± SEM, n=4-8). **(B)** Representative flow cytometry histograms indicating negative staining for iNOS and TNF-α, and positive staining for Arg-1 and CD206 in M2 cells regardless of exposure to basal media or MSCs-CM. **(C)** Representative images from choroidal explants exposed to conditioned media derived from M2 (M2-CM) or from M2 previously stimulated with MSCs-CM (M2-MSCs-

CM) for 24 hours. Exposure of explants to M2-MSCs-CM promoted significant vascular growth compared to M2-CM. The graphs represent the quantification of sprouting area (* $p<0.05$, values are mean ± SEM, n=3-5). Scale bar 200μm.

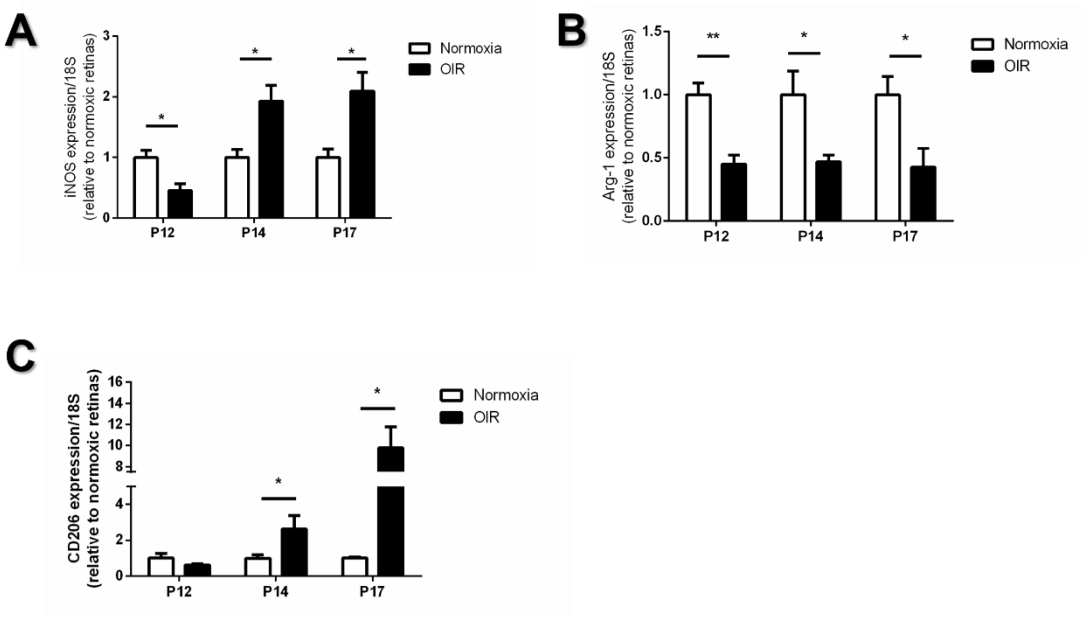
Figure 3. MSCs-CM shifted polarization of retinal myeloid cells toward M2 phenotype. **(A)** qPCR analysis of whole retinas from P17 OIR versus P17 normoxic retinas. OIR retinas exhibited significant high levels of M1-related markers (iNOS, IL-1β, TNF-α, IL-6) and low expression of M2-specific markers (Arg-1 and IL-10) compared normoxic retinas. Expression of CD206, a M2 marker associated with neovascular tufts, was markedly upregulated in P17 OIR retinas. (*$p<0.05$ vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(B)** qPCR analysis of P17 OIR retinas treated with MSCs-CM showed reduction in mRNA expression of iNOS, IL-1β, TNF-α and IL-6 compared to vehicle-treated OIR retinas. (*$p<0.05$, **$p<0.01$ s vehicle, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(C)** qPCR analysis of P17 MSCs-CM-treated OIR retinas demonstrated a significant increase in Arg-1 and IL-10 expression concomitant with downregulation of CD206, in comparison to vehicle-treated retinas. (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs vehicle, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(D)** Flow cytometry analysis of iNOS and Arg-1 expression on CD11b+ F4/80+ myeloid cells from P17 OIR retinas. Treatment of OIR retinas with MSCs-CM significantly lowered the ratio of iNOS/Arg-1 compared to vehicle treatment. (*$p<0.05$, values are mean ± SEM, n=3-4, pool of 2 retinas per n). **(E)** Flow cytometry analysis of M1 markers (iNOS and CD86) and M2 markers (CD206 and Arg-1) on CD11b+F4/80+ myeloid cells in treated OIR retinas. MSCs-CM reduced the number of iNOS+ and CD86+ M1 cells as well as CD206+ M2 phenotype in contrast to vehicle treated retinas. Upregulation in Arg-1+ M2 cells was noted

in MSCs-CM treated OIR retinas. (*$p<0.05$, values are mean ± SEM, n=3-4, pool of 2 retinas per n).

