THE ROLE OF ALTERED SPHINGOLIPID METABOLISM IN THE DEVELOPMENT OF DIABETIC RETINOPATHY

By

Harshini Chakravarthy

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ABSTRACT

THE ROLE OF ALTERED SPHINGOLIPID METABOLISM IN THE DEVELOPMENT OF DIABETIC RETINOPATHY

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Diabetic retinopathy (DR) is a vision-threatening microvascular complication of diabetes mellitus, and around 67% of patients have some degree of retinopathy after ten years of diabetes [1]. In spite of decades of investigations, the precise molecular mechanisms involved in the pathogenesis of DR have not been completely deciphered. The main aim of this dissertation is to provide an important molecular link connecting diabetic dyslipidemia with retinal vascular degeneration associated with diabetes. Our studies reveal a central pathway of sphingolipid metabolism involved in the development of DR, concurrently affecting function of bone marrow (BM)-derived inflammatory cells contributing to retinal inflammation and microvascular injury, and negatively affecting repair of retinal vasculature by BM-derived circulating angiogenic cells (CAC). Notably, we reveal that normalizing the pro-inflammatory and reparative functions of these BM-derived cells by modulating their lipid metabolism improves the outcomes of DR.

First, we explored the link between bone marrow and DR using a mouse model of diabetes, stably engrafted with GFP^+ bone marrow. We demonstrated that diabetes has a significant long-term effect on the BM-derived inflammatory monocytes as well as reparative circulating angiogenic cells (CAC) that circulate in the blood, localize to the retina and undergo further differentiation. Our findings indicate that BM-derived cells could play a central role in the development of DR.

Secondly, we investigated the role of altered sphingolipid metabolism in DR. We addressed the hypothesis that perturbation of a specific sphingolipid pathway in BM-derived cells may contribute to inflammation and vascular damage in the diabetic retina. We demonstrated that activation of an essential enzyme of sphingolipid metabolism, acid sphingomyelinase (ASM) in BM-derived cells plays a crucial role in retinal microvascular deterioration associated with diabetes. Inhibition of ASM in the diabetic BM prevented activation of BM-derived microglia-like cells and normalized retinal levels of proinflammatory cytokines. Notably, ASM also caused accumulation of ceramide on cell membrane of BM-derived reparative CACs, thereby reducing membrane fluidity and impairing CAC migration. Inhibition of ASM in these reparative cells improved membrane fluidity and homing of these cells to damaged retinal vessels in a mouse model of DR.

Finally, to demonstrate the effect of diabetes on the *in vivo* homing ability of CACs, we injected purified GFP-expressing CACs into the vitreous of healthy mice. We observed that increased numbers of diabetic CACs remained trapped in the retina without returning to their BM niche, implying impaired migration and homing efficiency of the diabetic CACs. However, inhibition of ASM in diabetic CACs improved their clearance from retina and homing into the BM niche, demonstrating that ASM upregulation in diabetes contributes to impaired migration and homing of reparative CACs.

Collectively, these findings indicate that modulation of sphingolipid metabolism in dysfunctional BM-derived cell populations could normalize the reparative/pro-inflammatory cell balance and can be explored as a novel therapeutic strategy for treating DR.

To my family

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KEY TO ABBREVIATIONS

ASM ..... **Acid sphingomyelinase**

ACCORD ..... **Action to Control Cardiovascular Risk in Diabetes**

AGEs ..... **Advanced glycation end-products**

BRB ..... **Blood-retinal barrier**

BM ..... **Bone marrow**

BMT ..... **Bone marrow transplantation**

BSA ..... **Bovine serum albumin**

Cer ..... **Ceramide**

C1P **Ceramide-1-phosphate**

CACs ..... **Circulating angiogenic cells**

COX-2 ..... **Cyclooxygenase-2**

DAPI ..... **4',6-diamidino-2-phenylindole**

DSE ..... **Debye-Stokes-Einstein equation**

DC ..... **Dendritic cells**

DCCT ..... **Diabetes Control and Complications Trial**

DR ..... **Diabetic retinopathy**

DHA ..... **Docosahexaenoic acid**

DOPC ..... **Dioleoyl phosphatidylcholine**

ER ..... **Endoplasmic reticulum**

ELISA ..... **Enzyme-linked immunosorbent assay**

s.e.m ..... **Standard error of the mean**

FITC-albumin ..... **Fluorescein isothiocyanate-albumin**

GCL ..... **Ganglion cell layer**

GFAP ..... **Glial fibrillary acidic protein**

GS ..... **Glutamine synthetase**

GM-CSF ..... **Granulocyte monocyte colony-stimulating factor**

HSC ..... **Hematopoietic stem cells**

HSPCs ..... **Hematopoietic stem/progenitor cells**

HEPES ..... **4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**

HDL ..... **High density lipoprotein**

HCD ..... **Higher-Energy collisional dissociation**

HIF-1α ..... **Hypoxia-inducible factor-1α**

INL ..... **Inner nuclear layer**

IPL ..... **Inner plexiform layer**

IGFBP ..... **Insulin-like growth factor-binding protein**

ICAM-1 ..... **Intercellular adhesion molecule-1**

IL-1β **Interleukin-1β**

Kg ..... **Kilogram**

LPS .....**Lipopolysaccharide**

LXR .....**Liver X receptor**

LDL .....**Low density lipoprotein**

M-CSF .....**Macrophage-colony stimulating factor**

MS .....**Mass spectrometry**

MSC ..... **Mesenchymal stem cells**

MCP-1 ..... **Monocyte chemoattractant protein-1**

iNOS .....**Nitric oxide synthase**

NEFA ..... **Non-esterified fatty acids**

NOD .....**Non-obese diabetic**

OPL ..... **Outer plexiform layers**

OIR .....**Oxygen induced retinopathy**

PBS ..... **Phosphate buffered saline**

PECAM-1..... **Platelet endothelial cell adhesion molecule-1**

PDGF-Rβ ..... **Platelet-derived growth factor receptor β**

PUFA ..... **Polyunsaturated fatty acids**

RT-PCR ..... **Real time polymerase chain reaction**

RFU ..... **Relative fluorescence units**

RGC ..... **Retinal ganglion cells**

SM..... **Sphingomyelin**

Sph..... **Sphingosine**

S1P **Sphingosine-1-phosphate**

s.d ..... **Standard deviation**

STZ ..... **Streptozotocin**

SDF-1..... **Stromal derived factor**

TCSPC ..... **Time-correlated single-photon counting**

TUNEL ..... **Transferase-mediated dUTP nick-end labeling staining**

TGF-β ..... **Transforming growth factor-β**

TNF-α ..... **Tumor necrosis factor- α**

TH .....Tyrosine hydroxylase

U ..... Units

VCAM-1.....Vascular cell adhesion molecule-1

VEGF .....Vascular endothelial growth factor

VLDL ..... Very low density lipoproteins

WT .....Wildtype

WHO ..... World Health Organization

Chapter 1. Introduction

1.1 Background and significance

Diabetes is a metabolic disease typically defined by elevated glucose levels in the blood, due to the pancreatic beta cells' failure to secrete insulin, or the body's failure to respond to the insulin produced. The total number of people with diabetes is projected to increase from 387 million in 2014 to 592 million in 2035 [2]. The World Health Organization (WHO) states that diabetes will be the 7th main cause of death in 2030 [3]. These statistics emphasize the importance of diabetes as a major global health problem. The total health care costs of a person with diabetes in the USA is more than three hundred billion dollars per year [4], highlighting the substantial financial burden that diabetes imposes on society.

Diabetes can be generally classified as type 1 or type 2 diabetes. Onset of type 1 diabetes is typically seen in children and adolescents. Here, the beta cells of the pancreas are destroyed by autoimmune attack, leading to a lack of insulin production. This is believed to occur in genetically predisposed subjects and is a result of an autoimmune attack by the body selectively destroying the insulin-producing beta cell mass. Onset of type 2 diabetes typically occurs in adults due to impaired metabolism, notably reduced insulin-mediated glucose uptake in skeletal muscle and increased rate of glucose production in the liver, which is called insulin resistance. Insulin resistance initially leads to compensatory beta cell mass expansion and increased insulin secretion in type 2 diabetes. Eventually, the beta cell numbers can decrease by 40% to 60%, and may also include insulin deficiency in later stages of the disease. Increased duration of diabetes increases the risk of developing complications like retinopathy, nephropathy, cardiovascular disease, stroke and neuropathy in both type 1 and type 2 diabetic patients.

DR is a disabling long-term complication of diabetes, affecting around 93 million people throughout the world, and 28 million of them have severe, sight-threatening retinopathy [5]. These numbers are anticipated to rise as the number of people diagnosed with diabetes continues to increase [6]. DR, including macular edema and neovascularization is the most prevalent reason for vision-loss in adults [7,8]. Visual loss and blindness resulting from DR impose a great social and economic burden on the patient and society.

Managing metabolic dysregulation induced by diabetes has been the primary method of slowing the development and progression of DR. Several major clinical trials (DCCT, ACCORD, UKPDS) have shown the benefit of tight glycemic control (HbA1c of less than 7%) in preventing onset and slowing the progression of DR [9,10]. However, extremely tight control of blood glucose (HbA1c of 6% or less) leads to increased mortality and cardiovascular risks, and hence is not recommended [11]. Over the last couple of decades, laser photocoagulation has emerged as a standard paradigm for managing patients with sigh-threatening DR. Panretinal photocoagulation (PRP) treatment has been shown to lower the risk of severe vision loss due to neovascularization in proliferative diabetic retinopathy and macular degeneration. Although proven beneficial, PRP may lead to complications such as loss of peripheral vision, subretinal fibrosis and may exacerbate macula edema [12].

Recently, a new class of drugs targeting the pro-angiogenic growth factor VEGF has become available in the management of DR. Intravitreal injections of anti-VEGF molecules such as Bevacizumab, Ranibizumab (antibodies against VEGF), Pegaptanib (RNA aptamer) and Aflibercept (recombinant fusion protein) have been found to be effective in treating patients with proliferative DR [13,14]. Limitations of anti-VEGF drugs include the relative short duration of their biological action as compared to the durability of photocoagulation, and repeated

intravitreal injections may be associated with tractional retinal detachment in some cases of severe proliferative DR [13]. Other treatment options such as vitrectomy and intravitreal injection of steroids have been known to stabilize vision, but these therapies are also invasive and are associated with various complications. Although current therapies have substantially reduced visual impairment from DR, these treatments alone cannot fully restore the damaged vasculature in the retina. Additional therapeutic strategies that target early retinal inflammation, microvascular damage and improve vascular repair are clearly needed.

1.2 Etiology of diabetic retinopathy

Understanding the pathogenesis of DR is crucial for development of new drugs, in order to provide more effective treatment options. Unlike other microvascular beds, such as lung or skeletal muscle, the inner retina has a limited blood supply, in spite of being a highly metabolically active tissue. The low density of microvasculature, combined with abundant metabolic activity due to a complex specialized network of neural, glial and vascular cells together contributes to making the retina exceptionally susceptible to the metabolic stress induced by diabetes [15]. Diabetes affects cell types in all the layers of the retina, leading to chronic low-grade inflammation, vascular abnormalities and gradual neurodegeneration. Clinically, the progression of DR is categorized as an early vasodegenerative stage, and a later proliferative stage with pathological growth of new, fragile blood vessels. In the vasodegenerative stage of DR, typically an increase in vascular permeability [16,17] neurodegeneration [18,19,20] and loss of capillary components like pericytes [21] and endothelial cells, causing acellular capillaries formation [22,23,24] are observed. The resulting loss of cellular support leads to microaneurysms, leakage of lipid exudates due to increased permeability, hemorrhages, loss of functional capillaries, and ensuing damage to the retina [17].

Chronic low-grade inflammation is another important feature of the early stages of DR [25,26,27]. In a normal retina, the blood-retinal barrier (BRB) regulates movement of ions, protein and water in and out of the retina, protects the retinal tissue from influx of blood-borne components and prevents leukocyte infiltration. Breakdown of the BRB is likely to be a result of increased expression of growth factors and cytokines in the retina, and upregulation of adhesion molecules on retinal vasculature, leading to adherence of leukocytes to the diabetic retinal vasculature (leukostasis). Several studies indicate that leukostasis may contribute to capillary non-perfusion, and eventual capillary death in the diabetic retina [28,29,30,31,32]. Retinal glia such as microglial cells, astrocytes and Müller cells are also thought to be early targets of vascular hyperpermeability, and increased glial reactivity may contribute significantly to the development of DR, by secretion of chemokines, neurotoxins and growth factors in the diabetic milieu [24,33,34,35,36].

As the severity of DR increases, vascular abnormalities, including fluid leakage, microaneurysms and hemorrhages occur with increasing frequency. In diabetic mice, the number of endothelial cells lining the retinal blood vessels can decrease by up to 38% after 20 months [37]. Non-proliferative DR could further progress to the advanced proliferative stage with uncontrollable growth of pathological new retinal blood vessels in response to local secretion of growth factors due to retinal non-perfusion. These new vessels are fragile and do not form tight BRB, leaking blood and contributing to edema. Eventually these abnormal vessels cover the field of view, contributing to loss of vision [38]. Untreated proliferative DR leads to fibrovascular tissue formation, which can adhere to the vitreous and cause traction retinal detachment, unless removed by vitrectomy.

The exact molecular pathways causing the pathological changes in retinal microvasculature at the early stage of DR and its progress into the proliferative stage are not fully understood as yet, but appear to involve retinal and systemic inflammatory abnormalities in response to hyperglycemia and dyslipidemia.

1.3 Animal models of diabetic retinopathy

In order to better comprehend the development and pathophysiology of DR, a variety of animal models have been developed. In general, mice and rats have been used routinely in many studies since they are small in size, have a short life span and share similarities in early DR with humans. Type 1 diabetes is usually induced in rodents by injecting with chemicals such as alloxan or streptozotocin (STZ) which destroy pancreatic β cells. Diabetic-like retinopathy can also be induced in rodents and dogs by feeding with galactose [39,40] Spontaneous hyperglycemia can be found in genetic animal models such as nonobese diabetic (NOD), $Ins2^{Akita}$, db/db mice and Bio-Breeding Zucker diabetic/Wor rats [41,42].

Chemically induced diabetes models reproduce early symptoms of DR such as increase in vascular permeability, increase in thickness of the vascular basement membrane, death of retinal pericytes and formation of acellular capillaries [17,18,21,22]. Non-vascular changes such as reactive gliosis and loss of retinal ganglion cells (RGC) are also seen in the rodent models of DR [24,33,34,43,44]. However, there are differences between species in the rate at which early stages of DR develop, and some histopathological responses to diabetes are controversial within the same species, possibly due to differences in STZ dosage or observation time points. For example, some studies demonstrate neurodegeneration at 10-14 weeks of diabetes, while others

find no evidence of RGC loss in STZ-induced diabetic mice, even after 9-10 months of hyperglycemia [24,43,45].

While many animal models of DR show the early symptoms of DR, most do not develop advanced proliferative retinopathy with neovascularization, probably due to their short life-span. Proliferative DR can be achieved via introduction of ischemia to the eyes, such as oxygen induced retinopathy (OIR), or overexpression of VEGF in photoreceptors in non-diabetic animals [46,47]. The choice of an appropriate animal model of DR should be driven by experimental design and focus of the study. However, it is important to keep in mind that no animal model can accurately mimic the complete pathophysiological progression of DR as observed in humans.

1.4 Inflammation in diabetic retinopathy

1.4.1 Leukostasis and increased vascular permeability

Inflammation is a protective reaction of the body to harmful stimuli, and recruits a wide range of chemical mediators, immune cells as well as activation of blood vessels. Acute inflammation is normally advantageous, but persistent chronic inflammation is undesirable. Inflammation was first proposed to contribute to the development of DR when high doses of salicylates decreased the incidence and severity of DR [48]. The crucial role of inflammation in the pathology of DR is now generally accepted [25,26]. Migration and adhesion of leukocytes to the diabetic retinal vasculature was shown to be considerably elevated, and some reports suggest that they may contribute to death of endothelial cells and capillary non-perfusion in DR [28,30,49]. Leukocytes use integrins CD11a or CD11b/CD18 to tether themselves to adhesion molecules such as VCAM-1, ICAM-1 and E-selectin on the surface of diabetic retinal endothelial cells

[28,50,51,52]. Inhibition or deletion of these proteins involved in leukocyte adhesion significantly inhibits vascular permeability and diabetes-induced capillary degeneration [25,50,51,53].

1.4.2 Pro-inflammatory cytokines and growth factors

An extensive body of preclinical and clinical studies has identified upregulation of pro-inflammatory cytokines and growth factors like IL-6, TNF-α, IL-8, IL-1β, MCP-1 and VEGF that play vital roles in propagating inflammation in early stages of DR. Vascular endothelial growth factor (VEGF) is an important mediator found in high levels in the diabetic retina, and contributes to increased retinal leukostasis [29,54] and to disrupting the blood-retinal-barrier in DR [55,56,57,58,59,60]. Although success has not been complete, targeting VEGF using anti-VEGF therapy represents a major advance in DR treatment. Therapeutic agents that can be injected directly into the vitreous to inhibit the action of VEGF are now available or undergoing clinical trials [61,62,63,64]. However, these treatments are invasive, expensive and more importantly, a large number of patients respond poorly to anti-VEGF therapy.

IL-1β and TNF-α are both potent inflammatory cytokines and their increased levels have been implicated in the pathology of DR. IL-1β is cleaved to its biologically active form by caspase-1. The activity of caspase-1 was demonstrated to be elevated in retinas of diabetic animal models as well as humans [65]. Moreover, blocking IL-1β or its receptor was demonstrated to protect diabetic mice from retinal vascular degeneration [66], indicating that activation of IL-1β is a vital component in developing diabetic retinal pathology. The pro-inflammatory cytokine, TNF-α is critical for leukostasis and BRB breakdown in the diabetic retina [67,68,69]. Both clinical and preclinical data have implicated TNF-α in the development of DR. Indeed, administration of a

TNF-α blocking antibody significantly increased visual acuity in a recent clinical study, building the case for TNF-α as a potential therapeutic target for DR [70].

Other inflammatory mediators such as iNOS, COX-2, Fas, complement factors, MCP-1 and transcription factors like NF-ƙB and HIF-1α have also been implicated in the pathogenesis of early stages of DR. Alterations as a result of hyperglycemia and inflammation are evident in specific cells like pericytes, endothelial cells, astrocytes, microglia and Müller cells in the diabetic retina. In retinal pericytes, continued exposure to high glucose significantly increased expression of VEGF, IL-1β, TNF-α, ICAM-1, NF-ƙB and TGF-β [71]. Retinal glia are among the first targets of increased vessel permeability, and further contribute to inflammation in DR by their secretion of various pro-inflammatory cytokines and growth factors.

1.4.3 Glial reactivity in the diabetic retina

The retina contains two types of glial cells, the macroglia which includes Müller cells and astrocytes, and the retinal microglia. The glial cells help maintain tissue homeostasis and preserve the integrity of the BRB in the normal retina. However under conditions of stress, reactive retinal glial cells can act as pro-inflammatory cytokine producers, contributing to the initiation of inflammation in the diabetic retina. Müller cells change from dormant to an activated state expressing elevated levels of GFAP- a characteristic of glial reactivity during early diabetes [33,34]. Diabetes is also associated with very early astrocyte loss is the retina, in addition to alteration in astrocyte morphology, suggesting that these glial cells may play and early and key role in inner retinal dysfunction in DR [33,72].

Microglia are resident macrophages of the neuroretina, and play a crucial role in the initiation of neural inflammatory responses. Substantial evidence shows that microglia become transformed

in DR, and it is hypothesized that microglial activation may contribute to retinal vascular damage and neuronal loss through release of inflammatory cytokines and neurotoxic factors [73]. Apart from these functional changes, phenotypic transformation of microglia in response to inflammation in experimental models of DR has been well documented, involving change from resting microglia with highly ramified processes to a reactive, amoeboid appearance with retraction of their branches upon activation (Figure 1) [33,35,43]. However, microglia can assume a large spectrum of alternative activation states, from amoeboid, microglia with thicker dendrites or less ramified microglia, depending on the nature and degree of injury to the tissue [74]. In addition, circulating monocytes can infiltrate the retina and adopt a microglia-like phenotype, secreting pro-inflammatory cytokines and contributing to retinal inflammation in diabetes [75,76,77,78]. Therefore, it is important to fully appreciate the functional roles of the variable phenotypes of retinal microglia and circulating monocytes in response to diabetic insult to enable therapeutic modification of their actions and prevent damaging inflammation in DR.

Figure 1.Transformation of resting microglia into more activated states.

From left to right, transformation of resting microglia with highly ramified morphology, into activated microglia with retraction of dendrites, thicker cell bodies and appearance of an ameboid morphology (Kreutzberg, 1996, *Trends in Neurosciences*).

An important question to consider is what leads to inflammation and cytokine secretion in DR. Several molecular mechanisms have been proposed to explain chronic low-grade inflammation in the diabetic retina, including hyperglycemia, dyslipidemia, oxidative stress, advanced glycation end products, and ER stress [18,31,79,80,81]. Insights from landmark clinical trials have demonstrated that control of both hyperglycemia and dyslipidemia is required to achieve maximal protection from DR [10,82]. Although hyperglycemia-associated damage has been studied extensively, the role of dyslipidemia in DR needs to be addressed in more detail.

1.5 Bone marrow pathology in diabetes

1.5.1 Inflammatory cells of the bone marrow

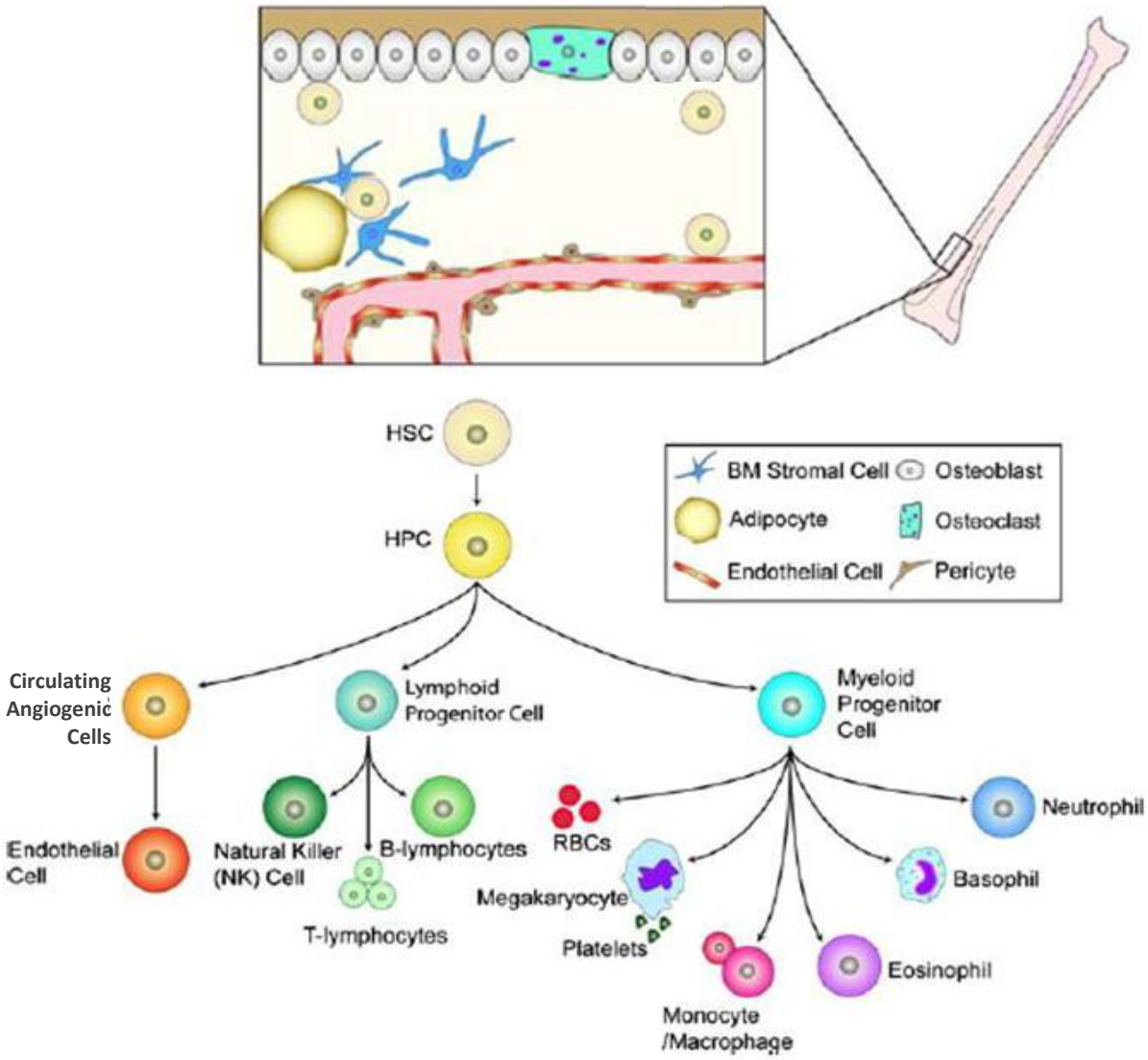
A stem cell niche is a distinct microenvironment in the body, supporting the habitation of a pool of stem cells which are maintained in an undifferentiated and self-renewable state. The bone marrow (BM) niche hosts two kinds of stem cells, the hematopoietic stem cells (HSC) and the mesenchymal stem cells (MSC). Apart from stem and progenitor cells, the BM also contains supportive stromal cells, osteoblasts, neuronal cells, vascular and perivascular tissue [83]. The HSCs differentiate into the myeloid and lymphoid progenitors that further differentiate into all the blood cells (Figure 2).

