Neural inflammation and the microglial response in diabetic retinopathy

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Abstract This chapter reviews the function of microglia and their potential roles in neural inflammation and pathological changes during diabetic retinopathy.

Keywords Diabetic retinopathy · Microglia · Leukostasis

Introduction

The term *neural inflammation* came into use approximately 15 years ago and generally refers to immune-related processes that take place in neural tissues. It differs from classical inflammation that takes place in other tissue types where redness (rubor), swelling (tumor), heat (calor), pain (dolor), and the accumulation of blood-born innate and adaptive immune cells occur. These processes are largely due to vasodilation, plasma exudation, endothelial activation, immune cell (leukocyte, granulocyte, and macrophage) adherence, and diapedesis leading to tissue invasion of the systemic immune system. The beneficial end result of inflammation is the clearance of injurious stimuli and/or damaged tissues. In contrast, the retina, like the central nervous system (CNS), is an immune-privileged tissue with a tight blood–tissue barrier and is not normally subject to the same vascular and plasma immune cell-mediated inflammatory mechanisms which occur in other organs. Instead, the retina contains innate immune cells that function to maintain tissue homeostasis and avoid potentially destructive inflammation. Most prevalent among these are cells called microglia. The

name is historical and a misnomer, for these cells bare no kinship to other glial cells and are in fact neural tissue-resident macrophages [1, 2]. By lineage marker expression, transcriptomics, and function, microglia are categorized as monocytes—a group that includes macrophages and dendritic cells. Although the developmental origins of microglia have been debated, it is widely accepted that they arise from hematopoietic stem cells [2, 3]. Microglia are key players in the initiation and resolution of neural inflammation. In fact, it has been argued that neural inflammation would be better described as “microglial activation” or perhaps as neural “pseudo-inflammation” in appreciation of the fact that astrocytes, as well as microglia, can also become activated and produce inflammatory mediators [4]. Considerable evidence suggests that microglia become altered in experimental models of diabetic retinopathy (DR), and it was hypothesized that microglial activation may trigger neuronal death and vascular dysfunction in DR [5].

Under ordinary conditions, microglia monitor the retinal environment and respond to perturbations so as to maintain tissue homeostasis. During neural inflammation, microglia undergo activation, modifying their morphology, expressing pro- and/or anti-inflammatory mediators, attacking infectious agents, engulfing apoptotic cells, and adopting an antigen-presenting phenotype. The existence of two distinctive systemic and neural immune systems suggests that the latter evolved by virtue of its beneficial ability to prevent a more extreme plasma immune cell-mediated response to acute infection or damage that would result in excessive collateral damage to irreplaceable neural tissue. However, neural inflammation processes may not be so well adapted to protracted maladaptive conditions and microglial activation may play a detrimental role in chronic neurodegenerative conditions. Because activated microglia are invariably associated with acute neural damage and with chronic

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neurodegeneration [6], it was surmised that these cells play a detrimental causal role in neuronal diseases [7, 8]. In fact, the understanding of the role of microglia in both acute and chronic CNS diseases is evolving to consider that microglial activation often represents a beneficial adaptive response to damage that is directed toward eventual resolution of disorder [9–11]. The view of microglia has gone so far as to consider them as necessary contributors to neuronal homeostasis such that dysfunction of their protective and corrective abilities could be causal in neurodegeneration [10]. However, it still seems probable that microglial activation may become maladaptive during chronic conditions that they did not evolve to cope with. The pathology of DR includes vascular dysfunction, neurodegeneration, and microglial activation. The cause, character, and implications of this activation are not well defined. Retinal microglia may undergo a beneficial adaptive response in the diabetic retina, only to eventually contribute to DR as the pathology worsens.