Supplementary Figures



Supplemental Figure 1



Supplemental Figure 2

Supplementary Figure Legends

Supplementary Figure 1. Naïve bone-marrow derived macrophages (BMDM) were polarized to M1 and M2 phenotype. (A) qPCR analysis of M1-polarized BMDM versus naïve BMDM (M0) showed significant increase in gene expression of pro-inflammatory cytokines iNOS, IL-1β, TNF-α, and IL-6. (*p<0.05, **p<0.01 vs M0 BMDM, values are mean ± SEM, n=4-6). **(B)** qPCR analysis of M2-polarized BMDM demonstrated upregulation of M2 markers Arg-1, CD206 and IL10 compared to unpolarized BMDM (M0). (*p<0.05, **p<0.01, ***p<0.001 vs M0 BMDM, values are mean ± SEM, n=5-8). **(C)** Representative flow cytometry histograms of iNOS, TNF-α, CD206 and Arg-1 expression in M0, M1 and M2 BMDM. Positive staining of iNOS and TNF-α was only detected in M1-polarized BMDM, whereas positive staining of CD206 and Arg-1 was limited to M2-polarized BMDM. Naïve BMDM M0 lacked expression of both M1 and M2 markers.

Supplementary Figure 2. Gene expression of M1 and M2 markers were modulated in OIR retinas. (A) qPCR analysis of P17 OIR retinas demonstrated a significant increase in M1-specific market iNOS expression compared to normoxic retinas. (*p<0.05 vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(B)** qPCR analysis of P17 OIR retinas demonstrated persistent reduction in Arg-1 during neovascularization phase, in contrast to normoxic retinas. (*p<0.05, **p<0.01 vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n). **(C)** qPCR analysis of CD206 levels in P17 OIR versus normoxic retinas indicated a steady increase in expression coinciding with the formation of neovascular tufts which peaks at P17. (*p<0.05 vs normoxia, values are mean ± SEM, n=4-5, pool of 2 retinas per n).

Chapter 6. General Discussion

6.1 Original contribution to knowledge

The following original contributions have been presented in this thesis:

1) MSCs derived from compact bone are capable of promoting vascular regeneration by simultaneously arresting vascular decay and neovascularization.
2) Intravitreal administration of MSCs or their secretome (MSCs-CM) share similar regenerative efficacy of retinal vascular regeneration.
3) MSCs-CM modulated the expression of various factors (growth factors, guidance cues, inflammatory mediators) in OIR retinas establishing a healthy microenvironment conducive for vascular regeneration.
4) MSCs-CM reduced vaso-obliteration by downregulating IL-1β and IL-1β-dependent Sema3A levels, and inhibited neovascularization by restoring Sema3E levels.
5) Myeloid cells secrete pro-inflammatory cytokine IL-17A in hypoxic conditions.
6) MSCs-CM curtailed expression levels of IL-17A.
7) Sema3E acts on Plexin D1-expressing myeloid cells downregulating IL-7A production.
8) MSCs-CM modulated cytokine expression in OIR retinas without impacting myeloid cell density.
9) *In vitro*, MSCs-CM skewed polarization of M1 macrophages to M2 phenotype but maintained the phenotype of M2-polairzed macrophages.
10) OIR retinas expressed high levels of M1 markers during neovascularization phase.
11) MSCs-CM reprogrammed pro-inflammatory M1 phenotype of retinal myeloid cells to anti-inflammatory M2-like state.

6.2 General Discussion

6.2.1 Semaphorins and retinal revascularization

Proper vascular growth depends on a fine balance between attractive and repulsive cues to reach non-perfused areas. An imbalance in these factors can cause vascular defects as evident in IRs. In the initial phase of retinopathy, local hypoxia stimulates exaggerated secretion of VEGF from the neurons in an attempt to promote angiogenesis and reinstate metabolic homeostasis. As the neurons become severely ischemic, production of Sema3A, a repulsive cue, predominates over VEGF[14]. Sema3A acts in an opposite manner to VEGF by repelling the nascent vessels from perfusing the retina. These opposing forces between VEGF and Sema3A explain the paradoxical formation of neovascular tufts. Interestingly, upregulation of Sema3A is mediated by the pro-inflammatory factor IL-1β[628]. Microglia of OIR retinas secrete high levels of IL-1β which sustains inflammation and indirectly induces microvascular injury via the release of Sema3A from adjacent neurons. Silencing Sema3A or IL-1 receptor arrests aberrant vessel growth and enables their perfusion into the ischemic retina[14, 628]. In this work, we have demonstrated that MSCs-CM suppressed IL-1β and subsequently IL-1β-dependent Sema3A, which in turn reduced the areas of vaso-obliteration.