The environment of the BM plays an important role in regulating self-renewal, proliferation and differentiation of stem and progenitor cells. In the normal state, a perfect equilibrium exists between the production of all the blood cells from the pool of stem/progenitor cells and the maintenance of an adequate reservoir of stem/progenitor cells in the niche [84,85,86]. HSCs usually differentiate into 90% lymphoid and 10% myeloid progenitors, but with age, or under

pathological conditions like diabetes, a shift in hematopoiesis occurs, resulting in an increase in

Figure 2. Hematopoietic stem cell differentiation in the bone marrow.

Adapted from Li Calzi *et al.* 2010.



pro-inflammatory myeloid-derived monocytes released into the circulation [76,87]. Several studies have found that myelomonocytic cells can infiltrate the diabetic retina, adopt a microglia-like phenotype and may add to inflammation by secreting pro-inflammatory cytokines and further activate resident microglia, astrocytes and Müller glia in the retina [75,77,78]. Furthermore, BM supernatants from diabetic mice demonstrate elevated amounts of macrophage colony stimulating factor (M-CSF) and the inflammatory cytokines, IL-1β, IL-27, and IFN-γ, as well as increased levels of the compensatory anti-inflammatory factors, TGF-β and IGFBP-3 [76,87]. The hematopoietic progenitors are also known to migrate from the BM to other niches including peripheral blood, spleen, umbilical cord blood, thymus and adult liver [88,89,90,91]. During ischemic injury, increased mobilization of monocytes from the spleen to inflammatory sites may contribute to vascular complications in diabetes [92,93].

1.5.2 Reparative cells of the bone marrow

The HSCs, apart from generating lymphoid and myeloid progenitors also give rise to vascular reparative cells, called circulating angiogenic cells (CACs) (Figure 2) [94]. These are a population of hematopoietic stem/progenitor cells (HSPCs), that circulates in the bloodstream with the ability to be mobilized to the location of endothelial damage and repair damaged blood vessels either by incorporation into damaged vessels and differentiating into endothelial cells to substitute the damaged endothelial cells, or via secretion of paracrine molecules to sustain the resident microvasculature [95,96,97]. Chemokines like stromal-derived factor (SDF-1) bind to the CXCR-4 receptor on HSPCs, playing a crucial role in regulation of their homing to the BM [98]. Mobilization of CACs from the bone marrow is stimulated by release of norepinephrine by peripheral noradrenergic neurons, which leads to osteoblast suppression [99]. This causes a

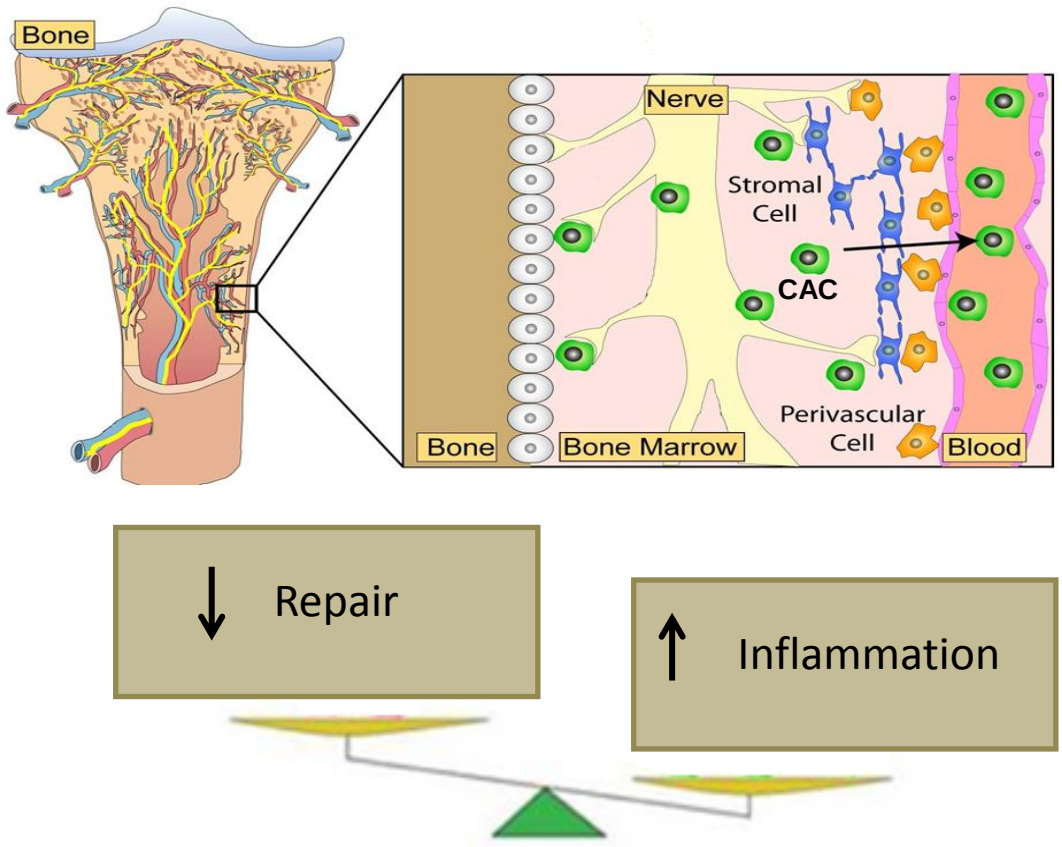
decrease in SDF-1 concentration in the BM niche and consequently, migration of CACs from the bone marrow [100].

Dysfunction of BM-derived CACs is a critical component of the pathogenic events associated with DR [101,102] (Figure 3). Recent studies indicate that diabetic human CACs have weakened proliferation, differentiation and migration capabilities, leading to ineffective vascular regeneration function [103,104,105]. Advanced diabetes is associated with BM pathology [76,106], which contributes to the inability of CAC to egress the BM, move into circulation and mobilize to areas of damage. Thus this trapping of CACs within the BM makes these cells unavailable for vascular repair [103].

We have previously demonstrated bone marrow pathology and CAC dysfunction precedes retinal vascular degeneration in DR [103]. Furthermore, the migratory prowess of diabetic CACs is severely altered due to their inability to respond to hypoxia with an increase in HIF expression or to migrate towards gradients of hypoxia regulated factors, such as VEGF-A and SDF-1 [107,108,109]. The migratory defect of these cells is a result of reduced bioavailability of NO as well as poor deformability [108]. Here, we explore the concept that altered sphingolipid metabolism changes the membrane of diabetic CACs leading to deficient deformability and migration to areas of vascular injury in a mouse model of DR.

Figure 3. Bone marrow pathology in diabetes.

We hypothesize that in diabetes, metabolic discrepancies in the bone marrow shifts hematopoiesis towards activation of more pro-inflammatory cell types, but also decreases the reparative capacity of progenitor cells. Adapted from Douglas *et al.* 2012.



1.6 Diabetic dyslipidemia

Dyslipidemia can be described as an abnormal level of lipids in plasma, due to disproportional metabolism, release and uptake by the adipose tissue, and inefficient removal from blood circulation. Insulin resistance promotes dyslipidemia by elevating LDL cholesterol, total cholesterol, free fatty acids and triglycerides, as well as decreasing HDL cholesterol [110]. Patients with diabetic dyslipidemia have a higher frequency of retinal irregularities, and raised cholesterol levels have been associated with progression of DR [111,112,113,114]. Evidence from large, randomized clinical studies reveals that the development of DR is strongly associated with dyslipidemia [10,82,114]. Analysis of data obtained from the Diabetes Control and Complications Trial (DCCT) indicates that severity of retinopathy positively correlates with size of serum lipoprotein classes (VLDL, LDL and HDL) in type 1 diabetic patients [82]. Another momentous clinical trial involving type 2 diabetic patients, the ACCORD EYE study showed that intensive control of dyslipidemia by targeting both triglyceride and cholesterol with simvastatin and fenofibrate reduced the odds of diabetic retinopathy progressing by about 40% over four years [10]. Since dyslipidemia involves complex metabolic dysregulation of multiple lipid classes including fatty acids, cholesterol, triglycerides and sphingolipids, the diabetes-associated changes in each lipid class are discussed below.

1.6.1 Triglycerides

Elevation in the levels of triglycerides over a period of time enhances the risk of developing diabetes, and this has been demonstrated in several studies [115,116,117]. Both type 1 and type 2 diabetic patients are found to exhibit increased triglyceride content in skeletal muscle and blood which further correlates with insulin resistance [118,119]. Raised serum triglyceride concentration is also linked to development and increased severity of DR [82,112,114,120],

while regulation of triglycerides by fenofibrates in type 2 diabetic patients with DR was demonstrated to diminish the requirement for laser therapy [121].

In the liver, fatty acids are converted to triglycerides, followed by their secretion as VLDL. In diabetes, larger quantities of fatty acids in the liver are reconstructed to form triglycerides, and released as large triglyceride-rich VLDL. Increased hepatic production and impaired clearance of VLDL results in increased production of small dense LDL particles, which is in turn inversely related to plasma HDL levels [122]. The severity of DR is shown to be positively associated with serum triglycerides and serum concentrations of LDL [82]. Triglycerides can be reduced by administration of fibrates, niacin and omega-3 fatty acids [123].

1.6.2 Cholesterol

Type 2 diabetic patients have elevated blood levels of cholesterol [112,114,120]. The benefits of intensive dyslipidemia therapy may be due to direct regulation of retinal lipid metabolism. Cholesterol is produced locally in the retina mainly in Müller cells and inner rod segments [124,125]. Endogenous cholesterol biosynthesis is essential for normal retinal function as blocking cholesterol biosynthesis in the healthy retina leads to progressive retinal degeneration [124]. Cholesterol can also be delivered to the healthy retina via the outer blood retinal barrier through an LDL receptor-mediated mechanism [126]. In the diabetic retina, an abnormal increase in LDL cholesterol uptake through the retinal microvasculature has been previously demonstrated [127]. Clinical trials have revealed that prevalence and progression of DR positively correlate with LDL-cholesterol and serum cholesterol levels, and negatively associated with HDL-cholesterol [82,112,114].

Reverse cholesterol transport is one of the mechanisms to eliminate cholesterol from the retina. Another way of removing cholesterol is by conversion to oxysterols, which are also activators of liver X receptor (LXRs) [125,126]. Activation of LXR has been recently shown to prevent DR in a mouse model of diabetes [128]. Activation of LXR could be a significant target for the treatment of DR, since they suppress inflammation and promote reverse cholesterol transport.

1.6.3 Fatty acids

Type 2 diabetic patients have elevated blood levels of esterified as well as non-esterified fatty acids (NEFA). Considerable alterations to fatty acid profiles are also observed in type 1 diabetes [129,130,131]. Inappropriate release of NEFA from adipose tissue in diabetes leads to increased hepatic triglyceride production and increased VLDL secretion, contributing to diabetic dyslipidemia [132]. Changes in elongation and desaturation of fatty acids are detected in both type 1 and type 2 diabetic patients [133,134]. Increased concentration of saturated, shorter fatty acyls correlates with small, dense lipoproteins, while increase in longer chain polyunsaturated fatty acids (PUFA) correlates with larger, less dense lipoproteins in serum [135,136].

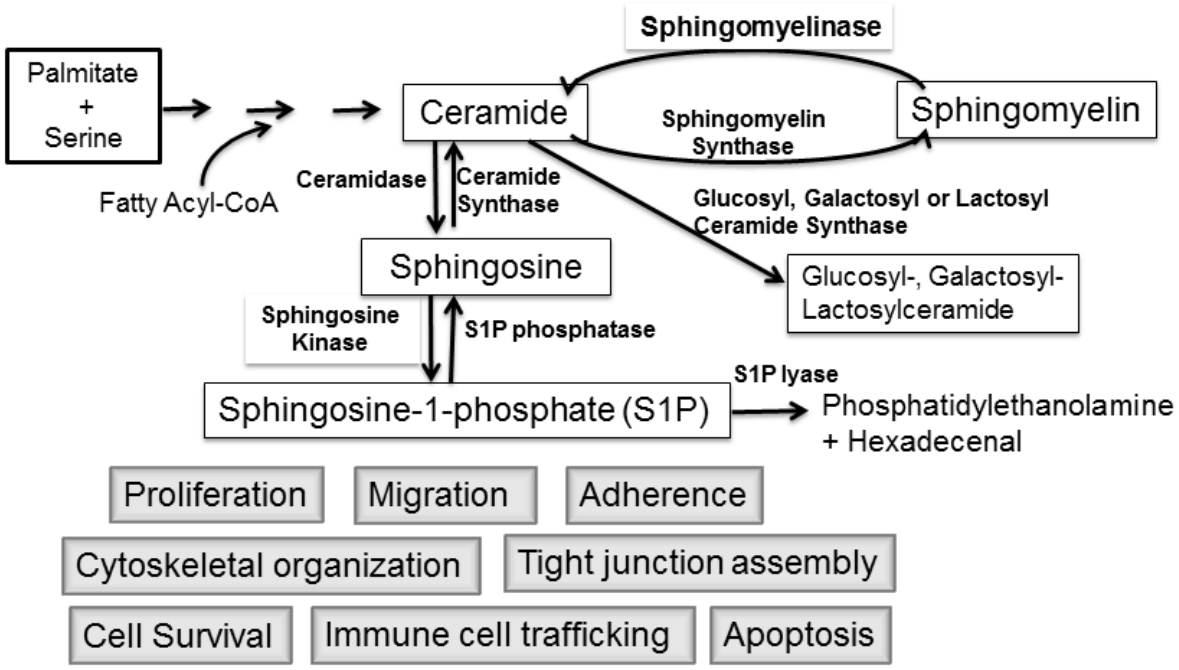
The retina has a fatty acid profile that is very distinctive from that of blood plasma or liver. A recent study demonstrated specific alterations in lipid metabolism in the retina induced by diabetes [137]. Retinal saturated and unsaturated fatty acids are created through a series of elongation (Elovl1-6) and desaturation (desaturases) reactions. Diabetes causes a significant reduction in the levels of elongase enzymes Elovl2, Elovl4 and Elovl6 [138]. A decrease in retinal levels of one of highest expressed retinal n-3 PUFA- docosahexaenoic acid (DHA) is observed due to diabetes-induced alterations in fatty acid remodeling in the retina, while supplementation of the diet with DHA was found to be protective against retinal vascular pathology [137,139,140].

1.6.4 Sphingolipids

Sphingolipids are an important class of biologically active lipids that have crucial roles in the regulating cell growth, death, inflammation, adhesion and migration. In order to identify the mechanisms of their various functions, we need to understand the complex pathways of bioactive sphingolipid metabolism and the regulatory enzymes that control their production and action. Ceramide holds a central position in sphingolipid metabolism and can be formed by *de novo* synthesis by the action of ceramide synthase and serine palmitoyl transferase, or through the breakdown of sphingomyelin by one of several sphingomyelinases (Figure 4). These include acid, neutral and alkaline sphingomyelinases. Ceramide can be cleaved into sphingosine by the action of ceramidases. Sphingosine is further acted upon by sphingosine kinase to generate sphingosine-1-phosphate (S1P). S1P lyase breaks down S1P irreversibly to produce hexadecenal and phosphatidylethanolamine. Ceramide can also be transformed by galactosyl or glucosyl ceramide synthase to give rise to glycosphingolipids, or acted on by ceramide kinase to form ceramide-1-phosphate (C1P). Sphingomyelin synthases act on ceramide, adding a phosphocholine headgroup to synthesize sphingomyelin.

Figure 4. Biologically active sphingolipids.

The scheme shows various roles of bioactive sphingolipids in biological processes. Ceramide can be produced *de novo* from serine and palmitate or by the breakdown of sphingomyelin by sphingomyelinases. Sphingosine and S1P can be generated by ceramidase and sphingosine kinase. Ceramide can be glycosylated to give rise to glycosphingolipids such as glucosyl-, galactosyl- or lactosylceramides. Bioactive sphingolipids are believed to play crucial roles in regulating many cellular processes.



Sphingolipids consist of only a small portion of total cellular lipids, but evidence indicates that dysregulation of sphingolipid metabolism is an important aspect of both type 1 and type 2 diabetes [141,142,143,144]. Sphingolipids have emerged as critical regulators of numerous functions in the cell like proliferation, inflammation, motility and apoptosis [145,146,147,148]. The delicate equilibrium of pro- and anti-apoptotic sphingolipids, called the sphingolipid 'rheostat' determines the survival fate of various cells (Figure 2). Changes in levels of sphingolipids could have several implications for inflammatory signaling in diabetes. Biologically active sphingolipids like S1P, glycosphingolipids and ceramide are believed to play a crucial role in the pathophysiology of diabetic complications [138,141,142,147,149,150].

1.6.4.1 Glycosphingolipids

Glycosphingolipids are ceramide metabolites that have been associated with inflammation, reduced insulin sensitivity and cellular stress. Inhibition of glycosphingolipid synthesis has been demonstrated to prevent insulin resistance in animal models of diabetes [151,152]. Simple glycosphingolipids such as hexosylceramides can be converted into more complex glycosphingolipids, such as gangliosides which have sialic acid added to their structure. GM3 ganglioside is known to modulate insulin receptor activity, thereby contributing to insulin resistance in diabetes [153]. The retina is normally responsive to insulin, exhibiting high basal levels of insulin receptor activity, which is reduced in diabetes [154]. Altered glycosphingolipid metabolism has been associated with the pathogenesis of DR, and blocking glucosylceramide synthase has been demonstrated to augment local insulin sensitivity and improve neuronal cell viability in the diabetic retina [142].

Numerous studies associate glycoconjugated sphingolipid metabolites with diabetic complications involving retina, liver and kidney [142,155,156,157]. These glycosphingolipid metabolites may contribute to proinflammatory cytokine signaling in diabetes, leading to cellular stress and apoptosis [142]. Glycosphingolipids such as lactosyl ceramide mediate upregulation of adhesion molecule expression on monocytes, inducing their adhesion and migration during inflammatory responses [158]. Lactosyl ceramide has also been shown to stimulate expression of CD11b/CD18 on human neutrophils and mediate NF-ƙB and ICAM-1 expression on vascular endothelial cells [159,160]. Ceramide metabolites such as gangliosides have also been shown to activate microglial response via PKC activation [161].

1.6.4.2 Sphingosine-1-phosphate

Ceramide can be broken down to sphingosine, which can then generate S1P by the action of sphingosine kinase. S1P has proliferation, migration and pro-survival effects [162], while ceramide is recognized to have pro-inflammatory and pro-apoptotic effects [163]. Hyperglycemia induces sphingosine kinase activity and increased S1P which contributes to increased adhesion of leukocytes to endothelial cells thereby mediating vascular damage [164]. Elevated levels of circulating S1P have been observed in animal models of type 1 diabetes, and inhibition of sphingosine kinase has been demonstrated to reduce retinal vascular permeability in an animal model of DR [149]. Inhibition S1P signaling using S1P receptor knockout mice or anti-S1P antibodies is also protective against neovascularization [165,166]. However, S1P and its targets in the diabetic retina have not been fully elucidated due to complexities such as multiple sphingosine kinases, receptors and presence of other intracellular targets. Sphingolipid metabolites such as C1P and S1P may upregulate SDF-1 secretion in the BM, leading to changes

in concentration gradients of critical chemokines such as SDF-1 which are known to regulate release and migration of progenitor cells from their niches [167,168,169].

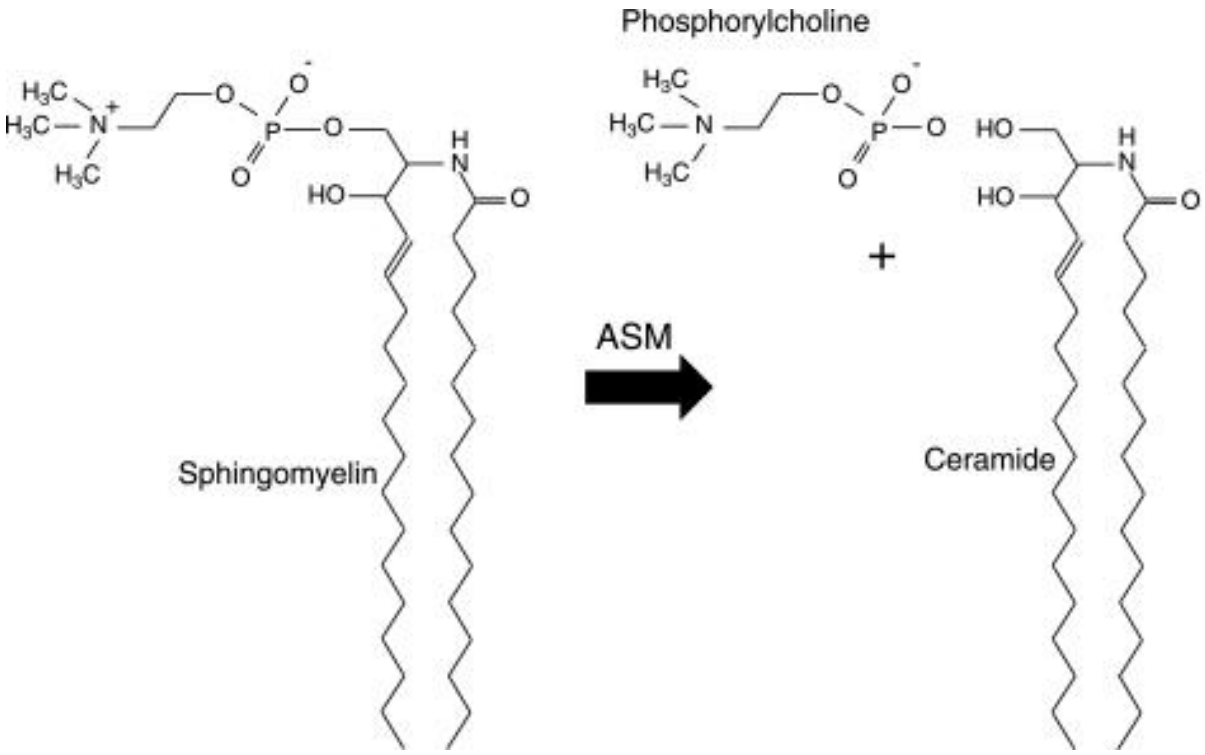
1.6.4.3 Ceramide

Ceramide is a pro-apoptotic sphingolipid accumulates in the cell by two principle mechanisms- in endothelial and immune cells, it is primarily synthesized by sphingomyelinases by the hydrolysis of sphingomyelin. In neuronal cells and adipocytes, it is mainly produced by *de novo* synthesis. Sphingomyelinases regulate the delicate balance between pro-apoptotic ceramide and sphingomyelin which is crucial in determining cell fate [138,147,170]. Acid sphingomyelinase (ASM), the enzyme converting sphingomyelin to ceramide, is a key element activated by diabetes in both BM-derived CACs and the retinal vasculature (Figure 5).

We and others have previously demonstrated that dysregulation of sphingolipid metabolism, leading to upregulation of ASM and higher rate of sphingomyelin-ceramide conversion plays a crucial role in endothelial damage in the diabetic retina [138,142,150,171], and that inhibition of ASM by dietary supplementation of docosahexaenoic acid (DHA) or by genetic manipulation prevents retinal inflammation and vascular degeneration associated with diabetes [150,172,173]. Similarly, we show that DHA-enriched feeding blocks upregulation of ASM in diabetic CACs, thereby normalizing CAC number and function in type 2 diabetic rats [172].

Figure 5. Acid sphingomyelinase (ASM) is a key enzyme in sphingolipid metabolism.

ASM hydrolyzes sphingomyelin to produce ceramide, an important molecular mediator in apoptotic and inflammatory cellular processes.



Ceramide has also been shown to alter the fate of inflammatory cells by several mechanisms. Alterations in membrane composition can arise due to the tendency of ceramide to self-associate, forming ceramide-enriched platforms which have been implicated in intracellular signaling cascades. Ceramide enrichment in membrane microdomains has been shown to facilitate cytokine receptor clustering and increase pro-inflammatory signaling in a variety of immune cells, including B lymphocyte activation, bacterial infection, release of cytokines during inflammation and induction of apoptosis [146,170]. Clustering of CD14 with CD11b/CD18 receptors, facilitated by ceramide promotes survival and immune activation of monocytes [174]. Ceramide in sphingolipid-rich membrane rafts regulates clustering of CD40, thereby initiating cytokine signaling in dendritic cells and B lymphocytes [175,176].

Ceramide has also been demonstrated to regulate superoxide release from neutrophils which have been previously shown to accumulate in the microvasculature of diabetic retinas [49,51,177]. Presence of ceramide in a membrane is also known to increase membrane rigidity or stiffness [178,179]. Interestingly, increase in membrane stiffness is believed to cause enhanced retention of neutrophils in capillaries during inflammation [180]. In the resident immune cells of the retina such as microglia and astrocytes, ASM activation has been associated with increased cytokine release. Complete blockade of IL-1β release is observed in glial cells isolated from ASM knockout mice [181].

1.6.4.4 Ceramide and membrane fluidity

In addition to pro-inflammatory mediator, we now explore the role of ceramide as a structural membrane component contributing to membrane fluidity. There is a well-established literature indicating that the presence of ceramide and cholesterol in a lipid bilayer leads to a change in the "viscosity" of the membrane [170,178,182]. Rigidity of cell membranes is a promising

biomarker indicative of underlying changes in cell mechanical properties associated with disease processes. Previous studies have demonstrated changes in membrane deformability of erythrocytes in cytoskeletal diseases, increased cell deformability of invasive cancer cells and changes in stiffness of neutrophils and monocytes in response to chemokine stimulation [183,184,185]. Changes in rigidity of the cell membrane is particularly important for CAC migration where egress from the bone marrow and extravasation from blood vessels into injured tissue depends on changes in membrane morphology and membrane protein dynamics, all of which are highly dependent on fluidity and deformability. We have previously shown that diabetes is associated with reduced deformability of human CACs, this reduced deformability is associated with decreased migratory prowess and that ASM activation leads to increased short-chain ceramide levels in diabetic CACs [108,172]. Therefore, modulating cell deformability can provide a way of improving their mobilization efficiency to sites of vascular injury.