Microglia originate from primitive macrophages during development. These cells enter the retina, differentiate, and distribute evenly into pluristratified arrays. The integration, differentiation, and distribution of microglia in the developing mouse retina was described nearly 30 years ago by Hume and colleagues [12]. Using immunohistochemical staining with the macrophage-specific F4/80 antibody, these authors described the process whereby monocytes extravasate into the developing retina to dispose of excess neurons and then differentiate into microglia. Perinatally, F4/80⁺ cells with highly amoeboid morphologies initially populate the ganglion cell layer as portions of these neurons undergo apoptotic death. The monocytes then migrate to the inner nuclear layer as these neurons undergo apoptosis. As the neuronal pruning diminishes, the monocytes undergo a morphological shift, developing processes of increased length and branching. In the mature retina, the microglia appear primarily in two stratified arrays within the inner plexiform layer and one stratified array within the outer plexiform layer [12, 13]. These fully ramified microglia are evenly distributed with extended process arbors that effectively span the space between adjacent microglia with minimal overlap [14]. These microglia are relatively long-lived. The exact lifespan of microglia in the mature retina as well as the mode of replenishment of these cells, either by proliferation or from bone marrow (BM)-derived monocytic cells, are matters of contention. The hematopoietic origins of these cells suggest that they may be continuously in equilibrium with BM-derived precursors. A study using radiation and bone marrow transplantation (BMT) to create chimeric mice with green fluorescent protein-labeled monocytes suggested that the complete replenishment of retinal microglia by BM-derived monocytes takes approximately 6 months [15]. However, the radiation depletion process may accelerate microglial turnover, as another study

suggested that replacement of retinal microglia by blood-born monocytes is negligible unless retinal damage occurs [16]. Alternatives to BMT, such as parabiosis, have shown that in the adult CNS microglial population is not maintained by extravasation and differentiation of BM-derived cells, but rather through self-renewal of proliferating resident microglia [17]. Thus, it is argued that the BMT procedure may greatly affect the experimental outcome it is meant to measure. Support of the hypothesis that the BMT procedure greatly affects the microglial turnover comes from results showing that irradiation prior to BMT contributes to monocyte recruitment to the CNS by causing cytokine expression in the nervous tissue and disruption of the blood–brain barrier (BBB) [18, 19]. Regardless, it is clear that BM-derived monocyte invade the damaged CNS and retina; what is unclear is whether they linger or differentiate into microglia, which occurs during development. A recent study suggested that BM-derived monocytes recruited to the CNS during experimental autoimmune encephalitis do not eventually contribute to the pool of resident microglia unless they were transplanted, and that this is due to the lineage uncommitted nature of the injected BM cells [20].

Microglia are often said to be in either a *resting* or *activated* state. Ramified microglia that occupy their respective territories were historically referred to as *resting microglia* [21]. This term is misleading, because these cells are not at rest, but rather, are actively monitoring their respective domains for clues to tissue well-being. Microglia are not still; their processes are constantly in motion [22]. Under normal homeostatic conditions, these cells are more accurately referred to as *surveilling microglia*. Such activity is not necessary for detection of diffusible paracrine signals, but would be of benefit for communicating with neurons within reach of their processes. Microglial processes transiently contact neuronal synapses, presumably monitoring their functional state [23]. In fact, neurons express several neuroimmunoregulatory (NIReg) proteins on their surface that function as ligands for microglial surface receptor proteins [24, 25]. These include, but are not limited to, fractalkine (CX3CL1), CD47, and CD200. These neuronal ligands correspond to specific receptors on microglia, namely, fractalkine receptor (CX3CR1), CD172 (SIRP-1 α), and CD200R. A lack of these ligands on neurons or a lack of the corresponding receptors on microglia greatly impacts microglial function and activation [26, 27]. These observations contributed to the conclusion that microglia are actively monitoring (or surveilling) neuronal health status and react when they sense an alteration in their environment.