In the second phase of OIR, the formation of neovascular tufts into the vitreous is associated with weak Sema3E expression[13]. Sema3E is another repulsive cue that counteracts VEGF-driven filopodia projections in ECs. Interestingly, ECs of the neovascular tufts selectively express Sema3E receptor Plexin-D1[19], perhaps to increase the odds of Sema3E-Plexin-D1 interaction given the scarce Sema3E levels. Intravitreal treatment with Sema3E reorients the growing vessels towards the retina by activating small GTPase RhoJ in ECs leading to cytoskeletal rearrangements. Similarly, treatment of OIR retinas with MSCs-CM suppressed disoriented angiogenesis

(neovascularization) by replenishing Sema3E. We further demonstrated that Sema3E acts on Plexin-D1-expressing macrophages to downregulate expression of pro-inflammatory cytokine IL-17A.

Semaphorins 3A and 3E play an important role in steering vascular growth. During development, both semaphorins are secreted by RGCs to act on adjacent nascent vessels to guide the vascular front to the avascular areas of the retina. Both these guidance cues exert anti-angiogenic effects by activating downstream signaling cascades which lead to cytoskeletal rearrangements and thus repulsion. Unlike Sema3A which exerts its biological effect by binding simultaneously to Plexin-A1 and Nrp-1, Sema3E signaling depends on the sole interaction with Plexin-D1[720]. During retinal ischemia, Sema3A is markedly upregulated in hypoxic RGCs where as Sema3E expression is curtailed. The mechanisms governing semaphorin expression remain elusive to date. Because of the differential expression of semaphorins observed in response to oxygen tension, hypoxia is considered as a compelling factor in regulating their expression. At low oxygen concentrations, HIF-1α translocates to the nucleus and binds to hypoxia responsive element (HRE) sequences in the promoter of target genes, including class-3 semaphorins, and hence triggering transcription[343-346, 721, 722]. However, the presence of HRE sequences doesn't necessarily correlate with gene upregulation secondary to hypoxia, as is the case with Sema3E, and hence further studies are warranted to understand the intracellular pathways which may drive selective gene expression. Of relevance, retinoic-acid-receptor-related orphan receptor-α (RORα), a lipid-sensitive nuclear receptor and transcription factor, was shown be overexpressed in ischemic retinas and play an important role in modulating Sema3E expression without impacting Sema3A mRNA levels[649]. RORα suppresses Sema3E transcription by binding to RORα responsive element (RORE) sequence found on the promoter of *Sema3E* gene, whereas silencing RORα promoted

vascular normalization. Interestingly, in RORα-deficient *Sg/Sg* mice, which exhibit spontaneous deletion in the *RORα* gene resulting in loss of activity, pro-inflammatory markers such as IL-1β, TNF-α and iNOS were significantly reduced while anti-inflammatory markers including IL-10 and Arg-1 were elevated[723]. These studies corroborate the findings presented in this thesis whereby restoration of Sema3E in ischemic retinas triggers an anti-inflammatory response permissive for vascular regeneration (Chapter 4). We have also demonstrated that MSCs-CM can directly skew the polarization state of myeloid cells towards an anti-inflammatory phenotype (Chapter 5), thus one might infer that the resorted levels of Sema3E can further substantiate resolution of inflammation; however, this will need to be further investigated. In addition to hypoxia, inflammation was also shown to have a direct effect on the expression of neuronal guidance cues. As discussed above, microglia-derived IL-1β acts directly on RGCs triggering significant release of Sema3A, which in turn misguides neovessels into the vitreous[14, 628]. Sema3A is further implicated in precipitating an inflammatory response by recruiting mononuclear phagocytes to ischemic retinas[717]. Blocking IL-1β or Sema3A mediates vascular regeneration.

Collectively, the study herein unravels a dual mechanism by which MSCs-CM mediate vascularization: suppressing Sema3A on one hand to mediate intraretinal vascular growth and restoring Sema3E on the other hand to inhibit extraretinal growth.