Several approaches have been used to measure deformability of cells like micropipette aspiration, atomic force microscopy, magnetic twisting cytometry and optical stretching [108,186,187,188]. Although precise, these techniques are low throughput and require highly skilled manual operation. Another approach to measure local viscosity in membrane structures is to quantitate the rotational diffusion behavior of a fluorescent probe molecule incorporated into the bilayer. The principle of this measurement is to photoselect a non-random distribution of chromophores in the membrane and then monitor the time required for this ensemble to re-randomize [189]. The functionality and time constant(s) associated with this relaxation provide direct insight into the viscosity of the bilayer membrane (Figure 6, 7). Using this technique, we demonstrate that increased levels of membrane ceramide due to ASM activation in diabetes are

Figure 6. Measurement of membrane fluidity using perylene.

Structure of the chromophore, perylene.

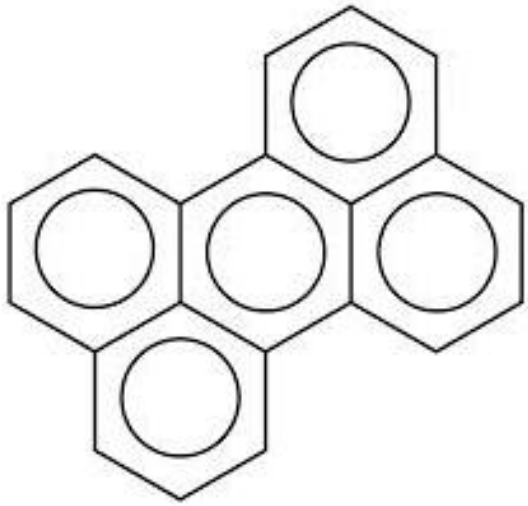
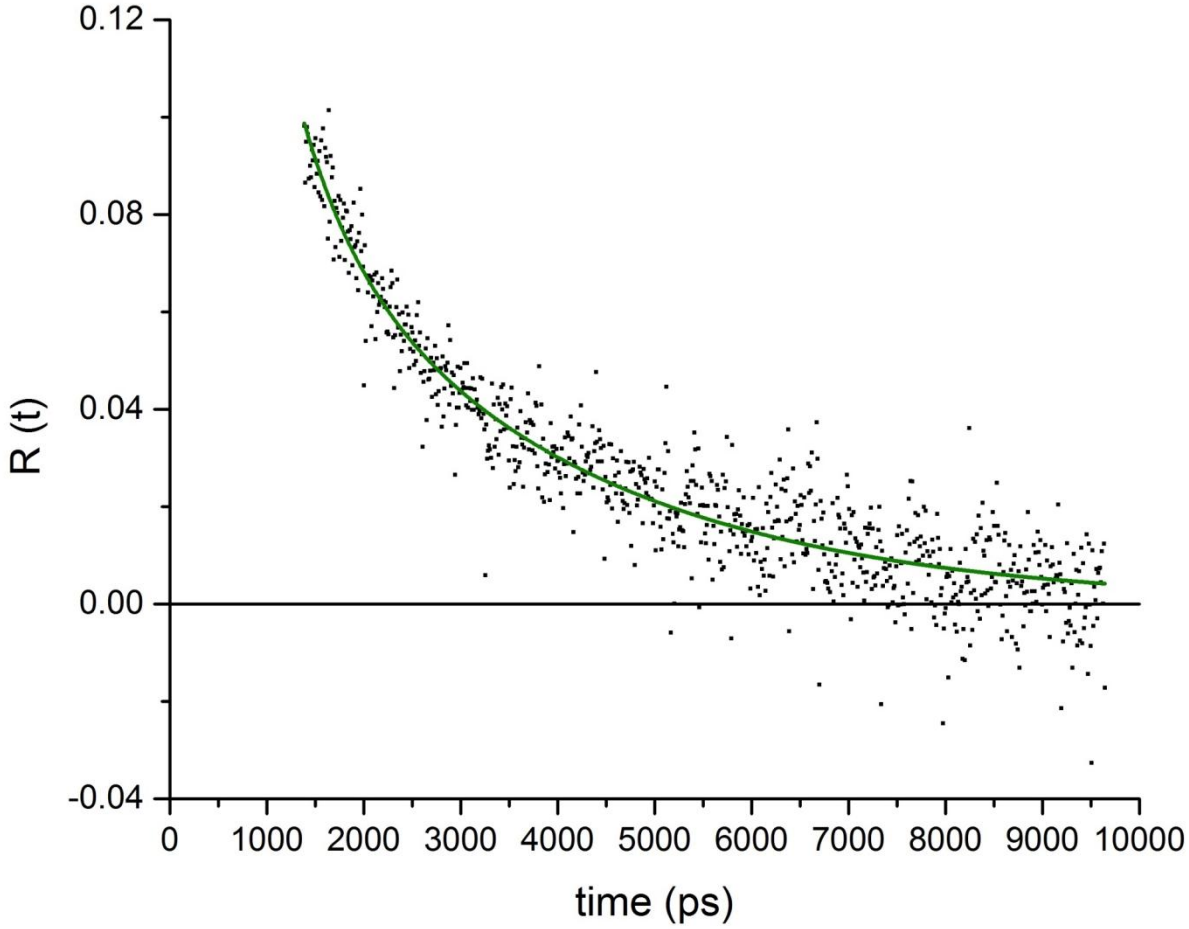


Figure 7. Anisotropy decay for perylene



associated with increased membrane rigidity, impaired migration and extravasation capacity of CACs, which in turn prevents effective homing from bone marrow to the site of vascular injury.

1.8 Objectives of the dissertation

Dyslipidemia and hyperglycemia accompanying diabetes are two of the chief metabolic aberrations that are likely to be involved in initiating the development of DR. There also exists a complex interplay between diabetes-induced retinal vascular injury, inflammation and impaired reparative function of bone marrow-derived CACs, collectively contributing to microvascular degeneration in the diabetic retina.

Accordingly, the principal hypothesis of this dissertation is as follows:

In diabetes, upregulation of acid sphingomyelinase (ASM) in the bone marrow plays a vital role in retinal vascular degeneration by two mechanisms:

- **by promoting inflammation and damaging vascular endothelial cells in the retina, through activation of bone marrow-derived pro-inflammatory cells; and**
- **by preventing efficient repair of injured retinal capillaries by bone marrow-derived circulating angiogenic cells (CACs).**

Therefore, preventing the early inflammatory damage and enhancing CAC-mediated repair by inhibition of ASM in the bone marrow can prevent diabetic retinopathy.

A schematic representation of the guiding hypothesis is shown in Figure 8.

Chapter I is a review of past and current relevant literature that is available on diabetic retinopathy, inflammation in DR, diabetic dyslipidemia, role of bone marrow-derived cells and

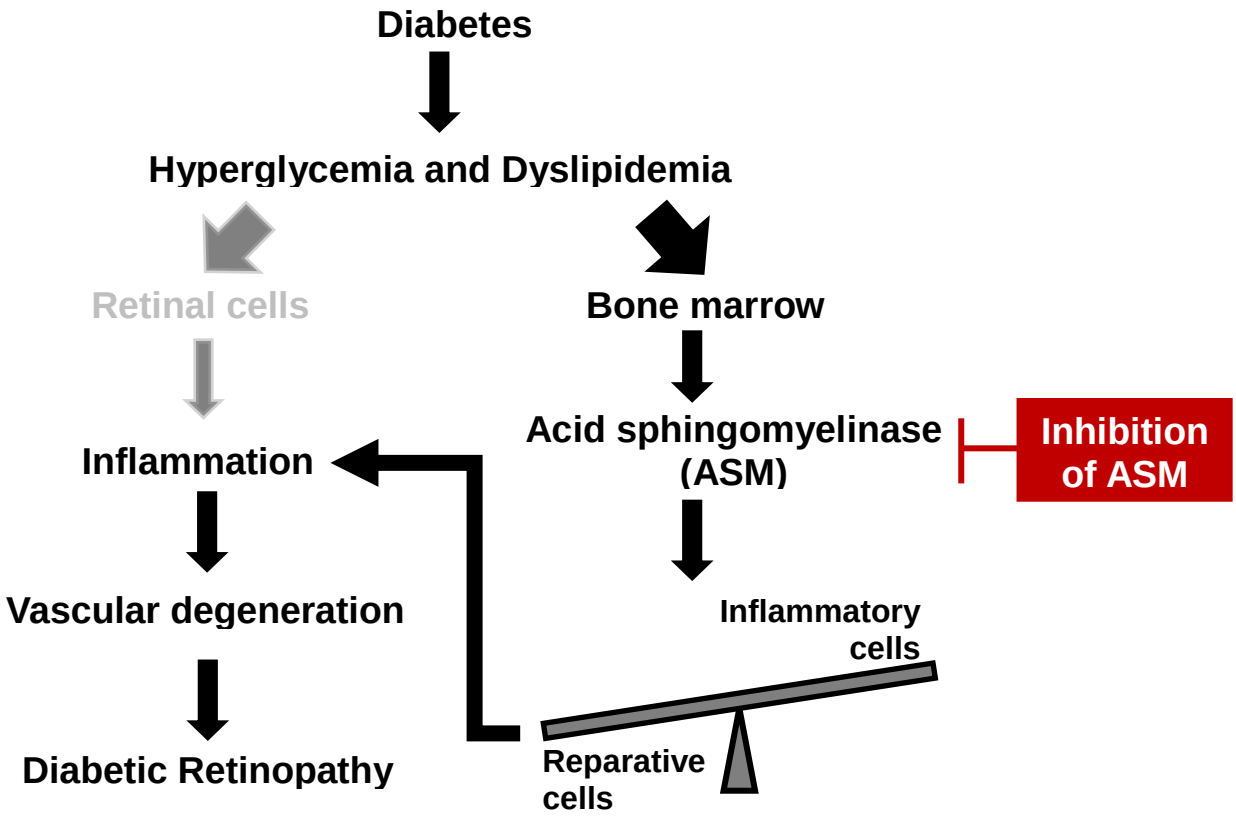
how they can be integrated for better understanding of the fundamental mechanisms that lead to the development of DR.

Chapter II describes the diabetes-induced shift from endothelial progenitor to pro-inflammatory bone marrow-derived cells in connection with the diabetic retina. The experiments described in this chapter are designed to test the hypothesis that diabetes induces a significant effect on the nature and proportion of bone marrow-derived cells that circulate in the blood and localize to the retina.

Chapter III investigates the role of acid sphingomyelinase in diabetes-induced increase in activity of bone marrow-derived pro-inflammatory cells and impairing function of vascular reparative CACs in a diabetic retinopathy mouse model. The experiments in this chapter illustrate that modulation of sphingolipid metabolism in dysfunctional bone marrow-derived cell populations could normalize the reparative/pro-inflammatory cell balance in diabetes.

Chapter IV demonstrates the beneficial effect of inhibiting acid sphingomyelinase in improving homing capacity of diabetic vascular reparative CACs *in vivo*.

Figure 8. Guiding hypothesis of the dissertation.



Chapter V summarizes all the described data, summarizes the conclusions that can be reached from these facts and outlines future directions of exploration associated with the present study.

The results of this work provide significant insights into the highly complex and poorly understood mechanisms that lead to retinal microvascular damage and inflammation in diabetes, and may encourage generation of new therapeutic strategies for complementing the available treatment for DR.

Chapter 2. Imbalances in mobilization and activation of pro-inflammatory and vascular reparative bone marrow-derived cells in diabetic retinopathy

This chapter is a modified version of a manuscript under review in PLoS One.

Authors: Harshini Chakravarthy[1], Eleni Beli[1], Svetlana Navitskaya, Sandra O'Reilly, Qi Wang, Nermin Kady, Chao Huang, Maria B. Grant, Julia V. Busik.

[1]These authors contributed equally to this work.

2.1 Abstract

Diabetic retinopathy is a sight-threatening complication of diabetes, and around 65% of patients are affected after ten years of diabetes. Diabetic metabolic insult leads to chronic low-grade inflammation, retinal endothelial cell loss and inadequate vascular repair. This is partly due to bone marrow (BM) pathology leading to increased activity of BM-derived pro-inflammatory monocytes and impaired function of BM-derived reparative circulating angiogenic cells (CACs). We propose that diabetes has a significant long-term effect on the nature and proportion of BM-derived cells that circulate in the blood, localize to the retina and home back to their BM niche. Using a streptozotocin mouse model of diabetic retinopathy with GFP BM-transplantation, we have demonstrated that BM-derived circulating pro-inflammatory monocytes are increased in diabetes while reparative CACs are trapped in the BM and spleen, with impaired release into circulation. Diabetes also alters activation of splenocytes and BM-derived dendritic cells in response to LPS stimulation. A majority of the BM-derived GFP cells that migrate to the retina express microglial markers, while others express endothelial, pericyte and Müller cell markers. Diabetes significantly increases infiltration of BM-derived microglia in an activated state, while

reducing infiltration of BM-derived endothelial progenitor cells in the retina. Collectively, these findings indicate that BM pathology in diabetes could play a role in both increased pro-inflammatory state and inadequate vascular repair contributing to diabetic retinopathy.

2.2 Introduction

DR is an important long-term complication of diabetes, affecting around 93 million people and is a foremost cause of visual loss among working adults throughout the world [5]. The initial stages of DR are characterized by various clinical features including increased microvascular permeability, vessel leakage and appearance of microaneurysms [18]. Diabetic metabolic insult affects retinal vascular degeneration at several levels: First, by contributing to chronic retinal low-grade inflammation resulting in endothelial cell injury [142,150,173,190]; Second, by inadequate repair of the injured retinal capillaries by bone marrow (BM)-derived circulating angiogenic cells (CACs), which are exquisitely sensitive to the damaging diabetic milieu [103,107]; finally, by activating monocytes [191] and further promoting a pro-inflammatory environment in the retina [101]. Retinal endothelial cell injury, activated monocytes and failed attempts by CACs to repair injured retinal capillaries collectively result in progression to the vasodegenerative stage of the disease [30,104,105].

Efficient release of CACs from the BM and spleen into circulation and extravasation into blood vessels in the tissues is a critical component of their surveillance and vascular repair function. We and others have previously shown that BM neuropathy precedes retinal vascular degeneration in DR, and that human diabetic CACs have weakened proliferation and migration capabilities, resulting in their ineffective contribution to vascular repair [103,104,105]. Here, we studied how diabetes affects the release of CACs from BM and spleen into systemic circulation. Besides hosting the CACs, the BM is an important niche for several cells types such as stem

cells, stromal supporting cells, myeloid and lymphoid precursors. Some of these cell types are recruited to the retina from the BM for retinal remodeling. The hematopoietic progenitors are also known to migrate from the BM to other niches such as peripheral blood and spleen [88,89]. Interestingly, spleen acts as an important reservoir during CAC trafficking and as a storage site for lymphocytes, dendritic cells (DC) and monocyte populations [89,92]. Leukocytes can be potentially activated by interaction with BM-derived DC, which secrete cytokines in response to immune stimulation and determine the nature of the leukocyte response during inflammation [192,193,194]. Aberrant activation of immune cells, as well as decreased mobilization of CACs from BM and spleen may contribute to vascular complications in diabetes [92,93,195,196].

The BM is also the source of myeloid-derived circulating monocytes, which contribute to DR-associated inflammation. We have previously demonstrated that diabetes induces a shift in hematopoiesis resulting in a reduction of reparative cells (CACs) and an increase in pro-inflammatory monocytes that are released into circulation [76,87,103]. Furthermore, BM supernatants from diabetic mice demonstrate increased levels of macrophage colony stimulating factor (M-CSF) and inflammatory cytokines, IL-1β, IL-27, and IFN-γ, as well as increased levels of the compensatory anti-inflammatory factors, TGF-β and IGFBP-3 [76,87]. Just like CAC dysfunction, immune cell imbalance and inflammation are critical participants in the pathogenic events associated with DR [101,102]. However, targetable signaling pathways of inflammation in DR remain largely unknown. Previously, we have showed that diabetes leads to increased accumulation of inflammatory monocytes in the retina [87]. It has been shown recently that pro-inflammatory BM-derived cells like neutrophils and monocytes play a critical role in retinal endothelial cell death and capillary degeneration in diabetes [32]. However, the influence of diabetes on a range of other types of BM-derived cells, their migration to niches such as spleen

and peripheral blood, and their association with retinal vasculature has not been explored in detail.

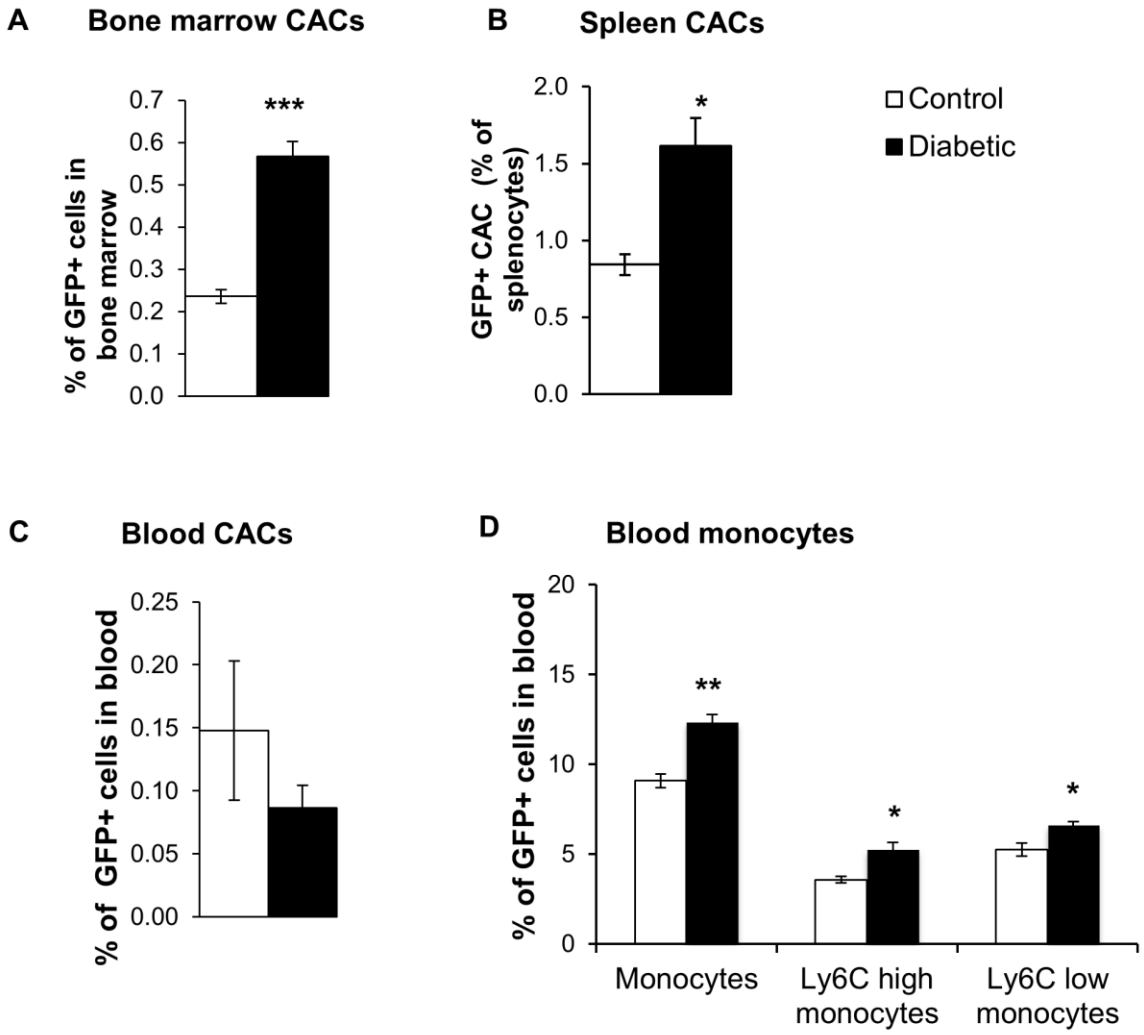
In this study, we propose that diabetes has a significant long-term effect on the nature and proportion of BM-derived cells that circulate in the blood and localize to the retina. To test this hypothesis, we generated chimeric mice with long-term, stable reconstitution of their BM with GFP^+ cells. After four months to allow for stable reconstitution of the BM, diabetes was induced by streptozotocin (STZ) injections and the retinas were analyzed for the type and number of BM-derived cells after 8 weeks of diabetes.

2.3 Results

- Diabetes alters release of BM-derived cells in circulation: To track the movement of BM-derived cells in diabetes, we created chimeric mice on a C57BL/6J background by transplanting with GFP^+ age-matched BM at 8 weeks of age. 98% reconstitution of transplanted BM was confirmed by flow cytometry. After 2 months of diabetes, we observed impaired release of GFP^+-expressing CACs, shown as a significantly higher number of these cells trapped in the BM and spleen of diabetic mice compared to controls (Figure 9A, B). Lower numbers of BM-derived CACs were observed in the blood of diabetic chimeras, as compared to controls (Figure 9C). In contrast to a decrease in CACs, we observed a significant increase in $CD11b^+$ GFP^+ circulating monocytes with surges of $Ly6C^{hi}$ as well as $Ly6C^{lo}$ monocyte populations in the blood of diabetic mice (Figure 9D).

Figure 9. Diabetes alters release of BM-derived GFP$^+$ cells into circulation.

(A) GFP$^+$ CACs as a percentage of total GFP$^+$ bone marrow cells, N= 4-8 (B) GFP$^+$ CACs as a percentage of total splenocytes, N= 4-8 (C) GFP$^+$ CACs as a percentage of total GFP$^+$ cells in blood, N= 4-8. GFP$^+$ CACs in blood, bone marrow and spleen are gated as Lin$^-$ CD34$^+$ CD309$^+$ cells. (D) GFP$^+$ monocytes (CD11b$^+$ Ly6C$^+$, CD11b$^+$ Ly6C^{hi} and CD11b$^+$ Ly6C^{lo}) as a percentage of total GFP$^+$ cells in blood. N= 4-5, *** p<0.001, ** p< 0.01 and * p<0.05.



- Diabetes alters immune responses of BM-derived DC and splenocytes: To determine whether diabetes affects activation of BM-derived DC and splenocytes, we analyzed cytokine secretion by diabetic and control cell populations in response to LPS stimulation. Elevated levels of proinflammatory cytokines, TNF-α and IL-1β were secreted by BM-derived DC and splenocytes derived from diabetic mice (Figure 10), indicating that diabetes may lead to aberrant activation of BM and splenic derived immune cells.
- Characterization of BM-derived cells in the control retina: Characterization of the vasculature-associated GFP^+ cells in retinas of chimeric mice by immunofluorescent staining of flat-mounted retinas indicated that BM-derived cells predominantly express markers of perivascular microglia, pericytes, Müller cells or vascular endothelial cells (Figure 11A-D). A majority of the BM-derived cells infiltrating the retina expressed the pan-microglial marker, Iba-1 (Figure 11F). BM-derived astrocytes were not observed in the neural retina or associated with the retinal vasculature (Figure 11E).

  To further analyze localization of the different BM-derived cell types within the retinal cell layers, immunohistochemical staining of retinal sections was performed. GFP^+ cells in the retina localized predominantly to the ganglion cell layer (GCL), inner nuclear layer (INL), inner (IPL) and outer plexiform layers (OPL), where the retinal vasculature is located (Figure 12). The BM-derived cells in the retina were immunoreactive for Iba1 (labelling microglia, Figure 11A), PDGF-Rβ (labelling pericytes, Figure 12B), glutamine synthetase (labelling Müller cells, Figure 12C) and collagen IV (labelling endothelial cells, Figure 12D). However, GFP^+ cells in the retinas were not recognized by antibodies against GFAP (labelling astrocytes, Figure 12E) and neuronal markers such as tyrosine hydroxylase (labelling amacrine cells, Figure 12F) and rhodopsin (labelling rod photoreceptors, Figure 12G). The

observed fate of BM-derived cells in the retina is in agreement with a previous study characterizing BM-derived cells in the mouse retina [197].

Flow cytometry of GFP^+ cells in control retinas of chimeric mice was done to further confirm and accurately quantify immunohistochemical data. We observed that 93% of the GFP^+ cells in control retinas were $CD45^-$ cells (Figure 13B). The CD45 marker is expressed by almost all differentiated hematopoietic cells except CACs, endothelial cells, erythrocytes and platelets [198,199], and its expression is reduced on microglia [78,200]. 20% of $CD45^- GFP^+$ cells in control retinas expressed endothelial markers, collagen IV, Tie2 and PECAM-1 (Figure 13C), while 33% of them expressed the microglial markers, $CD45^{dim}$ $CD11b^+$ (Figure 14A).

- Effect of diabetes on BM-derived cells in the retina: To determine whether diabetes has an effect on infiltration of circulating BM cells into the retina, we examined the retinas of chimeric mice at 2 months of diabetes. We observed similar total numbers of GFP^+ cells as well as GFP^+ $CD45^-$ infiltrating diabetic and control retinas (Figure 13A, B). However, only 13% of GFP^+ cells expressed endothelial cell markers compared to 20% in control retinas (Figure 13C), indicating that infiltration and/or differentiation of BM-derived progenitor cells into retinal endothelial cells is deficient in the diabetic retina.