The balance of calmativ NIReg signals, danger signals, neurodegeneration, and inflammatory mediators determine microglial phenotype and activity. In addition to the reassuring signals sent by NIReg interactions, microglia are equipped with an array of molecular pattern recognition

receptors and scavenger receptors that allow them to recognize and react to the presence of “nonself,” such as pathogens, “altered self,” such as apoptotic cells, and neurotoxicity [1]. Microglia are capable of detecting minute quantities of pathogen-associated molecular pattern molecules. For example, various Toll-like receptors (TLR) allow microglia to detect lipopolysaccharide and other peptidoglycans released from bacterial cell walls (TLR1, TLR2, TLR4, and TLR6) and to detect nucleic acids associated with bacteria and viruses (TLR3, TLR7, and TLR9) [28]. Microglia also react to gamma interferon (IFN- γ), which is released from virally infected cells [29]. Microglia detect the presence of sterile disease, such as dying neurons, by virtue of danger/damage-associated molecular pattern (DAMP) molecules. These signals may also be referred to as alarming NIREgs. Microglia also detect dying neurons by the release of internal proteins, including heat shock proteins, chromatin, high-mobility group box 1 protein, S100 proteins, and A β -amyloid [30]. These are detected by DAMP receptors such as the receptor for advanced glycation end product (RAGE) and macrophage antigen complex 1. Another signal clue is the presence of extracellular nucleotides and adenosine that are detected with purinergic receptors (P2X and P2Y) and adenosine receptors. Microglia also recognize and can be activated in response to plasma proteins such as fibronectin, vitronectin, plasmin, and plasminogen [31, 32]. In addition, microglia are highly sensitive to many interleukins, cytokines, and chemokines that modulate their phenotype, behavior, and motility.

Microglia can adopt a wide range of activated phenotypes depending on external conditions. The term *microglial activation* initially referred to the process whereby microglia react to detection of diseased tissue or infection by reversion to a macrophage-like phenotype with an amoeboid morphology, increased phagocytic activity, and production of inflammatory interleukins, cytokines, chemokines, proteases, nitric oxide (NO $\cdot$), and reactive oxygen species. In fact, fully activated microglia can actually cause neuronal death [4, 33]. Given that microglia with altered morphologies invariably coincide with acute neural damage and neurodegeneration, it was surmised that they are detrimental and that inflammatory microglial activation represented an opportune therapeutic target for neuroprotection [33]. With the realization that macrophages can adopt both inflammatory (M1) and anti-inflammatory (M2) phenotypes also came the recognition that microglial activation is not black and white. Rather, it seems that microglia can convert from their surveilling phenotype into a spectrum of alternative activation states, depending on the type and extent of tissue damage or infection [34]. Microglial *alternative activation* states are, by definition, not inflammatory. For example, after responding to neuronal damage and accomplishing the phagocytosis of dead neurons, microglia can adopt a

clearly anti-inflammatory phenotype described as *acquired deactivation* [35]. This phenotype includes expression of anti-inflammatory mediators, including TGF- β 1 and interleukin (IL)-10 [35, 36]. This may serve as a mechanism of signaling to the vasculature and beyond the barrier that there is no need for an additional systemic inflammatory response. By accomplishing the clearance of damaged tissues and providing anti-inflammatory cues, such microglia would prevent prolonged and excessive damage caused by invading monocytes, granulocytes, and leukocytes, plus help to prevent an autoimmune response to neuronal antigens. It seems unlikely that the ability of microglia to deal with chronic neurodegeneration would provide a selective advantage, so it is still very feasible that long-term alternative microglial activation could increase the likelihood of eventual maladaptive inflammatory microglial activation.

Evidence for inflammation in DR

Analysis of vitreous from patients with a number of vitreoretinal diseases found that out of 20 inflammatory mediators examined, only three factors, IL-6, IL-8 (CXCL8), and monocyte chemotactic protein-1 (MCP-1, aka chemokine C-C motif ligand 2, CCL2), were significantly elevated in all vitreoretinal diseases (diabetic macular edema (DME), proliferative diabetic retinopathy (PDR), branch retinal vein occlusion (BRVO), central retinal vein occlusion (CRVO), and rhegmatogenous retinal detachment (RRD)) compared with control group [37]. This study included patients with DME ($n=92$), PDR ($n=147$), as well as BRVO ($n=30$), CRVO ($n=13$), and RRD ($n=63$). As a control, vitreous from a total of 83 patients with either idiopathic macular hole or idiopathic epiretinal membranes (ERM) were examined. Levels of IL-6, IL-8, and MCP-1 were highly correlated in all the pathologies, including DME and PDR. As would be expected, vascular endothelial growth factor (VEGF) was significantly elevated in patients with PDR and CRVO, but surprisingly, not in DME. In PDR patients, the elevation of VEGF was significantly correlated with IL-6, IL-8, and MCP-1, while no significant correlation was observed in CRVO patients. In patients with DME, the glucocorticoid triamcinolone acetate (TA) reversed edema while markedly and significantly lowering vitreous levels of IL-6, MCP-1, platelet-derived growth factor subunit A (PDGF-A), and VEGF, but not IL-8, 4 weeks after intravitreal injection [38]. Thus, TA could treat edema by virtue of its anti-inflammatory effects as well as its ability to diminish VEGF levels.