6.2.2 Inflammation in IRs

Inflammation plays an integral role in the pathogenesis and progression of IRs. Elevated levels of pro-inflammatory factors were detected in the vitreous of patients[224, 640, 641, 665] and animal models[82, 149, 628, 693] of IRs, among which is IL-1β. In ischemic retinas, microglia are activated and produce copious levels of IL-1β which perpetuates its expression in an autocrine fashion and

subsequently sustain microglial activation thereby exacerbating retinal inflammation. High levels of IL-1β cause vascular decay and repulsion by inducing secretion of RGC-derived Sema3A[14, 628].

IL-17A, a pro-inflammatory cytokine, belongs to the IL-17 family which are known for participating in acute and chronic inflammatory responses[724]. There is mounting evidence for the role of IL-17A in retinal degenerative diseases[725, 726]. High concentrations of IL-17A have been documented in ROP and DR patients[640, 641, 727]. In animal models of DR, neutralizing IL-17A function abrogated retinal neovascularization[700, 728]. Th17 cells are considered the major source of IL-17A, though it can also be produced by other cell types including macrophages and microglia [709], while IL-17A receptor (IL-17RA) is ubiquitously expressed in various retinal cell types including neurons[729]. In Chapter 5, we demonstrated that retinal myeloid cells and bone marrow-derived macrophages produce IL-17A under hypoxic conditions. Consistently, resident microglia secrete a wide range of pro-inflammatory cytokines when activated while blocking microglial activation inhibited hypoxia-induced vascular damage. Furthermore, IL-17A propagates local inflammation by inducing the production of pro-inflammatory mediators, such as IL-1β and TNF-α, thus sustaining an inflammatory milieu[667]. Interestingly, IL-17A is a poor inducer of inflammation on its own, and therefore synergizes with other cytokines to elicit a robust inflammatory response[730-732]. In line with this concept, we have shown that treatment of macrophages with IL-17A potentiates expression of TNF-α, IL-1β and IL-6. Importantly, MSCs-CM-induced suppression of IL-17A expression concomitantly reduced expression of abovementioned cytokines.

IL-17A is also implicated in angiogenesis. Elevated levels of macrophage-derived IL-17A partakes in pathological angiogenesis by promoting EC proliferation and tube formation as well as upregulation VEGF expression[700, 725]. Moreover, hypoxic macrophages exhibit high levels of

ER stress precipitating IL-17A secretion. Inhibition of hypoxia-induced ER stress attenuated IL-1& production and curtailed retinal neovascularization[733]. Herein, treatment of OIR retinas with MSCs-CM or recombinant Sema3E mediated revascularization partially by suppressing IL-17. Collectively, the study presented in this thesis ascertains a causal role for IL-17A in retinal vascular degeneration.

6.2.3 Inflammatory response and macrophage/microglia polarization

Microglia are highly sensitive to local environmental changes[734]. In response to an acute insult, microglia are activated to promote regenerative and neuroprotective inflammation to restore retinal homeostasis. However, under sustained chronic inflammation, microglia become pathologically activated and secrete a wide range of immunomodulatory factors that contribute to tissue damage. As discussed above, abundant levels of myeloid-derived pro-inflammatory cytokines are documented in ischemic retinas. Given the functional plasticity of microglia, they can be classified as classical M1 or alternative M2 phenotype, similar to macrophages, based on the profile of the secreted factors. M1 cells are considered pro-inflammatory in that they produce IL-1β, IL-6, IL-12, among others, as well as express iNOS and co-stimulatory molecules. Whereas M2 are characterized by the expression of Arg-1, CD206, and Ym1, and secretion of anti-inflammatory cytokines IL-10, TGF-β, IGF-1.

Recently, numerous studies have begun investigating the role of M1/M2 polarization in retinal diseases. M1-polarized macrophages were found in abundant numbers in ischemic retinas as opposed to M2 phenotype [699, 700]. Similarly, we have shown that the expression levels of M1 markers are exaggerated in the neovascularization phase of retinopathy, Treatment of OIR retinas with MSCs-CM not only diminished M1 pro-inflammatory markers, but also skewed M1/M2 ratio

in OIR retinas towards M2 phenotype. This was further accompanied by the increased expression of IL-10 and Arg-1. Furthermore, microglial cells were found to increase in number, secondary to retinal inflammation, localizing in the superficial plexus of the retina and contribute to pathological vascularization [716, 735]. In the study presented herein, interestingly, MSCs-CM did not alter the cell density of retinal myeloid cells but rather modulated their activation state. This is in accordance with other studies which have highlighted the role of microglial activation in driving tissue damage[228, 690].