We then examined the effect of diabetes on infiltration of BM-derived microglia-like cells in the retina after 2 months of diabetes. Flow cytometry of retinal cells (selecting for GFP^+ $CD45^{dim}$ $CD11b^+$ cells) revealed that 41% of GFP^+ cells expressed microglial markers compared to 33% in control retinas. The significant increase in percentage of GFP^+ microglia-like cells in diabetic retinas indicates increased infiltration and/or differentiation of

microglia-like cells from BM to the diabetic retina (Figure 14A). High CD11b expression also correlates with increased microglial activation [74,201]. To further explore how diabetes affects activation of BM-derived microglia, we examined phenotype of the microglia in flat-mounted retinas. In the control mouse retinas, most of the BM-derived microglia-like cells (Iba^+ GFP^+) displayed a resting phenotype characterized by their highly ramified morphology. However in the diabetic retina, these BM-derived cells displayed a more amoeboid morphology with retraction of their dendrites, indicating microglial activation. (Figure 14B, C; white arrowheads).

We further studied the effect of diabetes on infiltration of BM-derived pericytes and Müller cells in the retina. From immunohistochemical staining of flat-mounted retinas and retinal sections, we observed that 10% of BM-derived cells in the retina expressed markers of pericytes (PDGF-Rβ) and Müller cells (glutamine synthetase) (Figure 11F). In the diabetic retina, the percentages of BM-derived cells expressing these markers did not change.

Figure 10. Diabetes alters response of BM-derived cells and splenocytes to LPS stimulation.

Increased secretion of cytokines IL-1β and TNF-α in (A) BM-derived dendritic cell-enriched population (B) splenocytes stimulated with LPS. N=4-5, * p< 0.05.

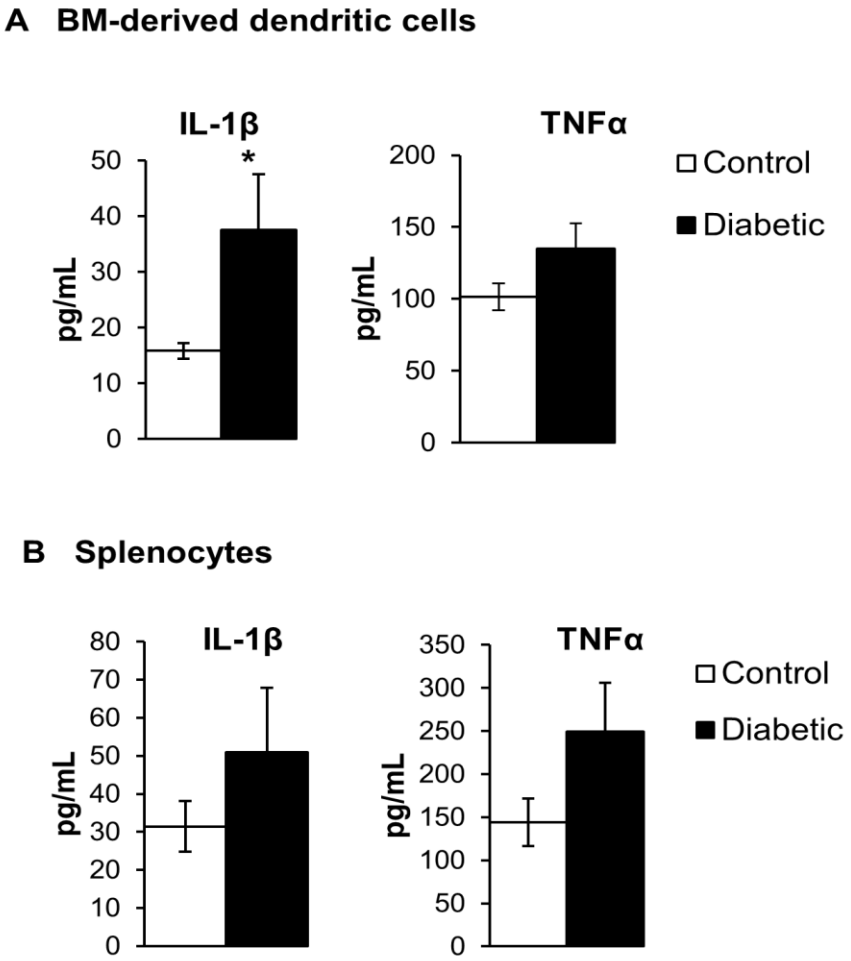


Figure 11. Characterization of GFP$^+$ cells in retina of GFP-BMT mice.

GFP$^+$ cells (green) colocalized with (A) microglial marker Iba-1 (red), (B) endothelial cell marker Collagen IV (red), (C) Müller cell marker Glutamine synthetase (red), (D) pericyte marker PDGFR-β (red) but not with (E) astrocyte marker GFAP (red). Colocalization of GFP$^+$ cells with the respective markers was observed as yellow stain. (F) Percentages of GFP$^+$ cells expressing markers for specific cell types, N= 4-5.

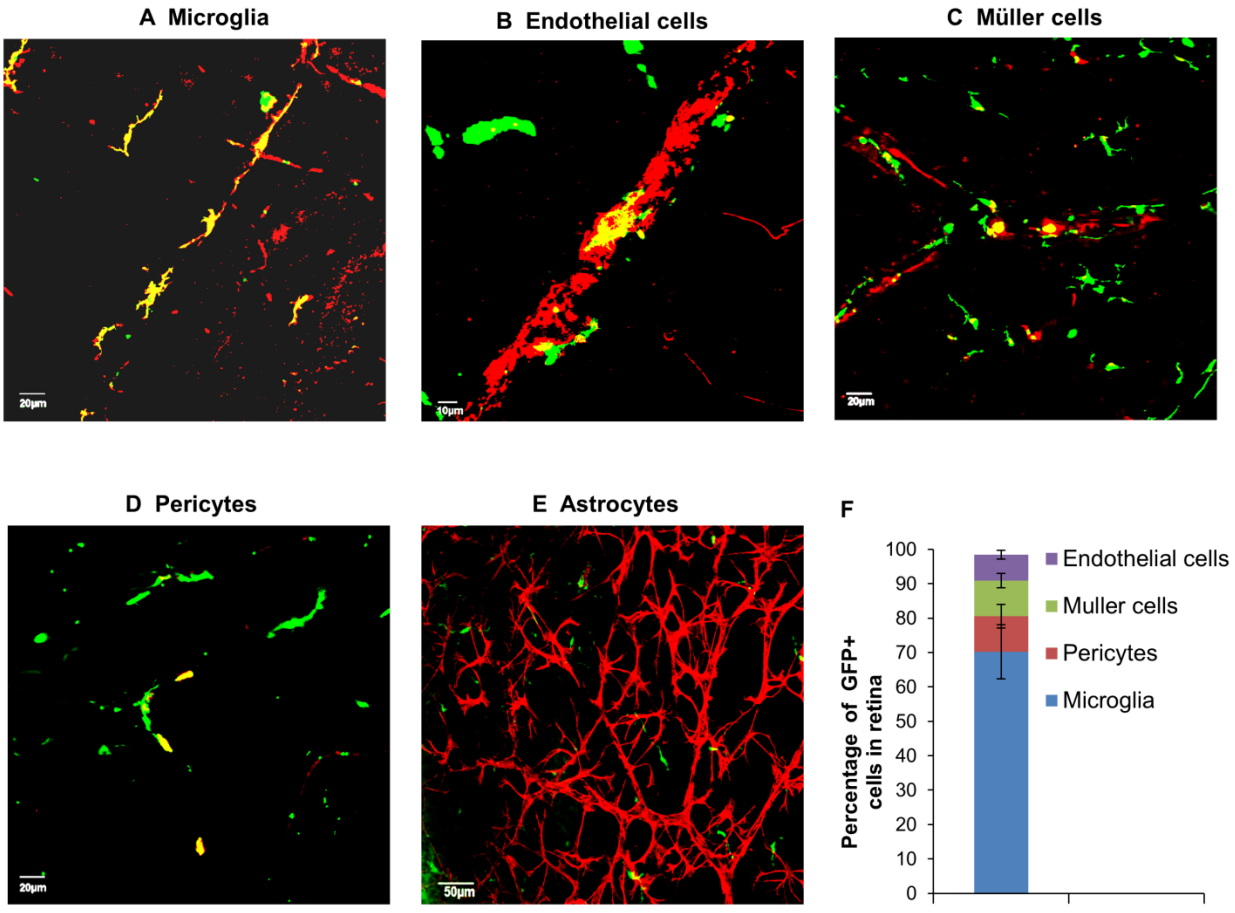


Figure 12. Characterization of BM-derived cells in retinal sections of GFP$^+$ BM chimeras at 2 months after transplantation.

Nuclei were counterstained with DAPI (blue). Overlays of confocal images of GFP$^+$ cells (green) immunoreactive for (A) Iba1 (red) labeling microglia, (B) Collagen IV (red) labeling endothelial cells, (C) Glutamine synthetase (red) labeling Müller glia, (D) PDGF-Rβ (red) labeling pericytes. Colocalization of GFP$^+$ cells with the respective markers was observed (yellow) in (A-D). (E) GFAP (red) labeling astrocytes, (F) Tyrosine hydroxylase (red) labeling amacrine cells, (G) Rhodopsin (red) labeling rod photoreceptors. No colocalization of GFP$^+$ cells with these markers was observed in (E-G). N= 4-5.

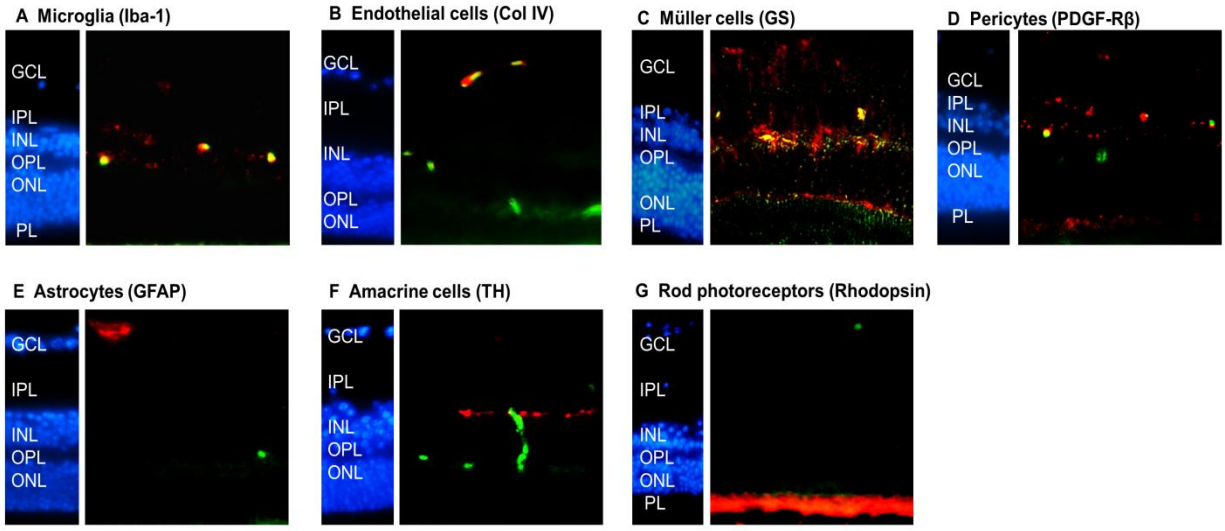


Figure 13. Diabetes alters numbers of BM-derived endothelial cells infiltrating retina.

(A) Number of BM-derived cells per mm^2 area of control or diabetic retina. (B) ~ 93% of GFP^+ cells detected in the retina are $CD45^-$ cells. Diabetes does not change the number of $CD45^-$ cells in the retina N=4. (C) GFP^+ endothelial cells (gated as $CD45^-$ Tie^+ $CD31^+$ cells) are expressed as a percentage of total GFP^+ retinal cells, and indicate a significant decrease in BM-derived endothelial cells in diabetic mice. N= 4-5, * $p< 0.05$.

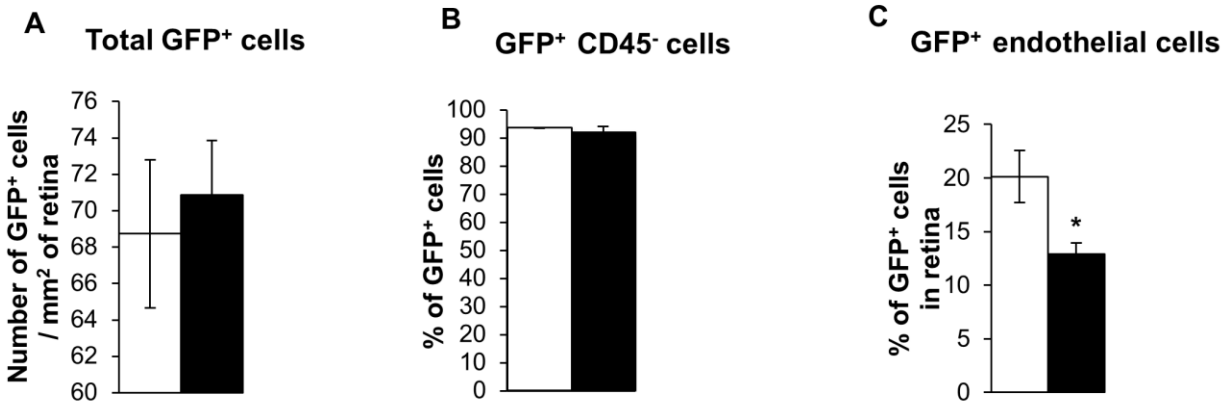
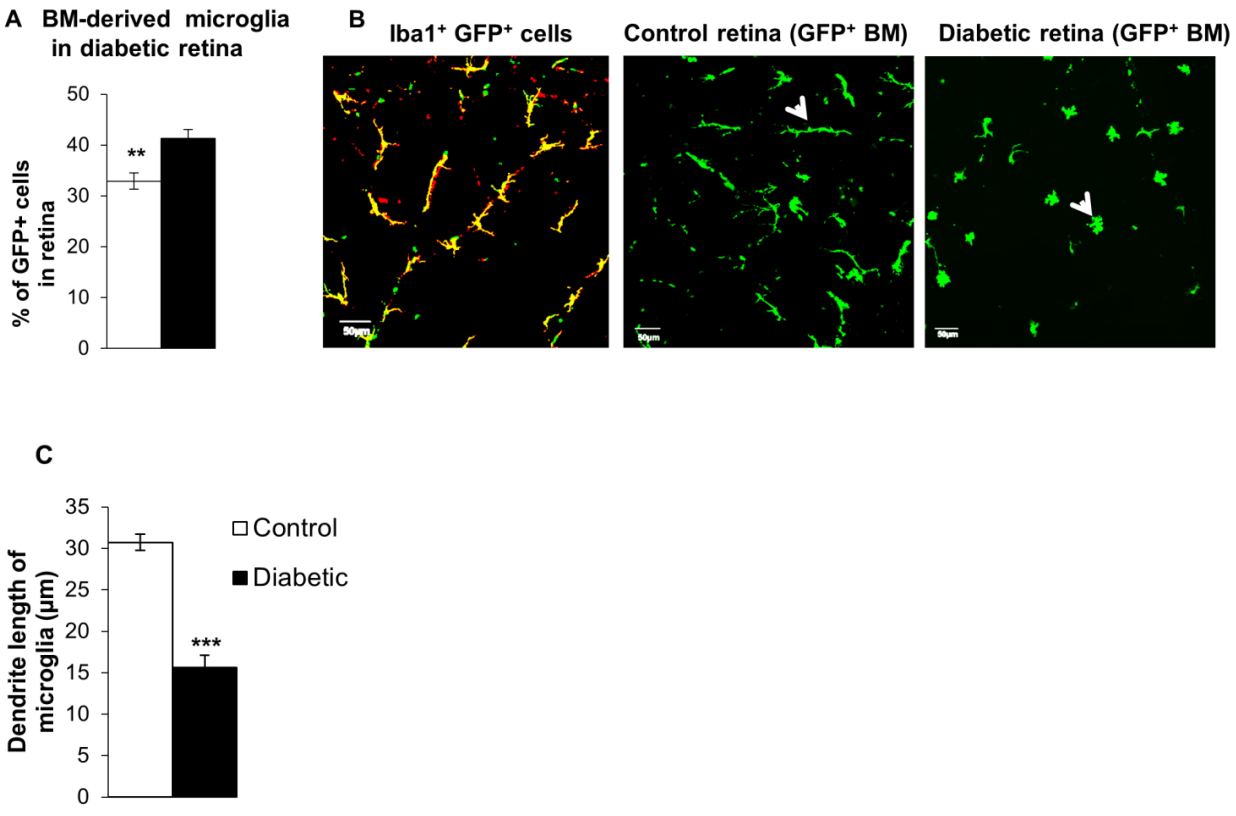


Figure 14. Diabetes alters BM-derived microglia in retina.

(A) GFP^+ microglia (gated as $CD45^{dim}$ $CD11b^+$ cells) are expressed as a percentage of total GFP^+ retinal cells in control and diabetic retina of chimeric mice , and indicate increase in BM-derived microglia in diabetic mice. N=3-5, ** p< 0.01. (B) Confocal images of retina isolated from control or diabetic GFP^+ BM-transplanted mice. Microglial marker $Iba\text{-}1^+$ staining (red) with GFP^+ (green) cells, showing colocalization (yellow) in retina. Increased retraction of processes observed in BM-derived microglia in diabetic GFP^+ chimeric mouse retina compared to ramified, resting phenotype of microglia in control retinas (white arrowheads). (C) Quantification of dendrite length of microglia in diabetic and control chimeric mouse retinas is shown. N=4-5, *** p< 0.001.



2.4 Discussion

Diabetes affects cells in all layers of the retina, leading to chronic low-grade inflammation [25], gradual neurodegeneration [20], loss of endothelial cells [21], leading to formation of acellular capillaries [22] and increased vascular permeability [16,17]. The resulting loss of cellular support leads to microaneurysms, leakage of lipid exudates due to increased vascular leakage, non-functional capillaries and consequently, damage to the retinal tissue [17]. Several molecular elements are involved in the pathology of DR, such as inflammation, accumulation of polyols and advanced glycation end-products (AGEs), chronic oxidative stress and vascular as well as neuronal dysfunction [18,31,44,79,80]. Diminished insulin signaling in retinal neurons may lead to neurodegeneration, which may further contribute to disruption of the blood-retinal-barrier in DR [20].

More recently, another mechanism, inadequate repair by deficient CAC in diabetes has gained interest in the development of DR. The combination of retinal cell damage, pro-inflammatory changes and failed attempts by BM-derived CACs to repair injured retinal capillaries eventually result in progression to clinically significant DR. With significant contribution of BM-derived cells to retinal pathology in DR, it is important to understand the effect of diabetes on BM-derived cells contributing to retinal inflammation, as well as the cells promoting retinal vascular repair.

In agreement with previous studies, our data demonstrate that BM-derived cells infiltrating the retina differentiate into various cell types like pericytes, endothelial cells, Müller cells and microglia (Figure. 11, 12) but not astrocytes or retinal neurons [32,197]. Diabetes did not affect the numbers or activation status of BM-derived Müller cells and pericytes, indicating that these cells may be recruited from BM for normal maintenance functions in both control and diabetic

retina [202]. However, diabetes significantly altered the numbers of BM-derived endothelial as well as microglia-like cells in the retina, suggesting that these BM-derived cell types may contribute to the pathogenesis of DR (Figure 13C, 14A).

Diabetes is known to activate monocytes, which play an important part in promoting a pro-inflammatory environment in the retina [101,191]. Circulating monocytes are classified as $Ly6C^{hi}$ or $Ly6C^{lo}$, depending on their expression levels of Ly6C on the cell surface. $Ly6C^{lo}$ monocytes perform surveillance functions and resolve inflammation, while $Ly6C^{hi}$ monocytes are characterized as inflammatory cells that can be actively mobilized to the diabetic retina, contributing to the observed pathology [87,203]. We and others have previously demonstrated a shift in the profile of BM cells and circulating BM-derived cells towards myeloid cells, contributing to diabetes-associated inflammation [30,31,87,204]. Indeed, reactive ($Ly6C^{hi}$) as well as the patrolling ($Ly6C^{lo}$) monocyte subsets of this BM-derived population (GFP^+ $CD11b^+$) were found to be elevated in the blood of diabetic chimeric mice (Figure 9D). Circulating monocytes can infiltrate the diabetic retina, assume a microglia-like phenotype and contribute to retinal inflammation by secreting pro-inflammatory cytokines and further activating resident glial cells in the retina [75,77,78]. In our study, we demonstrate increased infiltration of BM-derived pro-inflammatory cell types expressing microglial marker CD11b in the diabetic retina (Figure 14A). These data indicate that the BM microenvironment in diabetes induces a shift in hematopoiesis with generation of more pro-inflammatory monocytes released from BM into circulation, leading to accumulation of BM-derived inflammatory cells in the diabetic retina.

During embryonic development, resident tissue microglia are known to develop from a yolk sac and do not have myeloid origin [205]. Several studies demonstrated that in normal tissue, microglial regeneration occurs from tissue-specific microglial progenitors [206]. However,

microglial origin could be shifted towards BM-derived myeloid cells in ageing, inflammation or tissue damage [35,77,78]. In experimental models of DR, microglial activation is usually identified by morphological changes involving retraction of their highly ramified processes and appearance of an amoeboid shape with thicker dendrites and larger cell bodies [33,35]. We observed Iba^+ GFP^+ cells in control and diabetic retinas, however Iba^+ GFP^+ cells in control retinas had branched resting phenotype compared to clearly activated amoeboid phenotype in diabetic retinas, demonstrating for the first time that diabetes promotes activation of BM-derived microglia-like cells in the retina (Figure 14B, C).

The BM serves as a niche for hematopoietic stem cells which, apart from differentiating into lymphoid and myeloid progenitors, are also believed to give rise to CACs, a population of cells that circulates in the bloodstream with the ability to migrate to the site of endothelial injury and mediate repair of damaged blood vessels. These BM progenitors may migrate to the spleen, which serves as a reservoir for CACs, inflammatory monocytes and lymphocytes [88,89,92]. We and others have previously shown that diabetes affects mobilization of CACs into systemic circulation [93,103]. Studies by us and others also indicate that human diabetic CACs have weakened proliferation and migration capabilities, resulting in their ineffective contribution to vascular repair [103,104,105]. In this study, we show that effective release of CACs from BM and spleen into circulation is compromised in an animal model of diabetes (Figure 9A-C).

Further, we have demonstrated that 20% of BM-derived cells that infiltrate the retina express endothelial cell markers such as collagen IV, Tie2, PECAM-1 (Figure 13C). A previous study by Grant *et al* also demonstrated that adult hematopoietic stem cells are capable of migrating to the retina and differentiating into endothelial cells [95]. However, in the diabetic retina, we observed a significant reduction in the percentage of BM-derived cells expressing endothelial markers,

reinforcing the notion that migration and/or differentiation of progenitor cells derived from the BM, into retinal endothelial cells is deficient in diabetes (Figure 13C).

The reduction in number of BM-derived retinal endothelial cells in diabetes was accompanied by aberrant activation of splenocytes and BM-derived dendritic cells upon LPS stimulation. Immature DC can be activated by LPS and secrete cytokines that influence the leukocyte immune response [192,193,194]. Lymphocytes, dendritic cells and monocytes stored in the spleen may also contribute to inflammation in response to injury [92,192]. Here we show that LPS stimulation leads to increased secretion of pro-inflammatory cytokines such as TNF-α and IL-1β by splenocytes and BM-derived dendritic cells enriched from diabetic mice, indicating that these immune cells may also contribute to inflammation in diabetes (Figure 10).

Our study is in agreement with the main findings of a previous study by Li *et al*, on the role of pro-inflammatory marrow-derived cells in DR. However, there are important differences in the design of the two studies. In the Li *et al.* study diabetes was induced two weeks before BM transplantation [32]. As one of the goals of our study was to evaluate homing and migration of progenitor cells between different niches, we wanted to separate the effects of irradiation from the effects of diabetes and allowed for stable bone marrow engraftment (4-5 months) before induction of diabetes. This study design with BM transplantation before induction of diabetes assures that there are no effects of diabetes on homing efficiency of transplanted BM cells and subsequent re-population of stem cells in the BM niche.

In conclusion, this study identified a significant shift from reparative to pro-inflammatory BM-derived cells in the retina in diabetes. Reduced numbers of reparative BM-derived CACs in circulation, leading to reduction in the number of BM-derived endothelial cells in the retina was

observed in diabetic animals. In contrast, diabetes induced higher numbers in circulation, as well as retinal infiltration of pro-inflammatory myeloid cells giving rise to activated microglia-like population in diabetic retina. Control of BM-derived cell populations with normalization of the reparative/pro-inflammatory cells balance could represent a viable cell therapy option to enhance available DR treatments.

2.5 Methods

- Mice. All procedures involving animal models were permitted by Institutional Animal Care and Use Committee at MSU. Male C57BL/6J and C57BL/6-Tg(CAG-EGFP) mice were purchased from Jackson Laboratory. Mice were injected with 65 mg/Kg of streptozotocin (STZ) in 0.5% sodium citrate solution every day, for five consecutive days. The control mice were injected with the sodium citrate buffer. Mice with blood glucose levels higher than 13.8 mmol/L were identified as diabetic. Starting 14 days after STZ injections, mice were injected with insulin (0-2 units/day) to prevent acute weight loss, but maintaining them in the hyperglycemic range (around 20 mmol/L blood glucose).
- Generation of GFP^+ chimeric mice. The C57BL/6-Tg(CAG-EGFP) transgenic strain were purchased from Jackson Laboratory. The C57BL/6.GFP^+ chimeric mice were produced by irradiation of recipient 8-weeks old C57BL/6 mice with 1100 rads, and bone marrow (2 X 10^6 cells) was injected retroorbitally from donor GFP^+ mice. After 120-130 days to allow stable hematopoietic reconstitution, we performed flow cytometry to enumerate GFP^+ cells in the BM of chimeric mice. Diabetes was induced using STZ as described above.
- Tissue preparation and Immunohistochemistry. Mouse eyes were pierced with a 30-gauge needle, and fixed in freshly prepared 4% paraformaldehyde for 1 hour. The eyes were then washed in PBS 3 times, and retinas dissected. Isolated intact retinas were permeabilized in

HEPES-buffered saline witt 0.1% Tween-20 and 1% BSA, and incubated at 4°C overnight. Vasculature was stained with rabbit anti-collagen IV (Abcam) diluted 1:400, followed by PBS wash. Secondary antibody chicken anti-rabbit (Alexa Fluor 594, Invitrogen), at 1:1000 dilution was used. For characterization of vascular and perivascular GFP^+ cells in chimeric mice, retinas were further stained with primary antibodies: endothelial cells using 1:400 diluted rabbit anti-collagen IV (abcam); astrocytes using 1:200 diluted rabbit anti-GFAP (Cell Signaling); microglial cells using 1: 100 diluted goat anti-Iba1 (Novus Biologicals); pericytes using 1:100 diluted rabbit anti-PDGFR-β (abcam); and Müller cells using 1:300 diluted rabbit anti-glutamine synthetase (Novus Biologicals). After overnight incubation at 4°C and three PBS washes, respective chicken anti-rabbit or anti-goat secondary antibodies (Alexa Fluor 594, Invitrogen) diluted 1:1000 was used, stained specimens incubated for an hour, followed by a final wash in PBS. Retinas were flat-mounted with four slits, and kept on glass slides with Fluoromount mounting medium (Sigma).

- Tissue sectioning and Immunohistochemistry. Tissue samples previously fixed in Zinc Fixative (BD biosciences) were processed and vacuum infiltrated with paraffin using ThermoFisher Excelsior tissue processor, and embedded with ThermoFisher HistoCentre III embedding station. After cooling of the blocks, surplus paraffin was removed, and they were placed on a Reichert Jung 2030 rotary microtome exposing the sample. Then the blocks were cooled and finely sectioned at 4-5 microns. Sections were dried at a 56°C slide incubator to allow adherence to slides for 2 – 24 hours. Slides were then deparaffinized and rinsed in several changes of distilled water followed by Tris buffered saline pH 7.4. Sections were incubated with 10% blocking serum (chicken serum, Santa Cruz Biotechnology) in PBS for 20 minutes to prevent non-specific IgG binding, and then washed with PBS. For

localization of GFP^+ cell types in the different layers of the retina, retinas were stained with primary antibodies for vascular and perivascular cells such as endothelial cells, astrocytes, microglial cells and Müller cells using antibodies and dilutions described above. Further, retinas were also stained for retinal neuronal cells such as amacrine cells using 1:200 diluted rabbit anti-tyrosine hydroxylase (Millipore), rod photoreceptors using rabbit anti-rhodopsin (Sigma) at 1:200 dilution and ganglion cells using goat Brn-3a (Santa Cruz Biotechnology) at 1:50 dilution. After 1 hour incubation and three PBS washes, sections were incubated for an hour with Alexa Fluor 594-conjugated chicken secondary antibody against rabbit or goat (Invitrogen), diluted to 5 µg/ml, followed by a final wash in PBS. Retinal sections were mounted on coverslips with Fluoromount medium (Sigma).

- Sample processing and LPS treatment. Mice were euthanized and tibias and femurs were collected. Ice-cold PBS was used to flush bones, and single cell suspension was made. Ammonium chloride (STEMCELL technologies) was used to eliminate erythrocytes contaminating the bone marrow cells. For enrichment of dendritic cells, 1 million BM cells per well were incubated for 7 days at 37°C in R10 medium (RPMI 1640, 10% fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin and 55 µM β-mercaptoethanol) with supplementation of 10 ng/mL of GM-CSF (Peprotech). The culture medium was changed every 2 days by removing 50% of medium and replacing with fresh medium and supplements. Spleen was gently crushed, subjected to red blood cell lysis and then filtered through a 40µm nylon mesh. 1 million splenocytes per well were also maintained in R10 medium for 7 days. The dendritic cell-enriched population from BM and splenocytes were stimulated with 10 ng/mL of lipopolysaccharide (LPS, Sigma) for 24 hours. Culture

supernatant was collected and stored at -80°C. ELISA (eBiosciences) was performed to measure cytokine levels of TNF-α and IL-1β.

- Cell preparation and Flow cytometry. Cells from spleen and BM were obtained as described earlier. Heparinized tubes were used for blood collection, and Histopaque 1083 (Sigma) was used to isolate mononuclear cells, according to manufacturer's instructions. Eyes were collected, the retinas were isolated and disrupted mechanically by vigorous pipetting and digestion with 0.5 mg/ml collagenase D (Roche, Indianapolis, IN) and 750 U/ml DNase (Sigma) in HBSS for 15 min at 37 ^{o}C according to Kerr *et al* [207]. One million cells were stained with the appropriate antibodies on ice for 30 minutes according to standard cell surface staining protocol. The primary conjugated monoclonal antibodies that were used were purchased from BD biosciences or ebiosciences: PE-CD34 (RAM34), PerCPCy5.5 – Ly6A/E (D7), biotin- lineage (CD3e, CD45RA, GR1, CD11b, TER119), Alexa Fluor 700-CD45 (30-F11), APC-CD309 (Avas 12α1), PECy7-CD117 (2B8), streptavidin APC efluor780, PE-Tie2 (TEK4), PerCP efluor710-CD31 (390), APC Cy7-CD11b (M1/70), PerCPefluor 710-F4/80 (BM8), PE-Ly6G (1A8), PECy7-Ly6C (AL-21), APC-CD90.2 (53-2.1). Dead cells were identified using DAPI. Data was acquired with a LSR II instrument (BD) with three lasers at 488, 405 and 640 at the Flow Cytometry Core at Michigan State University and data were analyzed with FlowJo software (Tree Star, Inc.).
- Data Collection and Analysis. Digital images of flat-mounted retinas were generated using an Olympus FluoView 1000 confocal laser scanning microscope. For imaging retinal sections, a Nikon TE2000 fluorescence microscope with CoolSNAP HQ2 Photometrics camera was used. At least three fields in each retina were captured and analyzed. Colocalization of green (for GFP^{+} cells) and red (stained vascular endothelium or other retinal cells) fluorescence

was examined and area of fluorescence calculated using MetaMorph program (Molecular Devices).

- Statistical analyses. Data are presented as mean ± S.E.M. Results were evaluated for statistical significance by the Student's t-test or one-way ANOVA followed by Tukey's or Bonferroni's post-hoc test (GraphPad Prism5, GraphPad Software, San Diego, CA), where appropriate.

Chapter 3. Role of acid sphingomyelinase in shifting the balance between pro-inflammatory and reparative bone marrow cells in diabetic retinopathy

This chapter is a modified version of an article accepted in Stem Cells.

Authors: Harshini Chakravarthy, Svetlana Navitskaya, Sandra O'Reilly, Jacob Gallimore, Hannah Mize, Eleni Beli, Qi Wang, Nermin Kady, Chao Huang, Gary J. Blanchard, Maria B. Grant, Julia V. Busik.

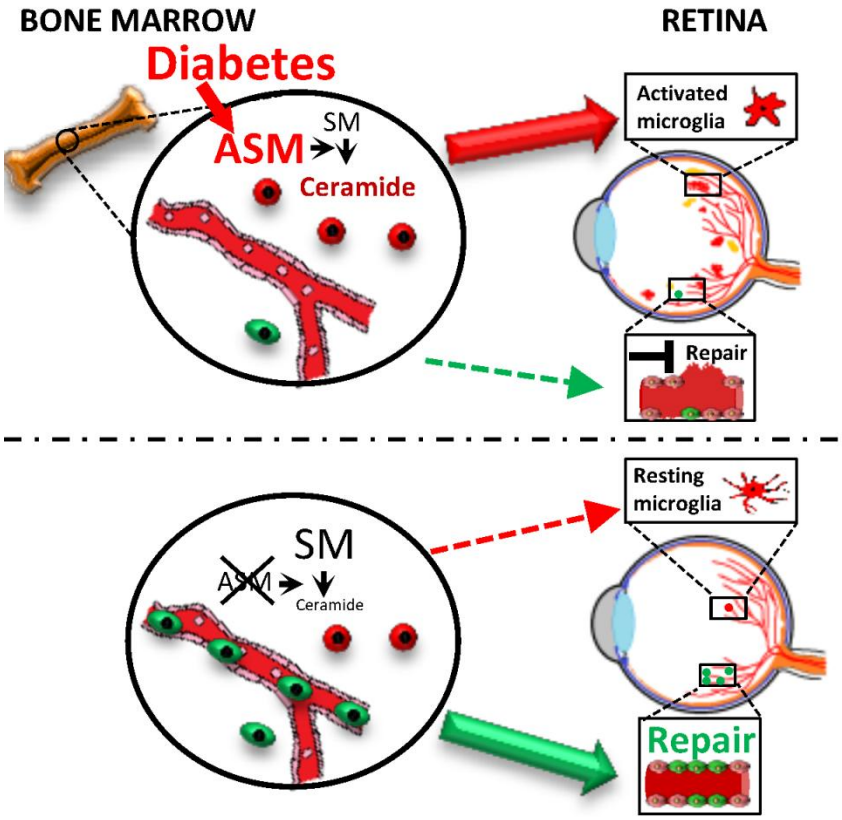
3.1 Abstract

The metabolic insults associated with diabetes lead to low-grade chronic inflammation, retinal endothelial cell damage and inadequate vascular repair. This is partly due to the increased activation of bone marrow (BM)-derived pro-inflammatory monocytes infiltrating the retina, and the compromised function of BM-derived reparative circulating angiogenic cells (CACs), which home to sites of endothelial injury and foster vascular repair. We now propose that a metabolic link leading to activated monocytes and dysfunctional CACs in diabetes involves upregulation of a key enzyme of sphingolipid signaling, acid sphingomyelinase (ASM). Selective inhibition of ASM in the BM prevented diabetes-induced activation of BM-derived microglia-like cells and normalized levels of pro-inflammatory cytokines in the retina. ASM upregulation in diabetic CACs caused accumulation of ceramide on their cell membrane, thereby reducing membrane fluidity and impairing CAC migration. Replacing sphingomyelin with ceramide in synthetic membrane vesicles caused a similar decrease in membrane fluidity. Inhibition of ASM in diabetic CACs improved membrane fluidity and homing of these cells to damaged retinal vessels. Collectively, these findings indicate that selective modulation of sphingolipid

metabolism in BM-derived cell populations in diabetes normalizes the reparative/pro-inflammatory cell balance, and can be explored as a novel therapeutic strategy for treating diabetic retinopathy.

Figure 15. Graphical Abstract

Top panel: Diabetes causes pathological upregulation of acid sphingomyelinase (ASM) in the bone marrow (left), leading to infiltration of pro-inflammatory monocytes (red cells) and impaired vascular repair by circulating angiogenic cells (green cells) in the retina (right). Bottom panel: Inhibition of ASM in the bone marrow restores the balance between pro-inflammatory and reparative cells and prevents diabetes-induced retinal vascular degeneration.



3.2 Introduction

Diabetic retinopathy (DR) is a sight-threatening complication of diabetes. Available DR treatment options are highly invasive and only help to slow progression of the disease. Hyperglycemia and dyslipidemia are major metabolic abnormalities in diabetes. Molecular pathogenesis of hyperglycemia and dyslipidemia-induced damage to the retina is not clear, but may involve several mechanisms including ER stress, accumulation of polyols and advanced glycation end-products (AGEs), oxidative stress, protein kinase C activation and low-grade inflammation [18,31]. Although clinical manifestations of DR are due to vascular abnormalities including increased microvascular permeability, vessel leakage, microaneurysms formation, and eventually capillary drop-out and pathological neovascularization [18], recent studies demonstrated that most retinal cell types contribute to pathogenesis of the disease. Activated retinal glial cells and pigment epithelial cells in diabetes express pro-inflammatory cytokines and VEGF, contributing to damage of endothelial cells lining the retinal vasculature [58,78,208]. Impaired retinal insulin receptor signaling contributes to metabolic stress to neurons, which may in turn impair blood-retinal barrier integrity [154].

In addition to direct effects on the retina, hyperglycemia and dyslipidemia can indirectly affect the retina through the induction of BM pathology. Hematopoietic stem cells in the BM give rise to lymphoid and myeloid progenitors that differentiate into blood cells. BM pathology in diabetes is proposed to cause activation of circulating myelomonocytic cells, leading to increased leukocyte adhesion, which contributes to retinal inflammation [30,32]. Several studies have found that myeloid-derived monocytes can infiltrate the diabetic retina, adopt a microglia-like phenotype and exacerbate inflammation by secreting pro-inflammatory cytokines. This cytokine

secretion can further activate resident microglia, astrocytes and Müller glia in the retina [75,77,78].

We have previously demonstrated that, in addition to increase in pro-inflammatory leukocytes, diabetes induces a reduction of vascular reparative cells [87,103]. Circulating angiogenic cells (CACs) are BM-derived vascular reparative cells known to be exquisitely sensitive to the diabetic milieu [105,108,172]. Release of CACs from BM into circulation and extravasation into tissues is a critical component of their physiological role as reparative cells. Indeed, there is growing interest in understanding stem/progenitor cell migration and developing interventions that increase homing efficiency of CACs to sites of tissue damage [108,169,209,210]. Recent studies indicate that CACs isolated from diabetic individuals are ineffective at vascular regeneration due to reduced migratory and proliferation abilities [105,108,209]. We recently demonstrated that reduced deformability of human CACs in diabetes is linked to decrease in migratory prowess of these cells [108]. Thus, modulating cell deformability can provide a way of improving mobilization efficiency of CACs to sites of vascular injury.

Hyperglycemia and dyslipidemia accompanying diabetes are likely to play crucial roles in the observed inflammation and impaired vascular repair in the retina, although the precise molecular steps leading to it are not completely determined. Data from clinical studies demonstrate robust associations between dyslipidemia and progress of DR [10,82]. Bioactive sphingolipids like ceramide, glycosphingolipids and sphingosine 1-phosphate (S1P) are believed to play crucial roles in development of DR [138,141,142,149,150]. Acid sphingomyelinase (ASM), the enzyme converting sphingomyelin to ceramide, is a key element activated by diabetes in both CACs and retinal vasculature. We have previously shown that ASM is important for cytokine signaling in

endothelial cells lining the retinal vasculature, and that inhibiting ASM prevents retinal inflammation and microvascular degeneration accompanying diabetes [150,172,173]. In this report, we describe a link between increased ASM activity in the BM cells, and shift in BM-derived inflammatory and reparative cell function in the diabetic retina.

3.3 Results

- ASM-deficiency in bone marrow prevents inflammation in diabetic retina: To address the specific role of ASM in affecting BM cells, C57BL/6J mice were transplanted with $ASM^{-/-}$, $ASM^{-/-}X\ gfp^{+}$, or WT age-matched BM. After 4 months to allow stable reconstitution, mice were made diabetic with STZ injections. Body weights and blood glucose levels for control and diabetic chimeras are shown in Table 1. By 2 weeks of diabetes, the weights of diabetic mice were significantly reduced and their blood glucose levels were significantly higher than controls. $ASM^{-/-}$ → WT and WT→ WT BM transplanted (BMT) diabetic mice had similar degree of diabetes throughout the study (Table 1).

  Previous studies showed that diabetes causes accumulation of BM-derived monocytes in the retina. These monocytes adopt a microglia-like phenotype and contribute to retinal inflammation by secreting pro-inflammatory cytokines [75,77,78]. Microglial activation is usually identified by morphological changes, involving retraction of highly ramified processes and assuming amoeboid shape with thicker dendrites and larger cell bodies [33,35,43]. We anticipated that $ASM^{-/-}$ phenotype in the diabetic BM may influence activation of BM-derived microglia-like population. To distinguish cells migrating to the retina from the BM, retinas from gfp^{+}.WT and $ASM^{-/-}$ X gfp^{+}.WT BM chimeras were examined after 3 months of diabetes. Although no significant difference in numbers of BM-

derived microglia-like cells was observed in chimeric mice, more activation of BM-derived gfp^+ microglia was seen in retinas of diabetic gfp^+.WT BM chimeras. $ASM^{-/-}$ X gfp^+.WT BMT prevented this increase in microglial activation in diabetic retinas, (Figure 16A) indicating that ASM upregulation in these cells may contribute to inflammation in the diabetic retina.

The pathogenesis of DR involves chronic low-grade inflammation, and studies show that activated glial cells may contribute to cytokine production in the diabetic retina [31,33,35,43]. The 2-fold or less changes in inflammatory markers in the diabetic retina reported here are consistent with low-grade inflammation associated with DR [78]. ASM deficiency in BM prevented diabetes-associated upregulation of inflammatory markers and growth factors like IL-1β, VEGF, ASM, ICAM-1, and VCAM-1 in retina of chimeric mice after 2 months of diabetes (Figure 16B).

In order to visualize retinal neuronal changes, we measured the thickness of the inner retinal layers and performed TUNEL staining of retinal sections. No gross differences in retinal structure and inner retinal thickness were observed in control, diabetic and diabetic mice with $ASM^{-/-}$ BM at 8 weeks of diabetes, in agreement with a previous study [45] (Figure 17B). The number of apoptotic neurons detected by TUNEL-positive cells was also similar in all groups (Figure 17A), which is in agreement with Feit-Leichman *et al* showing that neuronal apoptosis does not change significantly at 2-3 months of diabetes in C57BL/6J strain, despite other evidences of retinal vascular pathology [24].

- ASM-deficiency in bone marrow improves CAC release and homing to retinal vasculature: Apart from giving rise to all blood cells, HSC in the BM also differentiate into reparative

CACs, which circulate in blood and home to injured regions to mediate microvascular repair [95]. Accumulating evidence suggests that BM neuropathy affects CAC proliferation, release and migration in diabetes [103]. We observed impaired release of CACs, leading to higher number of CACs in BM of 2 month-diabetic WT chimeras (Figure 19A) as well as lower numbers of diabetic CACs in circulation (Figure 19B), which was prevented in diabetic $ASM^{-/-}$ BM chimeras.

To directly assess effect of ASM inhibition in CACs on *in vivo* homing capacity, we injected gfp^{+}-expressing CACs treated with ASM-siRNA into vitreous of 8-9 month diabetic WT mice. ~ 5-fold inhibition of ASM was achieved (Figure 18). Seven days post-injection, retinas were analyzed for colocalization of retinal vasculature with gfp^{+} progenitor cells. When healthy CACs were injected into the vitreous of diabetic mice, we observed their participation in vessel repair as demonstrated by yellow color, due to colocalization of green (gfp^{+} CACs) and red (retinal vasculature staining) (Figure 19C, left panel). Diabetic CACs lose this ability and do not migrate to the site of injury (Figure 19C, middle panel); however, diabetic CACs after ASM inhibition participated in repair to similar levels as control CACs (Figure 19C, right panel). These data indicate that specific inhibition of ASM in CACs restores their reparative capacity in a DR model.

- ASM inhibition reduces ceramide levels in CACs and BM cells: To assess effect of ASM deficiency on sphingolipid metabolism in BM cells, we characterized SM and ceramide species using mass spectra from lipid extracts of wildtype and $ASM^{-/-}$ BM cells and CACs. A dramatic increase in SM and concomitant decrease in ceramide was observed in $ASM^{-/-}$ BM cells and CACs compared to wildtype (Figure 20A, 20B, Table 2, Figure 21).

Ceramide metabolites such as glucosylceramides, sphingosine and S1P play important roles in insulin resistance, as well as cellular proliferation and migration [142,162]. We examined the effect of ASM deficiency on ceramide metabolites in $ASM^{-/-}$ BM cells and CACs compared to wildtype. While we observed a decrease in some hexosylceramide species, we did not observe any significant differences in sphingosine or S1P levels (Table 2).

We next determined the effect of diabetes on CACs ceramide levels in WT and ASM-/- BMT mice. As limited amount of material isolated from these stably engrafted long term diabetic mice does not allow for detailed mass spectrometry analysis, we utilized an alternative immunohistochemistry method using anti-ceramide antibodies. ~50% increase in ceramide level was observed in diabetic CACs. In contrast, ASM-/- BMT prevented this increase in ceramide levels in diabetic mice (Figure 20C).

- Ceramide levels alter membrane viscosity of synthetic vesicles and progenitor cells: Membrane deformability can be modulated by membrane lipid content [179,211]. We first determined the effect of ceramide on membrane viscosity of artificial vesicles by replacing 50% of sphingomyelin with equal length ceramide in model lipid bilayers. To measure membrane viscosity, we labeled synthetic vesicles with a fluorescent probe, perylene (Figure 22A)- nonpolar polycyclic aromatic hydrocarbon that incorporates selectively into bilayer nonpolar region with well characterized rotational diffusion behavior [189]. Viscosity of extruded synthetic vesicles was quantitated by rotational diffusion of perylene. We observed that replacing sphingomyelin with ceramide in synthetic vesicles caused a significant increase in viscosity of membranes (Figure 22A). We then determined the effect of ceramide on membrane viscosity of CACs. Membrane viscosity decreases significantly in $ASM^{-/-}$ CACs, indicating that loss of ASM prevents cell membrane rigidity in CACs (Figure 22B).

- $ASM^{-/-}$ BM transfer corrects diabetes-induced deficiency in CAC migration: Because migration of CACs is central to their function as reparative cells, we sought to understand if change in membrane rigidity due to ceramide is associated with diabetes-induced reduction in CAC motility. Increased membrane rigidity in CACs isolated from mice at 3 months of diabetes correlates with their reduced migration ability. However, membrane rigidity was normalized in diabetic CACs from $ASM^{-/-}$ BM chimeras, which further correlated with improved migration ability (Figure 22 C,D).
- Retinal vascular damage is prevented in diabetic $ASM^{-/-}$ BM chimeric mice: We examined whether the combination of improved CAC migration and reduced activation of BM-derived inflammatory cells could prevent retinal vascular degeneration in diabetic $ASM^{-/-}$ BM chimeras. We observed increased vascular permeability as assessed by leakage of FITC-albumin compared to control chimeras, and this increase was prevented in diabetic $ASM^{-/-}$ BM chimeras (Figure 23A).

  Acellular capillaries are a histopathological hallmark of DR. We observed a significant increase in acellular capillaries in diabetic as compared to control WT BM chimeric mice (Figure 23B). This increase was prevented in diabetic $ASM^{-/-}$ BM chimeras, supporting the hypothesis that ASM inhibition selectively in the BM protects from retinal vascular pathology in diabetes.

Table 1. Average body weights and non-fasting blood glucose levels of control and diabetic mice with WT or ASM$^{-/-}$ bone marrow transplantation.

Duration of diabetes (Weeks)	Treatment Group	Blood glucose (mg/dL)	Weight (g)
2	Control (WT bone marrow)	134 ± 6.9	26 ± 0.6
2	Diabetic (WT bone marrow)	361 ± 19.3*	21.2 ± 0.5*
2	Diabetic (ASM$^{-/-}$ bone marrow)	418 ± 21.0*	22.6 ± 0.7 †
8	Control (WT bone marrow)	140 ± 8.9	26.2 ± 0.6
8	Diabetic (WT bone marrow)	427 ± 46.6*	19.4 ± 0.3*
8	Diabetic (ASM$^{-/-}$ bone marrow)	426 27.4*	18.9 ± 0.6*

n= 7-9, Data expressed as mean ± s.e.m.

* Significantly different from age-matched control mice ($p < 0.001$).

† Significantly different from age-matched control mice ($p < 0.01$).

Figure 16. ASM deficiency in bone marrow prevents inflammation in diabetic retina.

A: Microglial marker Iba-1^{+} staining (red) with gfp^{+} (green) cells, showing colocalization (yellow) in retina. Control retinas show gfp^{+} microglia with highly ramified phenotype (white arrow). Diabetic retinas show gfp^{+} microglia with retracted processes (white arrowhead). Diabetic retinas from ASM$^{-/-}$ BM chimeric mice show ramified phenotype (white arrow). Quantification of dendritic length of microglia is shown on the right. n=8 per group. *** p<0.001 relative to diabetic. Scale bar is 50 μM. Duration of diabetes was 3 months.

B: Quantitative PCR for relative mRNA levels of indicated genes in diabetic retinas relative to controls. Duration of diabetes was 2 months. n= 7-10 per group. * p<0.05, **p<0.01 and ***p<0.001 relative to control. ##p<0.01 and ###p<0.001 relative to diabetic. Data are expressed as means ± s.e.m for all.

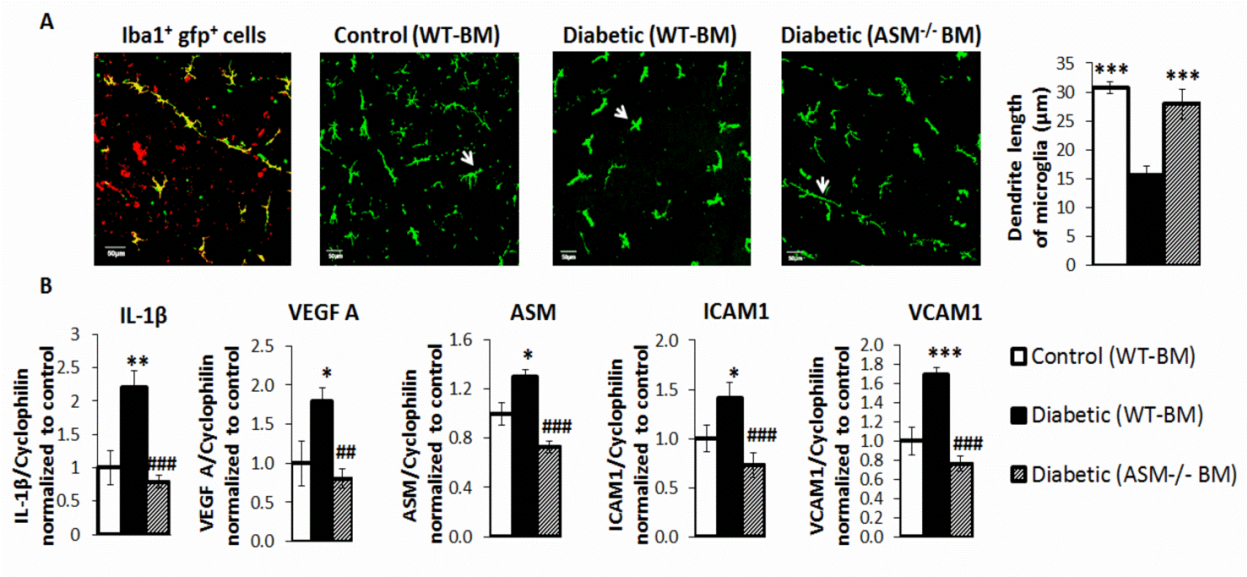


Figure 17. ASM deficiency in diabetic bone marrow does not lead to retinal neuronal changes.