IL-17A can also modulate macrophage polarization. In a streptozocin-induced retinopathy mouse model, elevated levels of IL-17A activated microglia and induced iNOS expression via STAT3 pathway[736]. Moreover, IL-17A deficiency in OIR retinas suppressed retinal neovascularization by shifting M1 polarization to M2, which in turn produced IL-10 establishing an anti-inflammatory milieu[700]. Results from chapters 4 and 5 suggest that MSCs-CM suppression of IL-17A, partly mediated via SEma3E upregulation, modulate microglial cytokine expression profile and alter the retinal microenvironment to an anti-inflammatory milieu permissive of healthy revascularization.

6.2.4 MSCs as treatment avenue for IRs

Our understanding of the underlying pathophysiological mechanisms of ocular vasculopathies has grown substantially; yet to date the most common treatment remains anti-VEGF. VEGF is considered a culprit of pathological angiogenesis and thereby blocking its function in patients with retinopathy demonstrated some beneficial functional outcomes. Safety and efficacy concerns related to anti-VEGF treatment[9] encouraged active research in finding alternative therapies that would promote healthy vascular growth but also modulate inflammation

and support neurons[737, 738]. Accordingly, MScs are a promising treatment modularity for IRs since they possess broad mechanisms of action.

Our data revealed that MSCs can promote healthy revascularization by simultaneously arresting vascular decay and promoting intraretinal vascular growth. This effect is mediated by targeting the neurovascular unit. MSCs-derived factors can act directly and indirectly on multiple retinal cell types. Herein, we have shown that MSCs-CM indirectly abrogated expression of IL-17A and other inflammatory cytokines by replenishing neuron-derived Sema3E. Concomitantly, MSCs-CM can act directly on myeloid cells and modulate their pro-inflammatory profile towards an anti-inflammatory phenotype. Of note, treatment with MSCs-CM increased VEGF levels in OIR retina despite vascular regeneration, confirming the role of alternate factors in neovascularization. These results underscore the therapeutic potential of using MSCs-CM to regulate multiple pathological processes in tandem driving tissue regeneration.

6.3 Summary and Future Perspectives

In summary, the present work underscores the potential use of MSCs to treat ischemic retinopathies. MSCs-CM promote vascular regeneration by promoting proper vascular growth to repopulate the damaged regions of the retina and by inhibiting neovascularization into the vitreous cavity. The vaso-reparative effect was mediated by modulating the crosstalk between neurons and myeloid cells, and by establishing an anti-inflammatory microenvironment conducive for vascular regeneration. This is the first study to unravel a novel mechanism by which MSCs replenish Sema3E levels and in turn inhibit IL-17A production. Moreover, we elucidated the effect of MSCs in establishing an anti-inflammatory microenvironment by directly targeting retinal myeloid cells.

The results presented herein were based on the intravitreal injection of the supernatant of MSCs, which is composed on RNAs, proteins, lipids and extracellular vesicles[739]. Because the supernatant was concentrated using a 10K molecular weight cut off, presence of small-size molecules and extracellular vesicles (EVs) can't be excluded. Recently, numerous studies have emerged exploring the potential of EVs in retinal degenerative diseases[740, 741]. The main interest in EVs lies in their nano-size and their vesicular cargo. Because of their small size, EVs can cross the blood-retina barrier[742] to reach cells of the outer retinal and the choroid. This would be of interest for treating choroidal diseases. Of note, our lab has recently shown that ROP patients develop choroidal thinning[743, 744], and thereby it would be worth investing the therapeutic effect of MSC-derived EVs in repairing choroidal vascular damage.

Chapter 7. Comprehensive Bibliography

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Appendix

Appendix – A List of publications

A. Part of the thesis:

- **Noueihed B**, Rivera JC, Dabouz R, Abram P, Omri S, Lahaie I, Chemtob S. *Mesenchymal Stromal Cells Promote Retinal Vascular Repair by Modulating Sema3E and IL-17A in a Model of Ischemic Retinopathy.* Front Cell Dev Biol. 2021 Jan 21;9:630645. PMID: 33553187
- **Noueihed B**, Rivera JC, Omri S, Chemtob S. *Immunomodulation of myeloid cell dynamics by Mesenchymal Stromal Cells in oxygen-induced retinopathy model*. (In preparation)

B. Not part of the thesis:

- Villain G, Poissonnier L, **Noueihed B**, Bonfils G, Rivera JC, Chemtob S, Soncin F, Mattot V. *miR-126-5p promotes retinal endothelial cell survival through SetD5 regulation in neurons.* Development. 2018 Jan 8;145(1):dev156232. PMID: 29180574.
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