A: TUNEL staining of retinal sections does not show significant changes in neuronal viability in retinas of chimeric control (left), diabetic (middle) and diabetic $ASM^{-/-}$ BM (right) chimeric mice (black arrowheads). Quantification is shown below.

B: Thickness of retinal layers was measured in retinal sections. No significant change in inner retinal thickness was detected. GCL- ganglion cell layer, IPL- inner plexiform layer, INL- inner nuclear layer, OPL- outer plexiform layer, ONL- outer nuclear layer, PL- photoreceptor layer, n=4-5 mice per group, scale bar is 20 μM, duration of diabetes was 8 weeks, data expressed as mean ± s.e.m.

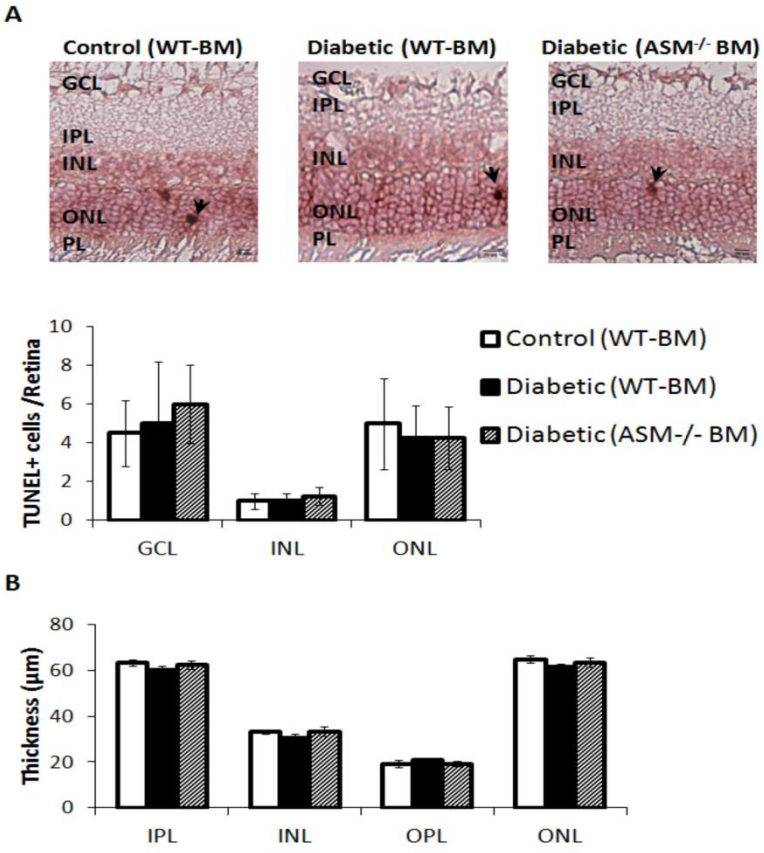


Figure 18. ASM inhibition in CACs.

~ 5-fold inhibition of ASM using siRNA treatment, quantified by real-time PCR and Western blot. n=10-12 per group.

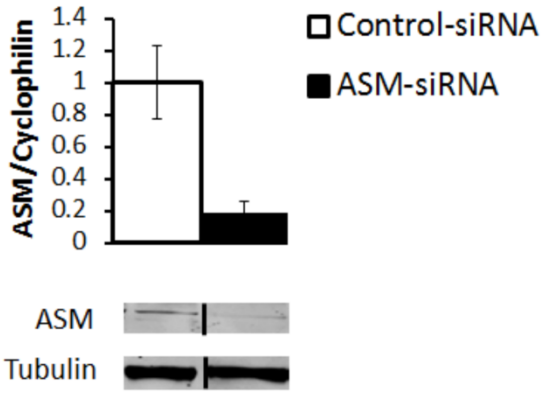


Figure 19. ASM inhibition improves CAC release and incorporation into retinal vasculature.

A: Percentages of CACs in BM of 2 months diabetic WT chimeric mice is increased compared to non-diabetic chimeras, and this is prevented in diabetic $ASM^{-/-}$ BM chimeras. n= 4-8 per group, ** p<0.01 relative to control, ##p<0.01 relative to diabetic.

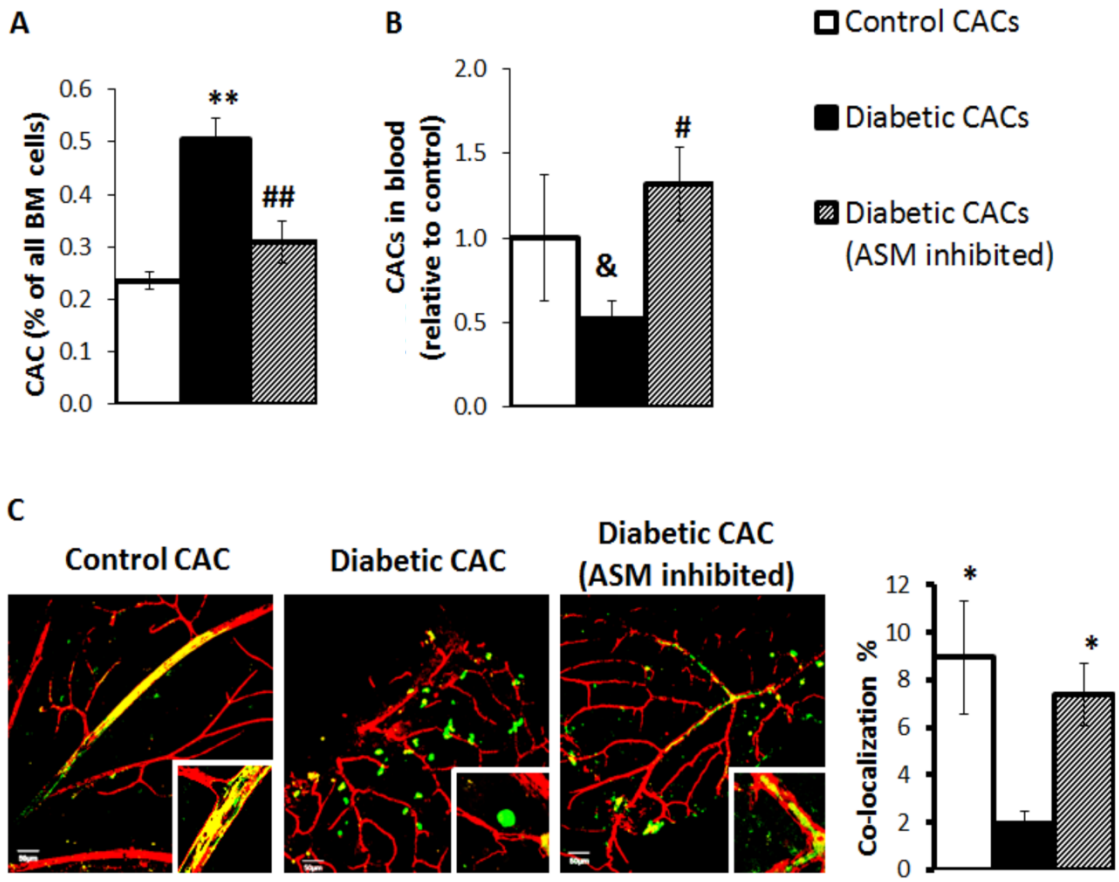


Figure 19 (cont'd)

B: Percentages of CACs in blood of 2 months diabetic WT chimeric mice is decreased compared to non-diabetic chimeras, and this is prevented in diabetic $ASM^{-/-}$ BM chimeras. n= 4-8 per group, & p= 0.07 relative to control, #p<0.01 relative to diabetic.

C: Diabetic animals received intravitreal injections of control (left), diabetic (middle) or diabetic CACs treated with siRNA for ASM (right). CACs (green) were isolated from gfp^{+} mice, retinal vasculature was stained with anti-collagen IV antibody (red), and colocalization (yellow) indicates vascular association. Diabetic CACs show reduced colocalization with vasculature (middle), while ASM inhibition improves vascular association of diabetic CACs (right). Quantification of colocalization (yellow) is shown on far right. Duration of diabetes was 8-9 months. * p< 0.05 relative to diabetic. Data are expressed as means ± s.e.m. Scale bars are 50 μM.

Figure 20. Effect of ASM inhibition on sphingolipid metabolism in bone marrow cells and CACs.

Total ceramide and ratio of total ceramide to total sphingomyelin (SM) were significantly decreased, and total SM significantly increased in ASM$^{-/-}$ BM cells (A) and CACs (B). n= 6 per group, ** p<0.01, *** p<0.001. Data are expressed as means ± s.d.

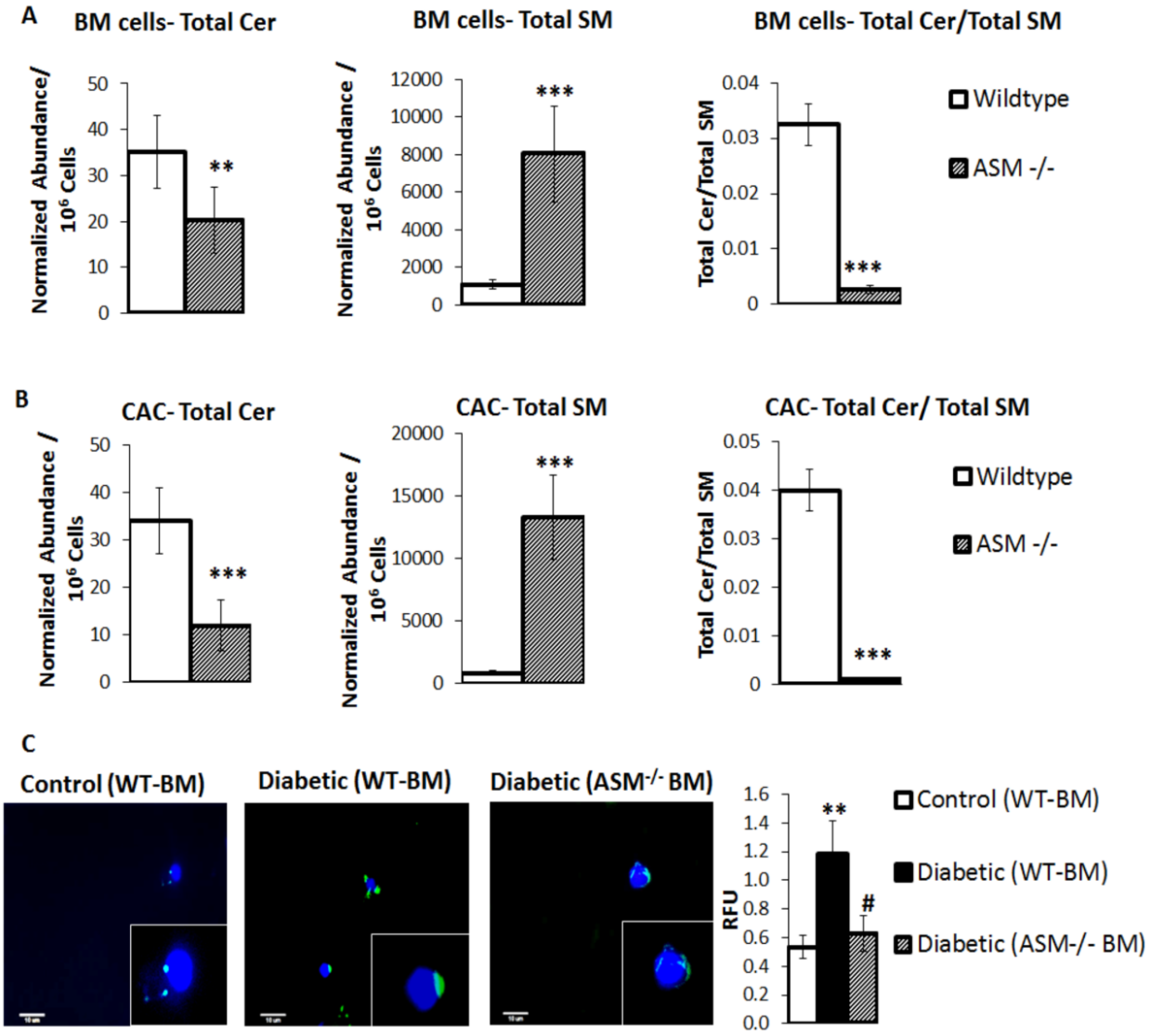


Figure 20 (cont'd)

C: Immunofluorescence using anti-ceramide antibody (green) demonstrates that diabetic CACs from WT BM chimeras (middle) have increased ceramide levels compared to control CACs (left). This increase is prevented in diabetic CACs from $ASM^{-/-}$ BM chimeras (right). Scale bar is 10 μM. Quantification of green fluorescence relative to DAPI nuclear stain is shown on far right. Duration of diabetes was 3 months. Data are expressed as means ± s.e.m. n= 4-5 per group. ** $p<0.01$ relative to control, #$p<0.05$ relative to diabetic. RFU, relative fluorescence units.

Table 2. Sphingolipids in wildtype and ASM$^{-/-}$ mouse CAC and bone marrow cells.

Significant increases or decreases in ASM$^{-/-}$ compared to wildtype are indicated by arrows pointing upwards or downwards respectively.

	Mouse CACs				Mouse bone marrow cells			
Lipid species Normalized abundance/ 10^6 cells	**Wildtype**	**ASM$^{-/-}$**	**Difference**	**P**	**Wildtype**	**ASM$^{-/-}$**	**Difference**	**P**
Cer (d18:0/14:0)					0.36	0.32		0.896
Cer (d18:0/16:0)	0.68	1.11		0.412	1.13	0.69		0.309
Cer (d18:0/18:0)	0.11	0.13		0.829				
Cer (d18:0/22:0)					0.07	0.04		0.155
Cer (d18:0/24:0)					0.05	0.01	↓	0.010
Cer (d18:1/14:0)	0.06	0.07		0.926				
Cer (d18:1/16:0)	7.22	3.62	↓	0.002	6.48	4.18	↓	0.029
Cer (d18:1/18:0)	1.47	0.50	↓	0.011	0.48	0.29		0.050

Table 2 (cont'd)

Cer (d18:1/20:0)	0.17	0.07		0.102	0.10	0.06		0.139
Cer (d18:1/22:0)	2.07	0.33	↓	7.1e-7	2.68	1.18	↓	0.002
Cer (d18:1/22:1)					0.08	0.06		0.576
Cer (d18:1/23:0)	0.37	0.03	↓	0.001	0.30	0.11	↓	0.032
Cer (d18:1/24:0)	6.00	1.17	↓	4.7e-6	7.81	4.34	↓	0.009
Cer (d18:1/24:1)	10.83	3.27	↓	9.2e-5	10.69	5.43	↓	0.001
Cer (d18:1/24:2)	4.81	0.85	↓	3.4e-5	6.05	3.65	↓	0.011
Hex-Cer (d18:1/16:0)	2.09	0.54	↓	0.031	1.08	0.31	↓	0.004
Hex-Cer (d18:1/18:0)	0.43	0.05		0.264	0.10	0.08		0.732
Hex-Cer (d18:1/20:0)	0.44	0.13		0.278	0.16	1.21		0.388
Hex-Cer (d18:1/22:0)	2.42	1.03	↓	0.020	1.14	1.29		0.759
Hex-Cer (d18:1/24:0)	5.21	1.62	↓	1.1e-5	3.13	1.02	↓	0.002
Hex-Cer (d18:1/24:1)	4.70	2.46	↓	0.043	2.13	0.92	↓	0.010

Table 2 (cont'd)

Hex-Cer (d18:1/24:2)	1.02	0.39	↓	0.022	0.55	0.33		0.127
SM (d18:0/14:0)	0.00	0.21	↑	0.039				
SM (d18:0/16:0)	23.59	292.73	↑	2.4e-6	29.30	181.15	↑	5.2e-5
SM (d18:0/17:0)	0.01	0.91	↑	0.005				
SM (d18:0/18:0)	0.83	30.59	↑	5.6e-7	0.82	17.97	↑	6.3e-5
SM (d18:1/14:0)	0.12	11.07	↑	4.8e-5	0.10	6.11	↑	1.7e-5
SM (d18:1/15:0)	1.33	15.97	↑	5.5e-6	1.44	8.79	↑	0.0001
SM (d18:1/16:0)	330.12	3480.86	↑	5.2e-6	371.04	2063.66	↑	5.1e-5
SM (d18:2/16:0)	3.02	32.01	↑	7.2e-6	2.85	18.02	↑	2.6e-5
SM (d18:1/17:0)	3.09	24.15	↑	2.7e-5	4.03	14.50	↑	0.002
SM (d18:1/18:0)	20.33	448.63	↑	2.1e-6	14.57	252.65	↑	4.4e-5
SM (d18:1/18:1)					0.10	13.23	↑	2.2e-5
SM (d18:2/18:1)	0.43	23.95	↑	6.2e-6	0.08	4.31	↑	2.8e-8

Table 2 (cont'd)

SM (d18:2/18:2)	8.13	130.33	↑	0.002				
SM (d18:1/19:0)	0.45	11.68	↑	5.7e-6				
SM (d18:1/20:0)	9.54	174.27	↑	2.7e-6	7.23	100.65	↑	6.6e-5
SM (d18:1/20:1)	0.12	16.21	↑	3.9e-6	0.11	9.12	↑	5.4e-5
SM (d18:2/20:1)					0.00	0.35	↑	2.8e-6
SM (d18:1/21:0)	7.56	23.80	↑	0.002	9.17	15.63		0.160
SM (d18:1/22:0)	34.48	969.78	↑	1.9e-6	43.07	606.24	↑	5.07e-5
SM (d18:1/22:1)	4.62	171.11	↑	3.1e-6	6.35	99.97	↑	5.4e-5
SM (d18:2/22:1)	2.66	76.66	↑	1.1e-5	3.52	43.41	↑	8.3e-5
SM (d18:1/23:0)	7.04	154.43	↑	4.2e-6	9.08	97.85	↑	0.0002
SM (d18:1/23:1)	1.19	41.09	↑	6.2e-6	1.37	23.91	↑	0.0001
SM (d18:1/23:2)					0.12	2.11	↑	0.004
SM (d18:1/24:0)	46.63	1455.81	↑	1.6e-6	76.79	947.45	↑	3.4e-5

Table 2 (cont'd)

SM (d18:1/24:1)	185.69	2727.35	↑	3.2e-6	252.45	1704.36	↑	6.3e-5
SM (d18:1/24:2)	163.95	2687.45	↑	6.7e-6	249.41	1654.85	↑	9.9e-5
SM (d18:2/24:2)					7.41	83.78	↑	3.6e-5
SM (d18:1/24:3)	5.32	149.91	↑	1.4e-5				
SM (d18:1/25:0)	0.08	28.25	↑	4.2e-6	0.30	17.32	↑	6.3e-5
SM (d18:1/25:1)	0.14	18.10	↑	1.0e-5	0.22	10.64	↑	0.0001
SM (d18:1/25:2)					0.05	3.39	↑	0.001
SM (d18:1/26:0)	0.01	13.64	↑	5.5e-6	0.03	8.30	↑	8.8e-5
SM (d18:1/26:1)	0.13	21.16	↑	5.9e-6	0.37	12.57	↑	5.7e-5
SM (d18:1/26:2)	0.50	37.07	↑	7.5e-6	1.61	22.38	↑	5.5e-5
SM (d18:1/26:3)	0.28	48.13	↑	0.002				
Sph16:0	5.32	8.65	↑	0.048	6.51	5.82		0.602
Sph16:0-P	0.00	0.00		0.061	0.00	0.01		0.149

Table 2 (cont'd)

Sph16:1	5.91	5.85		0.946	6.75	5.05		0.188
Sph16:1-P	0.00	0.00		0.585	0.00	0.01		0.439
Sph18:0	5.65	8.20		0.469	7.51	6.23		0.636
Sph18:0-P	0.00	0.01		0.377	0.00	0.01		0.269
Sph18:1	7.59	10.28		0.627	8.59	6.00		0.591
Sph18:1-P	0.01	0.00		0.845	0.01	0.01		0.844
Sph18:2	19.54	19.68		0.993	9.51	11.59		0.796
Sph18:2-P	0.00	0.00		0.653	0.00	0.00		0.054
Sph20:0	9.82	12.32		0.454	10.66	7.48		0.223
Sph20:0-P	0.00	0.00		0.223				
Sph20:1	12.32	7.85		0.442	11.26	4.43		0.177
Sph20:1-P	0.05	0.00		0.196	0.05	0.02		0.405
Sph20:2	0.61	0.59		0.940	0.43	0.27		0.520
Sph20:2-P	0.00	0.01		0.488	0.01	0.00		0.434

(CAC- Circulating angiogenic cells, ASM- Acid sphingomyelinase, Cer: Ceramide, Hex-Cer: Hexosyl ceramide, SM: Sphingomyelin, Sph: Sphingosine, Sph-P: Sphingosine phosphate)

Figure 21. Mass spectra of sphingolipids.

Orbitrap high resolution/accurate mass positive ionization mode Higher-Energy Collisional Dissociation (HCD)-MS/MS spectra of (A, C) Sphingomyelin d18:1/24:1 at m/z 813.6844, and (B, D) Ceramide d18:1/24:1 at m/z 648.6289 from WT or ASM $^{-/-}$ CAC. Sphingolipid identities were initially assigned based on accurate mass measurements from full scan MS experiments, and sphingoid base constituents were determined by MS/MS.

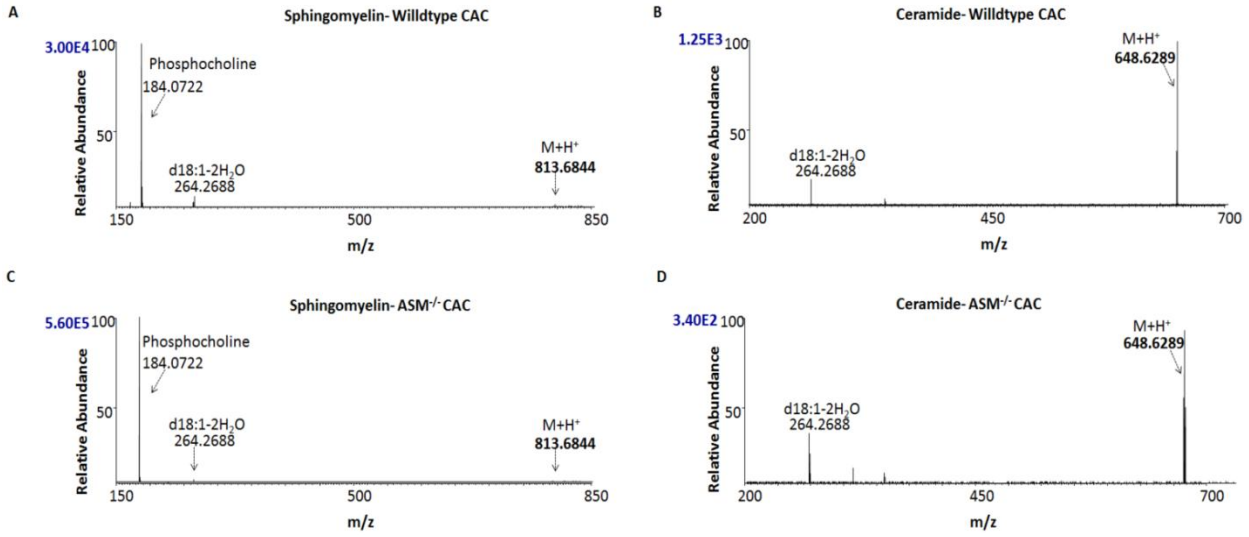


Figure 22. Change in ceramide and ASM levels alter membrane viscosity of diabetic CACs.

A: Replacing SM with ceramide increased viscosity of synthetic membranes. *** p< 0.001, n=5 per group. On Left: Extruded synthetic vesicle by electron microscopy. Data are expressed as means ± s.d.

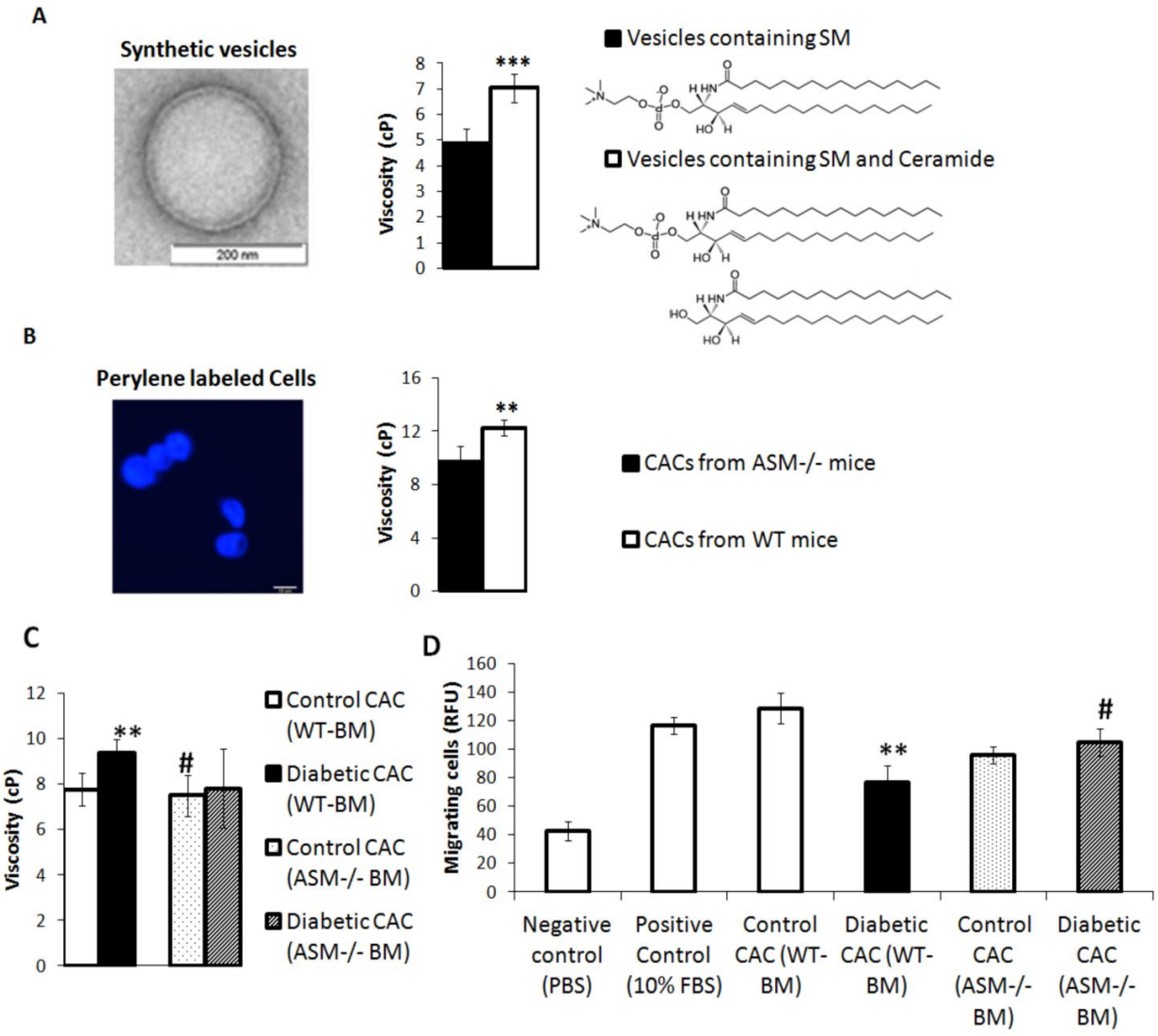


Figure 22 (cont'd)

B: CACs isolated from $ASM^{-/-}$ mice have decreased membrane viscosity compared to CACs from WT mice. n=4-5 per group, ** $p<0.01$ relative to control. Data are expressed as means ± s.d. On left: Cells labeled with fluorescent probe, perylene.

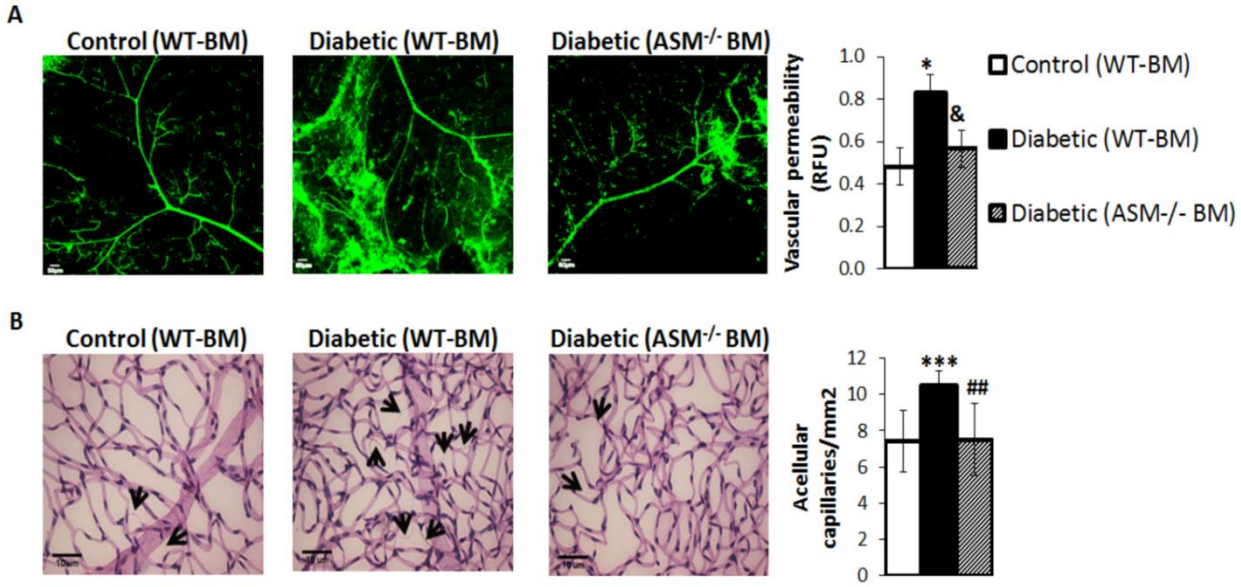
C: Increase in membrane viscosity observed in diabetic CACs from WT chimeras was normalized in 3 months diabetic CACs from $ASM^{-/-}$ BM chimeras. Data are expressed as means ± s.d. n=4-5 per group, ** $p<0.01$ relative to control, #$p<0.05$ relative to diabetic.

D: Migration towards SDF-1 is improved in diabetic CACs isolated from $ASM^{-/-}$ BM chimeras compared to diabetic CACs from WT chimeras. Data are expressed as means ± s.e.m. n=5-7 per group, **$p<0.01$ relative to control. #$p<0.05$ relative to diabetic. Duration of diabetes was 3 months.

Figure 23. Prevention of retinal vascular damage in diabetic ASM-/- BM chimeric mice.

A: Diabetic retinas (2 months duration) have increased vascular permeability compared to control as demonstrated by high retinal fluorescence intensity for FITC-albumin (middle). The increase is prevented in diabetic retinas from $ASM^{-/-}$ BM chimeric mice (right). Quantification of retinal fluorescence intensity for FITC-albumin is shown on far right. n=10-12 per group. Data are expressed as means ± s.e.m. * $p<0.05$ relative to control and & $p = 0.08$ relative to diabetic. Scale bar is 50 μM.

B: Increase in number of acellular capillaries (black arrows) in 3-5 months diabetic retina (middle) is prevented in $ASM^{-/-}$ BM chimeras (right). Quantification of acellular capillaries is shown on far right. n= 10-14 per group, Data are expressed as means ± s.d. *** $p<0.001$ relative to control and ## $p<0.01$ relative to diabetic. Scale bar is 10 μM.



3.4 Discussion

Landmark clinical trials, DCCT and ACCORD Eye demonstrated that control of hyperglycemia and dyslipidemia is required to achieve maximal protection from DR [10,82]. Although hyperglycemia-associated damage has been investigated in detail, few studies addressed the effect of dyslipidemia in DR. Dyslipidemia is a complex metabolic dysregulation of multiple lipid classes including fatty acids, cholesterol, triglycerides and sphingolipids.

Previous studies by others and us indicated that dysregulation of sphingolipid metabolism, with upregulation of ASM and higher hexosyl ceramide production plays a crucial role in diabetic dyslipidemia-induced retinal damage [138,142,150]. However, whole-body ASM deficiency leads to retinal dysfunction and neurodegeneration in $ASM^{-/-}$ mice [212,213]. A recent study by Dannhausen *et al.* demonstrates activation of microglia and loss of retinal function in $ASM^{-/-}$ mice [212].

In addition to previously reported direct retinal effects, ASM upregulation could also affect the BM niche, disturbing the balance between pro-inflammatory and reparative BM cell populations. This affords us an opportunity to inhibit ASM only in the BM cells, thus avoiding the unwanted neurodegenerative effects due to systemic ASM inhibition. To achieve selective BM inhibition in this study, we have generated chimeric mice by $ASM^{-/-}$→WT or WT→WT BM transplantation. BM transplantation ensures that all the cells derived from the BM are ASM deficient, while ASM activity is maintained in other tissues, including retina.

Dysfunction of BM-derived CACs is a critical component of the pathogenic events associated with DR [101,102]. Recent studies indicate that human diabetic CACs have weakened proliferation, differentiation and migration capabilities, resulting in ineffective contribution of

these CACs towards vascular repair [103,104,105]. Advanced diabetes is associated with BM pathology which contributes to the inability of CAC to egress the BM, enter the circulation and home to areas of injury [76,106]. Thus, this trapping of CACs within the BM makes these cells unavailable for vascular repair [103]. Furthermore, the migratory prowess of diabetic CACs is severely altered due to their inability to respond to hypoxia with an increase in HIF expression or to migrate towards gradients of hypoxia regulated factors, such as VEGF-A and SDF-1 [107,108,109]. The migratory defect of these cells is a result of reduced bioavailability of NO as well as poor deformability [108].

We have previously demonstrated that blocking upregulation of ASM in diabetic CACs leads to improved colony forming ability, indicating an improved capacity of these reparative cells to adhere and differentiate in culture [172]. Recently, ceramide metabolites such as C1P and S1P were found to upregulate SDF-1 secretion from BM stromal cells, potentially altering chemokine gradients that guide the release of progenitor cells from their BM niche [167,168,169]. In our study, we demonstrate that increased ceramide levels alters membrane fluidity of diabetic CACs leading to impaired CAC release from the BM and reduced migration to areas of retinal vascular injury in a mouse model of DR.

In addition to reparative CAC dysfunction, diabetes affects BM-derived inflammatory myelomonocytic cells, which circulate in blood and contribute to diabetes-associated inflammation. Previous studies showed that diabetes causes accumulation of inflammatory monocytes in the retina [30,87]. Pro-inflammatory BM-derived cells, like neutrophils and monocytes, were recently demonstrated to play important roles in retinal endothelial cell death and capillary degeneration in diabetes [32]. However, the exact contribution of BM-derived microglia to retinal damage during diabetes remains unclear. During embryonic development,

resident tissue microglia are known to develop from a yolk sac and do not have myeloid origin [205]. Several studies demonstrated that in normal tissue, microglial regeneration occurs from tissue-specific microglial progenitors [206]. This balance could shift towards myeloid cells with ageing, inflammation or tissue damage [35,77,78]. We observed Iba^{+} gfp^{+} cells in control and diabetic retinas, however Iba^{+} gfp^{+} cells in control retinas had branched resting phenotype compared to clearly activated amoeboid phenotype in diabetic retinas, indicating that diabetes-induced damage promotes myeloid-derived microglial activation. Furthermore, our data demonstrate that inhibition of ASM in BM prevents activation of these microglia-like cells. Reduced activation of BM-derived microglia may contribute to normalizing levels of pro-inflammatory cytokines and growth factors in retinas of diabetic $ASM^{-/-}$ BM chimeras.

In this study, we demonstrate that the combination of improved CAC migration and reduced activation of BM-derived inflammatory cells in ASM-/- BM chimeras prevents diabetes-induced retinal vascular degeneration. Vascular degeneration characterized by increase in number of acellular capillaries is typically determined after 6 months of diabetes; however BM transplantation and stable engraftment before the induction of diabetes resulted in older age of mice at the end of the experiment (9-11 months old). Older age likely contributed to the high numbers of acellular capillaries that we observed at 3-5 months of diabetes (Figure 23B).

ASM activation and ceramide production can alter cell fate through a variety of mechanisms. ASM activation has been associated with increased cytokine release in the resident microglia of the retina [181]. Ceramide may serve as a second messenger in initiating apoptosis of endothelial cells, while reduction of ceramide levels by inhibiting ASM has been demonstrated to prevent endothelial cell death, leading to reduced capillary apoptosis in tumor microenvironments [214,215]. Previously, we showed that inhibition of ASM by dietary supplementation of

docosahexaenoic acid (DHA) or by genetic manipulation prevents retinal vascular degeneration associated with diabetes [150,172,173]. However, as retinal effects of $ASM^{-/-}$ are also associated with neurodegeneration, in this study we wanted to separate the bone marrow and retinal/vascular effects of $ASM^{-/-}$. The retina and vasculature of the chimeric animals in this study were from the WT host, only the bone marrow was from the $ASM^{-/-}$ mice. Thus, although in the whole body $ASM^{-/-}$model endothelial cell death is inhibited, the model we used does not directly affect endothelial ASM expression levels and the effects we observed were mediated by changes in bone marrow progenitors.

In this study, we explore the role of ceramide as a structural membrane component contributing to membrane fluidity. Ceramide has a tendency to self-associate, forming ceramide-enriched platforms in the cell membrane [170]. These altered membrane microdomains have been demonstrated to enhance inflammatory signal transduction by facilitating receptor clustering, thereby promoting survival and immune activation of a variety of immune cells including monocytes [146,170,174]. Here, we further explore the concept that altered membrane fluidity of diabetic CACs leads to their impaired release and migration, making them unavailable for vascular repair. These roles of ceramide are not conflicting – these effects are likely happening simultaneously with decreased membrane fluidity, endothelial cell apoptosis and pro-inflammatory pathways contributing to pathogenesis of DR.

Various sphingolipids, such as glycosphingolipids, sphingosine and S1P have been shown to play important roles in the pathogenesis of DR [141,142,149,150]. Ceramide can be metabolized into S1P, which has proliferation, migration and pro-survival effects [162]. Elevated levels of S1P are shown to contribute to retinal vascular pathology in animal models of diabetes [141,149]. However, we did not observe any changes in sphingosine or S1P levels in $ASM^{-/-}$

mouse BM cells or CACs, indicating that although these bioactive lipids may be involved in other aspects of diabetes-induced damage, they cannot explain the correction of deficient migration or normalization of pro-inflammatory pathways observed in $ASM^{-/-}$ BMT mice in this study.

Apart from the important role of ceramide in inflammatory responses, there is also a well-established literature indicating that presence of ceramide and cholesterol in a lipid bilayer causes a change in "viscosity" of membranes [170,178,182]. We have previously shown that diabetes is associated with reduced deformability of human CACs, this reduced deformability is associated with decreased migratory prowess [108] and that ASM activation leads to increased short-chain ceramide levels in diabetic CACs [172] . In this study, we show that increased levels of membrane ceramide due to ASM activation in diabetes are associated with increased membrane rigidity, impaired migration and extravasation capacity of CACs, which in turn prevents effective homing from BM to sites of vascular injury.

In conclusion, we demonstrated that pathological activation of ASM in BM-derived cells plays a crucial role in diabetes-induced retinal microvascular degeneration by activating microglia-like BM-derived cells that infiltrate the retina, and preventing efficient retinal vascular repair by reparative CACs. Our studies provide novel insights into the highly complex mechanisms that lead to retinal vascular degeneration in diabetes and suggest that strategies targeting normalization of ASM in BM-derived cell populations, thereby controlling the balance between reparative and pro-inflammatory cells could represent a viable cell therapy option to enhance available DR treatments.

3.5 Methods

- Mice. Male C57BL/6J and C57BL/6-Tg(CAG-EGFP) mice were procured from Jackson Laboratory. $ASM^{-/-}$ mice used as donor strain were obtained from Dr. Kolesnick (with permission of Dr. Schuchman). C57BL/6.$ASM^{-/-}$, C57BL/6.gfp^{+} and C57BL/6.$ASM^{-/-}$ X gfp^{+} chimeras were produced as described in Chapter 2. Wildtype BM donor cells transferred into wildtype recipient chimeras were used as control. Diabetes was induced as described in Chapter 2.
- Retinal vascular permeability. Two months after induction of diabetes in the C57BL/6.$ASM^{-/-}$ chimeric mice, *in vivo* vascular permeability in the retina was measured. Briefly, mice were injected with FITC- albumin (0.5 mg in 100 µL PBS. After two hours, blood was collected from each mouse and centrifuged to obtain plasma; the animal was perfused with 1% formaldehyde and enucleated. Retinas were removed, flat-mounted with four slits and kept on glass slides with Fluoromount mounting medium (Sigma). Images were acquired using an Olympus FluoView 1000 scanning laser confocal microscope. Retinas were disrupted mechanically and cleared by centrifugation. FITC-albumin in supernatant was quantified using spectrofluorometer and normalized to plasma fluorescence.
- Acellular capillaries. Mice eyes were enucleated and retinal vasculature isolated by elastase digestion [150]. Retinal blood vessels were stained using hematoxylin and eosin. Acellular capillaries in midretina were systematically counted by three investigators as described previously [150]. All samples were analyzed in a masked manner.
- Quantitative real-time PCR. RNA was isolated from retina and real-time quantitative PCR was done as described previously [150]. Mouse gene-specific primers for ASM, IL-1β, VEGF, ICAM-1 and VCAM-1 were used. All results were normalized to cyclophilin.

Table 3. Sequences of specific primers used for quantitative real-time PCR.

Gene	Forward	Reverse
Mouse ASM	aaccctggctaccgagtttaccaa	tggcctgggtcagattcaagatgt
Mouse ICAM-1	acactatgtggactggcagtggtt	tgaggctcgattgttcagctgcta
Mouse VCAM-1	cccaggtggaggtctactca	ccagatggtcaaagggataca
Mouse VEGF A	tcaccaaagccagcacataggaga	ttacacgtctgcggatcttggaca
Mouse IL-1β	aagggctgcttccaaacctttgac	atactgcctgcctgaagctcttgt
Mouse Cyclophilin	attcatgtgccagggtggtga	ccgtttgtgggtccagca
Human ASM	caacctcgcgctgaagaa	tccaccatgtcatcctcaaa
Human Cyclophilin	aaggtcccaaagacagcaga	cttgccaccagtgccattat

- Western Blot Analysis. Samples were lysed using lysis buffer composed of 50 mM HEPES, 1 mM EGTA, 1.5 mM $MgCl_2$, 150 mM NaCl, 10% glycerol and 1% Triton X-100, pH 7.5). Phosphatase inhibitor (1 mM Na_4PP_i, 10 mM NaF, 100 µM glycerophosphate, 1 mM Na_3PO_4) and protease inhibitor (Sigma) were added fresh to the solution. A protein quantification assay (Bio-Rad Laboratories) was performed to determine the protein concentration for each sample. Proteins were loaded on NuPAGE gels containing 10% Bis-Tris (Life Technologies, Carlsbad, CA) and run for 1.5 hours at 100 V. Resolved proteins were transferred to nitrocellulose membrane and stained with anti-ASM antibody at 1:1000 dilution (Cell Signaling), followed by Alexa-Fluor secondary antibody (Molecular Probes, Life Technologies). Immunoreactive bands were visualized using the Odyssey digital imaging program. ImageJ software was used for quantification of scanned blots.
- CAC isolation and migration. Mice were euthanized and tibias and femurs were collected. Ice-cold PBS was used to flush bones, and single cell suspension was made. Ammonium chloride (STEMCELL technologies) was used to eliminate erythrocytes contaminating the bone marrow cells. Next, negative selection using magnetic beads (STEMCELL Technologies) was used to isolate hematopoietic stem/progenitor cells from mouse bone marrow, and positive selection for Sca-1 (STEMCELL Technologies) was used to obtain Lin^- Sca^+ progenitor cells.

  Enriched progenitor cells were kept in a cell culture incubator with 5% CO_2 at 37°C overnight, in EGM-2 media with SingleQuot supplements and growth factors added (Lonza) to enable recovery from the enrichment process. The next day, cells were tagged with a fluorescent dye, Calcein-AM (BD Biosciences), and placed on the filter sites (5 µm pore size) at the top of a migration chamber (Neuro Probe). The wells below were loaded with 100

nM SDF-1, 10% FBS as positive control or PBS as negative control. The migration set-up was incubated at 37°C in humidified air with 5% CO_2 for 4 hours. To determine the number of migrated cells, fluorescence emitted at 550 nm was measured using a microplate reader. Samples were analyzed in triplicate and data expressed as percentage relative to positive control ± SEM. [172]. The cells isolated by this protocol were formerly called EPCs (endothelial progenitor cells). The terminology has now been updated to CAC (circulating angiogenic cells), which is more reflective of the function of these cells [216].

- Unilamellar Vesicle formation. Unilamellar vesicles were formed by extrusion. Batch 1 was composed of 49 mol% DOPC, 1 mol% perylene, 10 mol% cholesterol and 40 mol% sphingomyelin (chicken egg), and batch 2 of 49 mol% DOPC, 1 mol% perylene, 10 mol% cholesterol, 20 mol% sphingomyelin, 20 mol% ceramide. Perylene was purchased from Sigma Aldrich and other vesicle constituents from Avanti Polar Lipids. Constituents were present in chloroform or ethanol (perylene), aliquots were evaporated to dryness and taken up in 2.86 mL milli-Q water. The resulting mixture was put through five freeze-thaw-vortex cycles and then extruded 11 times through a polycarbonate filter [189]. The extruded vesicles were measured by DLS and were determined to be ca. 200 nm in diameter.
- Membrane Fluidity measurements. Fluorescent probe perylene was utilized in the fluidity measurements. Perylene is a nonpolar polycyclic aromatic hydrocarbon that incorporates selectively into the membrane lipid bilayer. In this measurement a non-random distribution of chromophores in the membrane is photoselected and the time required for this ensemble to re-randomize is monitored [189]. The functionality and time constant(s) associated with this relaxation provide direct insight into the viscosity of the bilayer membrane. 10,000 CACs were labeled with perylene [189] (10 μM) for 15 minutes. Polarized fluorescence

measurements for labelled cells as well as extruded vesicles were collected using time-correlated single-photon counting (TCSPC) instrument, as described previously [189]. The instrument response function is ca. 35 ps fwhm. Perylene emission transients (470 nm) were collected for polarizations parallel (I_{II} (t)) and perpendicular ($I_{\perp}$ (t)) to vertically polarized excitation pulse (435 nm). The polarized emission transients are used to construct the induced orientational anisotropy decay function, r(t), which decays with a single exponential functionality and time constant of decay, τ_{OR}, is given by the modified Debye-Stokes-Einstein (DSE) equation,

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = r(0)\exp(-t/\tau_{OR})$$

$$\tau_{OR} = \frac{\eta V f}{k_B T S}$$

Where η is viscosity of the medium, V is hydrodynamic volume of perylene (225 $Å^3$), k_BT is a thermal energy term, f and S are constants to account for frictional interactions between perylene and its surroundings, and the non-spherical shape of perylene. For all measurements, V, f, S and T remain constant and changes in τ_{OR} reflect changes in η.

- ASM gene inhibition. For inhibiting ASM gene expression, 5 X 10^5 CACs per 100 µL of electroporation medium (Lonza) was treated with 300 nM ASM siRNA and electroporated using Nucleofector (Amaxa Biosystems). The siRNA-treated cells were incubated at 37°C in 5% CO_2 in a humidified cell culture incubator for 48 hours. RNA was isolated from the CACs, and real time PCR was performed to confirm inhibition of the ASM gene. ASM protein was analyzed using Western Blot. ~ 5-fold inhibition of ASM gene expression was obtained.

- Reendothelialization of retinal vasculature. 10,000 Lin^- Sca^+ gfp^+ progenitor cells were injected intravitreously using 33-gauge Hamilton syringe, into diabetic and control wildtype mice. After seven days to allow progenitor cells homing to retinal vessels, mice were sacrificed and eyes were removed. Mouse eyes were pierced with a 30-gauge needle, and fixed in freshly prepared 4% paraformaldehyde for 1 hour. The eyes were then washed in PBS 3 times, and retinas dissected. Isolated intact retinas were permeabilized in HEPES-buffered saline with 0.1% Tween-20 and 1% BSA, incubated at 4°C overnight. Vasculature was stained with rabbit anti-collagen IV (Abcam) diluted 1:400, followed by PBS wash. Secondary antibody chicken anti-rabbit (Alexa Fluor 594, Invitrogen), diluted 1:1000 was used.
- Immunofluorescent staining.

  Ceramide in BM cells: CACs were treated with anti-ceramide (Sigma) antibody and corresponding secondary antibody (Invitrogen).

  Microglia in the retina: Retinal flat-mounts from gfp^+ chimeras were stained for microglia using 1:100 diluted goat anti-Iba-1 (Novus Biologicals). BM-derived microglia cells were defined by gfp^+, Iba-1^+ staining and shape. Mean counts of Iba^+ gfp^+ cells were taken from 3 points in the retina at 20x magnification. Length of primary dendrites of microglia was quantified using MetaMorph software. All measurements were done in a masked fashion. Nikon TE2000 fluorescence microscope with Photometrics CoolSNAP HQ2 camera was used for imaging. Images were quantified using MetaMorph software (Molecular Devices, Downingtown, PA).
- Transferase-mediated dUTP nick-end labeling staining. Paraffin-embedded sections were subjected to TACS-XL Basic In Situ Apoptosis Detection (Gaithersburg, MD), performed

according to manufacturer's instructions. Number of TUNEL-positive cells in the inner and outer nuclear layers (INL, ONL), and ganglion cell layer (GCL) were counted, and data expressed per retina [150].

- Morphometric analysis. Retinal sections were stained with hematoxylin and eosin (H&E), and thickness of IPL, INL, OPL and ONL was measured using images captured from four microscopic fields with Nikon TE2000 microscope and color camera. Calibrated lines were drawn perpendicular to each layer of the retina using Metamorph software, and average thickness of each retinal layer was expressed in μm.
- Flow cytometry. Flow cytometry was performed using LSR II instrument (BD). Data were analyzed with FlowJo software (Tree Star, Inc.). Antibodies were purchased from eBioscience.
- Mass Spectrometry. 1-5 million cells were subjected to monophasic lipid extraction as previously described [217]. 10 μL of lipid sample was directly infused by nanoelectrospray ionization (nESI) into a high resolution / accurate mass Thermo Scientific model LTQ Orbitrap Velos mass spectrometer (San Jose, CA) using an Advion Triversa Nanomate nESI source (Advion, Ithaca, NY). High resolution mass spectra and Higher-Energy Collision Induced Dissociation (HCD-MS/MS) product ion spectra were acquired in positive ionization mode to confirm lipid headgroups and elucidate the sphingosyl backbone/acyl chain compositions of selected sphingolipid ions using the FT analyzer operating at 100,000 mass resolving power. Lipid identifications were achieved using the Lipid Mass Spectrum Analysis (LIMSA) v.1.0 software linear fit algorithm, in conjunction with a user-defined database of hypothetical lipid compounds for automated peak finding and correction of ^{13}C

isotope effects. Relative quantification between samples was performed by normalization of target lipid ion peak areas to appropriate internal standards.

- Statistical analyses. Data are presented as mean ± s.e.m. Results were analyzed for statistical significance by Student's t-test or one-way ANOVA followed by Tukey's or Bonferroni's post-hoc test (GraphPad Prism5, GraphPad Software, San Diego, CA), where appropriate.

Chapter 4. Inhibition of acid sphingomyelinase improves homing efficiency of diabetic circulating angiogenic cells

4.1 Introduction

The BM is an important niche for several cells types such as stem cells, stromal supporting cells, myeloid and lymphoid precursors, and also host the circulating angiogenic cells (CACs). The functioning of the BM in inflammation and repair in the diabetic retina is increasingly gaining interest. Circulating hematopoietic-derived progenitors are found to contribute around 10% of the endothelium in new vessels that develop in response to injury, demonstrating the important role of the BM in vascular repair [218]. Efficient vascular repair by these circulating progenitors is governed by their ability to maintain circadian patterns of release from the BM and extravasate into blood vessels in the tissues. It has been demonstrated in previous studies that long-standing diabetes causes deterioration of BM niches, leading to a progressive depletion of progenitor cell population in the BM [87]. We have also demonstrated that retinal vascular degeneration in diabetes is associated with BM neuropathy, leading to trapping of diabetic progenitor cells in the BM, and affecting circadian release of these cells into circulation [103].

Homeostatic recirculation of cells back to the BM niche is an equally important aspect of their role in maintaining the BM progenitor microenvironment [219,220,221]. The process of homing and retention of the circulating progenitors in BM niches requires complex regulation, involving upregulation of specific receptors such as CXCR-4 and formation of concentration gradients of chemokines such as SDF-1 [222,223]. Expression of specific integrins such as α4β1, β2 and αvβ3 by CACs are major determinants of CAC adhesion to endothelial cells, homing and mobilization from the BM [224,225].

In Chapters 2 and 3 we have demonstrated that diabetes significantly reduces the proportion of CACs released from the BM into circulation, and also decreases the number of BM-derived endothelial cells found in the diabetic retina. Further, we show that modulation of sphingolipid metabolism in the diabetic CACs improves homing of these reparative cells to damaged retinal microvasculature, as a result of improved membrane fluidity. However, the effect of altered sphingolipid metabolism on the ability of diabetic CACs to home from the tissues back to the BM niche has not been adequately studied. In this study, we demonstrate that diabetic CACs injected into the vitreous are incapable of migrating back to their BM niche, indicating that the *in vivo* homing efficiency of diabetic CACs is dramatically reduced. We also describe a link between increased acid sphingomyelinase (ASM) activity and impaired homing ability of diabetic CACs.

4.2 Results

In order to examine the effect of altered sphingolipid metabolism on the *in vivo* migration and homing capability of diabetic CACs from the retina to the BM niche, we injected control, diabetic or ASM-inhibited diabetic GFP^+ CACs into the vitreous of mice with healthy retinal vasculature (Figure 24). We observed that increased numbers of diabetic CACs remained trapped in the vitreous as compared to control CACs, implying impaired migration and homing efficiency in the diabetic CACs. Interestingly, ASM inhibition improves clearance of diabetic GFP^+ CACs from the vitreo-retinal space (Figure 25A, B).

To further confirm that diabetic CACs are defective in homing back to their BM niche, we examined the BM cells of the tibia and femurs of mice that received intravitreal injections of diabetic CACs in comparison to animals that received control CACs. Quantification of GFP^+ cells in the BM by flow cytometry demonstrated that homing of intravitreally injected diabetic

GFP^+ CACs to the BM of recipient mice was significantly reduced compared to control GFP^+ CACs. However, inhibition of ASM improved the migration and homing capacity of diabetic CACs injected in healthy recipient mice (Figure 25C).

Figure 24. Scheme for testing homing efficiency of ASM-inhibited diabetic CACs to the BM.

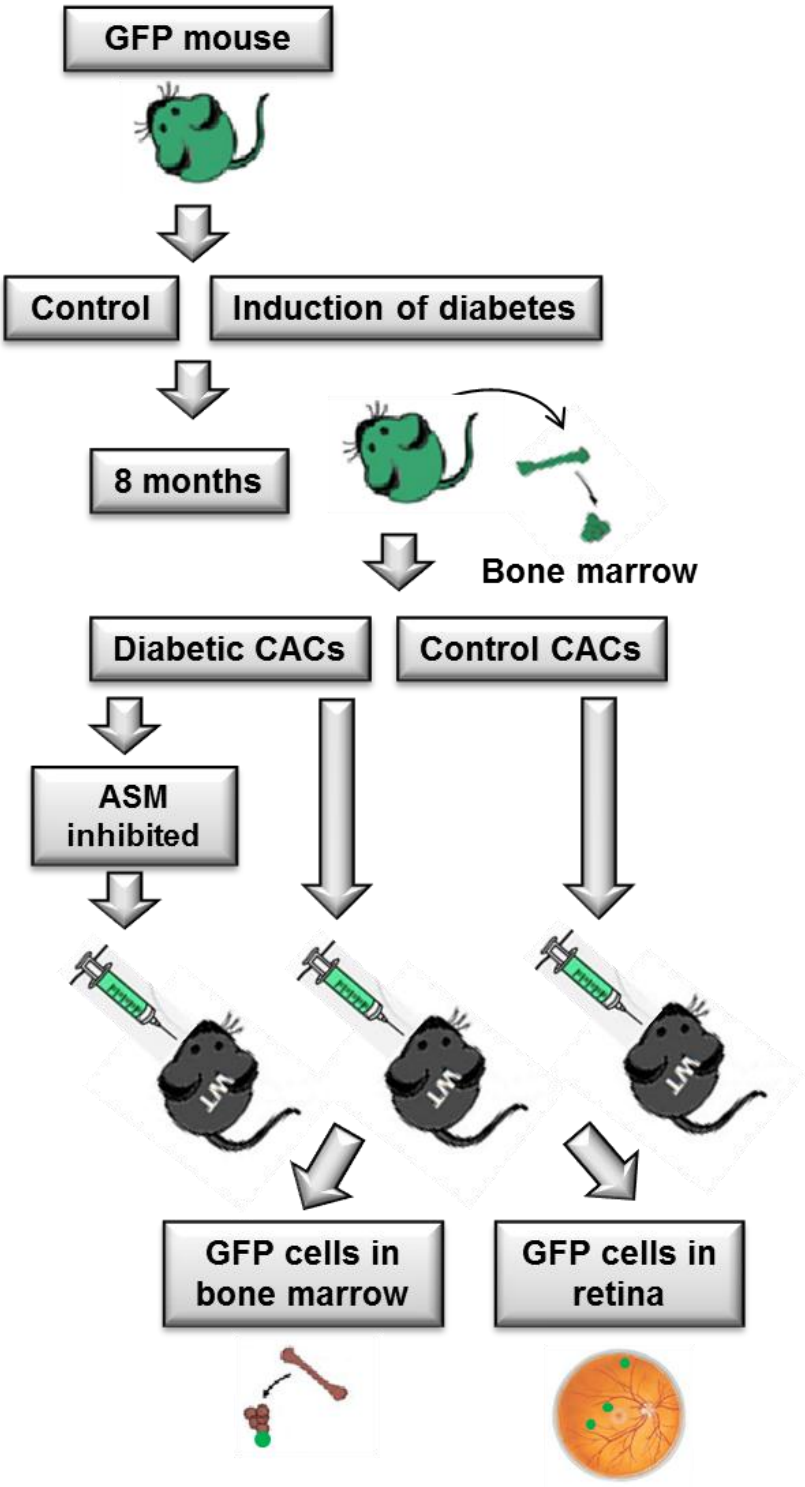


Figure 24 (cont'd)

Male C57BL/6-Tg(CAG-EGFP) mice were made diabetic by STZ injections. After 8 months of diabetes, 10,000 CACs were isolated from BM of control and diabetic GFP^+ mice and ASM inhibited in diabetic CACs by siRNA. Control, diabetic or ASM-inhibited diabetic CACs were injected into the vitreous of healthy mice. Seven days after injection, the retinas and BM were collected and analyzed by confocal microscopy for presence of GFP^+ CACs.

Figure 25. ASM inhibition improves homing efficiency of diabetic CACs.

(A) Control, diabetic or diabetic ASM-inhibited GFP^{+} CACs (green) injected into the vitreous of mice with healthy retinal vasculature stained red using anti-collagen IV antibody.

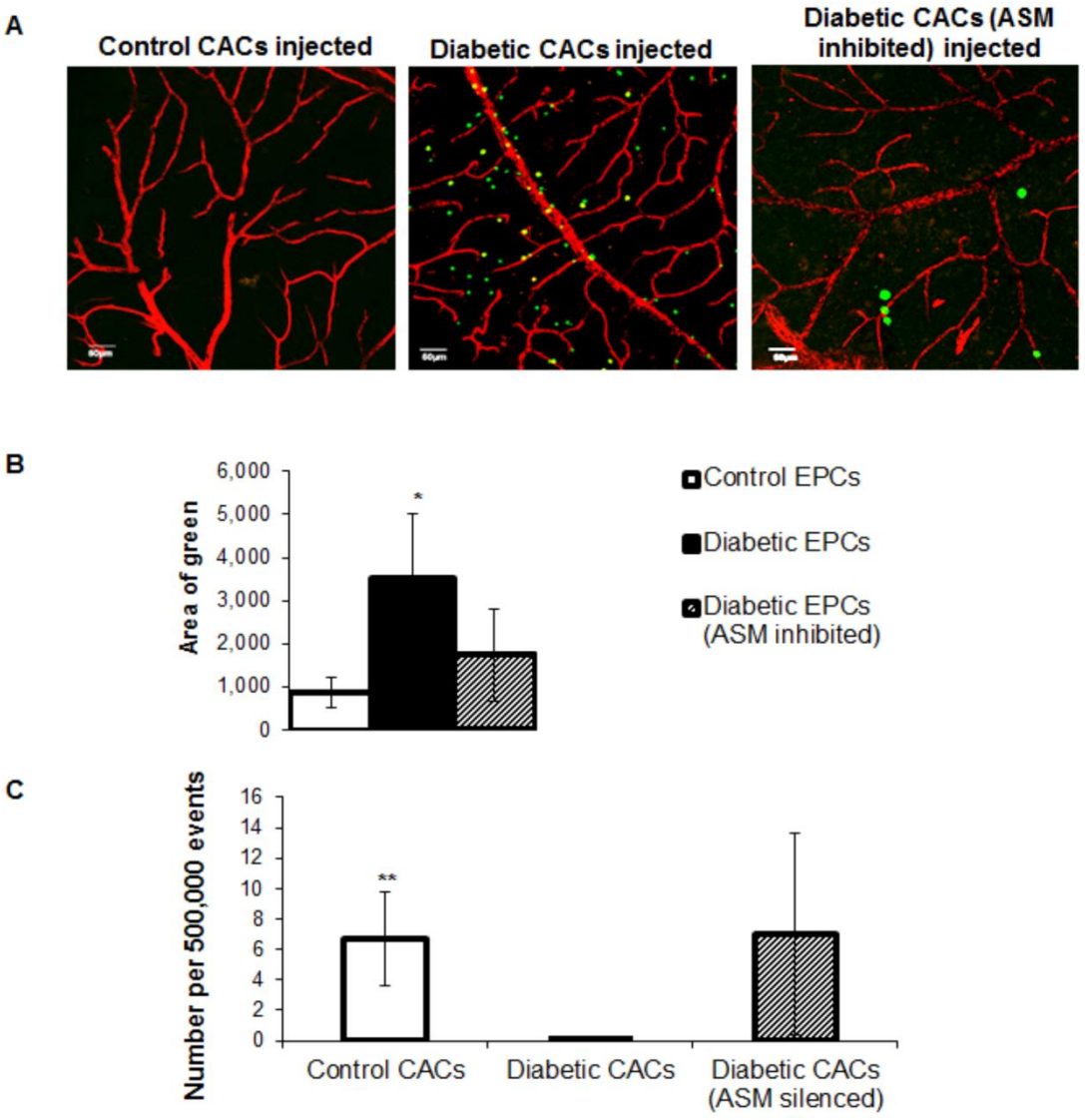


Figure 25 (cont'd)

(B) Quantitation of area of green CACs observed in confocal retinal images shows reduced clearance of diabetic CACs from retina, while ASM inhibition improves clearance of diabetic CACs, N=10-12. (C) Quantification of GFP^+ cells from bone marrow of tibia and femurs of recipient mice shows homing of diabetic GFP^+ CACs to the recipient bone marrow was significantly lower compared to control GFP^+ CACs, and is improved in diabetic CACs with ASM inhibition, N= 4-6. * $p< 0.05$, ** $p<0.01$.

4.3 Discussion

In the previous chapters, we demonstrated how BM-derived pro-inflammatory cells as well as impaired function of reparative CACs contributes significantly to retinal pathology in DR. We then demonstrated how upregulation of a central enzyme in the sphingoipid pathway, ASM and concomitant higher ceramide production contributes to dysfunction of diabetic CACs, leading to impaired retinal vascular repair. Here, we describe how ASM upregulation in diabetic CACs impairs their homing from the healthy retinal tissue back to their niches, using the BM niche as an example of a major reservoir for CACs.

We have previously demonstrated that modulation of sphingolipid metabolism in the diabetic retina prevents endothelial cell activation and ensuing retinal vascular injury [138,142,150]. Sphingolipid metabolites derived from ceramide, such as C1P and S1P may influence patterns of release and migration of progenitor cells from their niches, by altering critical chemokine levels in the BM niche [167,168,169]. We also demonstrated that preventing ASM upregulation in the diabetic BM leads to increased numbers of CACs in circulation and diminished function, as measured by migration, proliferation, and colony forming ability [172]. In this study, we further expand our research to demonstrate that aberrant ASM activation in diabetic CACs leads to impaired homing of diabetic CACs from healthy retina back to their BM niche.

CACs arising from the BM, spleen and other niches normally circulate in blood and migrate to regions of endothelial damage to mediate microvascular repair [95]. In a healthy animal, chemokine gradients such as SDF-1 and up-regulation of CXCR-4 receptors on CACs play crucial roles in regulating release, surveillance and homing of reparative CACs to sites of retinal vascular injury which is disturbed in diabetes, as we have previously described [103,222,223]. In order to maintain their population, these cells migrate from various tissues back to their niches,

governed by expression of integrins and chemokine gradients [219,222,225]. To observe the effect of altered sphingolipid metabolism in diabetes on the *in vivo* migration and homing capacity of CACs, we injected GFP^+ CACs isolated from 8-month diabetic, ASM-inhibited diabetic and age-matched control mice, into the vitreous of wild type mice with healthy retinal vasculature. Seven days post injection, the retinas were collected and analyzed for presence of residual GFP^+ CACs in the vitreo-retinal space (Figure 24). As there was no retinal damage in the control recipient mice, as expected, normal control CACs migrated back into the blood stream and were homing into the bone marrow niche. However, we observed that a significant number of diabetic GFP^+ CACs remained in the vitreo-retinal space, unable to migrate. With ASM inhibition, we observed improved clearance of diabetic GFP^+ CACs from the vitro-retinal space in the recipient mice (Figure 25A, B). This improvement in migration and homing efficiency of diabetic CACs was also apparent in the observed increase in ASM-inhibited diabetic GFP^+ CACs circulating and homing back to the BM niche (Figure 25C).

In a healthy animal, there is a distinct circadian pattern in the release, surveillance and homing of reparative CACs to sites of retinal vascular injury which is disturbed in diabetes, as we have previously described [103]. In agreement with the migration data in Chapter 3 we further demonstrate here, that migration and homing of diabetic CACs from healthy retina back to the BM is impaired. Further, we demonstrate that modulation of sphingolipid metabolism in diabetic CACs by inhibiting ASM leads to improved homing of these reparative cells to the BM niche. Collectively, our data robustly supports the premise that altered sphingolipid metabolism in the diabetic BM plays a vital role in the development of DR.

4.4 Methods

- Mice. Male C57BL/6J and C57BL/6-Tg(CAG-EGFP) mice were purchased from Jackson Laboratory. Mice were injected with 65 mg/Kg of streptozotocin (STZ) in 0.5% sodium citrate solution every day, for five consecutive days. The control mice were injected with the sodium citrate buffer. Mice with blood glucose levels higher than 13.8 mmol/L were identified as diabetic. Starting 14 days after STZ injections, mice were injected with insulin (0-2 units/day) to prevent acute weight loss, but maintaining them in the hyperglycemic range (around 20 mmol/L blood glucose).
- Isolation of hematopoietic progenitors/CACs from mice. Mice were euthanized and tibias and femurs were collected. Ice-cold PBS was used to flush bones, and single cell suspension was made. Ammonium chloride was used to eliminate erythrocytes from the bone marrow cell pellet. Next, the cells were enriched for mouse hematopoietic stem/progenitor cells by negative selection followed by a Sca1 positive selection kit (STEMCELL Technologies) to obtain Lin^{-} Sca^{+} progenitor cells.
- Homing of CACs from retina. Lin^{-} Sca^{+} progenitor cells were obtained from GFP^{+} 9 month-diabetic and control mice, as described above. The cells were maintained overnight in EGM-2 media with SingleQuot supplements and growth factors (Lonza) to allow their recovery from the isolation process. Next day, cells were washed using PBS, counted and 10,000 cells were injected intravitreously using a 33-gauge Hamilton syringe, into healthy wild type mice. After 7 days, the wild type mice were sacrificed, and their eyes removed.
- Tissue preparation and Immunohistochemistry. Eyes were pierced with a 30-gauge needle and fixed in freshly prepared 4% paraformaldehyde at 25°C for 60 minutes. The eyes were then washed in PBS 3 times, and retinas dissected. Isolated intact retinas were permeabilized

at 4°C in HEPES-buffered saline with 1% BSA and 0.1% Tween-20. For characterization of vascular GFP^+ cells in chimeric mouse retinas, vasculature was stained with primary antibody for endothelial cells, rabbit anti-collagen IV (abcam) diluted 1:400 in PBS with 2% non-immune goat serum, incubating overnight at 4°C, followed by a change into PBS for 6-8 hours. Secondary antibody chicken anti-rabbit (Alexa Fluor 594, Invitrogen), diluted 1:1000 was used, followed by a final wash in PBS. The retinas were flat-mounted with four slits, and positioned on glass slides with Fluoromount mounting medium (Sigma).

- Cell preparation and Flow cytometry. Mice were euthanized and tibias and femurs were collected. Ice-cold PBS was used to flush bones, and single cell suspension was made. Ammonium chloride solution (STEMCELL technologies) was used to remove erythrocytes from the cell pellet. Cells were stained with DAPI to discriminate dead cells. Data was acquired with using an LSR II instrument (BD) at the Flow Cytometry Core at Michigan State University and data were analyzed with FlowJo software (Tree Star, Inc.).
- Statistical analyses. Data are presented as mean ± s.e.m. Results were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test (GraphPad Prism5, GraphPad Software, San Diego, CA).

Chapter 5. Summary and future perspectives

The information presented in this dissertation demonstrates for the first time that diabetes alters sphingolipid metabolism in the bone marrow (BM), leading to increased activity of BM-derived proinflammatory monocytes and impaired function of BM-derived reparative circulating angiogenic cells (CACs). We propose that diabetic dyslipidemia specifically altered sphingolipid metabolism in the BM, has a significant long-term effect on the nature and proportion of BM-derived cells that circulate in the blood, localize to the retina and home back to their BM niche.

Using a streptozotocin mouse model of diabetic retinopathy with GFP BM-transplantation, we first demonstrated that BM-derived circulating proinflammatory monocytes are increased in diabetes while reparative CACs are trapped in the BM, with impaired release into circulation. Importantly, diabetes significantly increases infiltration of BM-derived microglia in an activated state, while reducing infiltration of BM-derived endothelial progenitor cells in the retina.

In the next study, we show that diabetes resulted in activation of acid sphingomyelinase (ASM), a key enzyme of sphingolipid signaling in the bone marrow. Inhibition of ASM in the BM-derived cells revealed a previously unidentified role for this enzyme in altering membrane fluidity of BM cells, thereby leading to an imbalance in BM-derived inflammatory and reparative cell function in the diabetic retina. Inhibition of ASM in the diabetic BM prevented activation of BM-derived microglia-like cells and normalized pro-inflammatory cytokine levels in the retina, while inhibition of ASM in diabetic CACs improved membrane fluidity and homing of these cells to damaged retinal vessels.

The third investigation in my research was to explore the role of altered sphingolipid metabolism on the *in vivo* homing capacity of diabetic CACs. We demonstrated that control CACs injected into the vitreous are very efficient at migrating back to their BM niches, whereas diabetic CACs have lost this ability. Importantly, inhibition of ASM significantly improved the in vivo homing capability of the diabetic CACs.

Taken together, these findings indicate that modulation of sphingolipid metabolism in dysfunctional BM-derived cell populations could normalize the reparative/pro-inflammatory cell balance and can be explored as a novel therapeutic strategy for treating diabetic retinopathy. However, further investigations would be beneficial to establish whether altered membrane structure is the central mechanism in the ASM-mediated impairment of diabetic CAC function. The following experiments are suggested for future study:

1. We have demonstrated the effect of ASM inhibition on membrane ceramide levels and membrane fluidity of diabetic CACs in a mouse model of diabetic retinopathy. However, in humans, the CAC population best characterized is the $CD34^+$ cells; hence it is important to confirm the effect of pharmacological inhibition of ASM on membrane ceramide levels and membrane fluidity in $CD34^+$ cells isolated from diabetic patients.
2. Other ceramide metabolites such as S1P and C1P can upregulate secretion of chemokines such as SDF-1 by stromal cells in the BM niche [167,169]. This can potentially alter chemokine gradients that are known to guide the release and migration of CACs from their niche. Although we did measure chemokine levels, we did not find them affected by diabetes or ASM inhibition in this study. Recently, we and others demonstrated that chemokines regulating the release and mobilization of CAC, such as SDF-1, VEGF and MCP-1 are under

stringent circadian control [99,103]. Hence, a circadian study should be conducted, with collection of samples at distinct Zeitgeber times, which would reveal true differences in chemokine gradients with ASM inhibition in the diabetic BM.

3. It would be useful to identify whether increase in membrane rigidity of diabetic CACs correlates with duration of DR. For example, it may be worthwhile to examine relative membrane fluidity changes in early (6-8 weeks) versus late (8-9 months) stages of DR using a mouse model of diabetes.
4. Several studies have shown that as the cholesterol or ceramide content of a membrane increases, the membrane becomes more rigid [179,226]. In this study, we have used modulation of sphingomyelinase to control ceramide content in cell membranes. However, control of cholesterol levels could be tested as another approach to improve fluidity of cell membranes.
5. Although we have demonstrated that blocking ASM upregulation in diabetic CACs improves their homing and recirculation back to the BM niche (Chapter 4), it is important to identify changes in CAC homing patterns to extramedullary tissues such as spleen, thymus and liver to which they are known to migrate.

Overall, our studies suggest that modifying membrane lipid composition of diabetic BM-derived cells may be beneficial for preventing retinal vascular degeneration. Conceivably, measure of membrane rigidity could be used in the clinic as a mechanical biomarker to gain evidence of ongoing biological stress and tissue damage due to diabetic dyslipidemia. Further, correction of membrane rigidity by controlling membrane cholesterol or ceramide levels can be explored as a potential treatment strategy to improve migration and repair function of CACs in diabetic patients. Intravenous infusion of autologous CACs corrected for membrane rigidity *ex vivo* could

be used as a therapeutic strategy to enhance repair *in vivo* [209]. Cell therapy is ideal for treatment of diabetic patients as microvascular disease does not lend itself to revascularization procedures such as stents and angioplasty but is amenable to cell therapy. The interest in cell therapy is apparent as $CD34^+$ cells are the most commonly used cells for human regenerative studies. There are currently 388 trials using $CD34^+$ cells listed on Clinical Trials.gov, including one trial using these cells for retinal vascular repair in diabetes (NCT01736059).

In conclusion, we demonstrated for the first time that pathological activation of ASM in BM-derived cells plays a crucial role in diabetes-induced retinal microvascular degeneration by activating microglia-like BM-derived cells that infiltrate the retina and preventing efficient homing and retinal vascular repair by reparative CACs. Our studies provide novel insights into the highly complex mechanisms that lead to retinal vascular degeneration in diabetes and suggest that strategies targeting normalization of ASM in BM-derived cell populations, thereby controlling the balance between reparative and pro-inflammatory cells could represent a viable cell therapy option to enhance available DR treatments.

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