In streptozotocin (STZ)-treated rats diabetic for 6 months, retinal IL-6 and tumor necrosis factor (TNF)- α protein levels were increased and anti-inflammatory IL-10 was decreased [39]. Surprisingly, IL-1 β levels were not increased.

Several other studies of T1D models have demonstrated increased TNF- α levels in retinas [40]; however, the extents of elevations are nowhere near those observed during acute inflammation. In a genome-wide analysis of gene expression in STZ-induced diabetic rats after 3 months of diabetes, the confirmed gene changes associated with inflammation did not include interleukins and cytokines most often associated with tissue inflammation [41]. Instead, the confirmed inflammatory genes diabetes-altered retinal expression included MCP-1/CCL2, chemokine C–C motif receptor 5 (CCR5), cell surface glycoprotein CD44, janus kinase 3, signal transducer and activator of transcription 3 (STAT3), laminin, tissue inhibitor of metalloproteinase-1 and 27-kDa heat shock protein 1, chitinase 3-like 1, galactose-binding soluble lectin 3 and its binding protein, complement component 1 inhibitor, as well as PDGF. IL-1 β mRNA levels increases were detected in the array analysis, but not confirmed. With the exception of CCL2, none of the confirmed genes can be considered to be conventional inflammatory mediators. Notably, CCL2 mRNA was increased as much as 80-fold in diabetic rat retinas [41].

Recently, Huang and coworkers demonstrated that retinal leukostasis was abrogated in diabetic mice with disruption of the TNF- α gene [42]. Although diabetes did not significantly increase, and even decreased TNF- α mRNA expression in retinas of wild-type mice, lack of its expression in the knockout animals greatly reduced vascular leakage after 3 and 6 months of diabetes and neurodegeneration after 3 months of diabetes. Interestingly, vascular leakage after 1 month and 6 weeks of diabetes was not affected by the absence of TNF- α , suggesting that vascular permeability at early points in the progression of experimental DR was not dependent on TNF- α or leukostasis. Similarly, Wang and coworkers demonstrated that specific disruption of the VEGF-A gene in Müller cells abrogated the effects of diabetes on leukostasis, inflammation, and vascular leakage [43]. In this study, vascular permeability was investigated in mice after 6-month duration of hyperglycemia. Interestingly, TNF- α expression was found to be elevated in the retinas of diabetic wild-type mice but not in VEGF-A knockouts. Similar results were obtained by Lin and coworkers when they observed that Müller cell-specific disruption of the hypoxia-inducible factor 1 gene resulted in diminished expression of VEGF and ICAM-1, as well as reduced leukostasis and vascular permeability in mice made diabetic with STZ [44].

Microglial activation in diabetic retinopathy

There is very little information regarding the role of microglia in clinical DR. In 1991, Weller and coworkers published an immunohistochemistry (IHC) study of

surgically removed preretinal traction membranes from patients with PDR, as well as patients with idiopathic and traumatic proliferative vitreoretinopathy (PVR) [45]. These authors concluded that microglia were prevalent in ERM from patients with idiopathic PVR, significant in those from traumatic PVR patients and inconsequential in those from patients with PDR. However, the lectin and antibodies used to identify microglia were only suggestive and do not differentiate between microglia and macrophage cells. Patients with PVR exhibit markedly elevation levels of MCP-1 (CCL2), which also correlate with PVR severity [46]. CCL2 could attract macrophages from the circulation or attract microglia from the retinal tissue. Additionally, it is now known that bone marrow-derived resident monocytes/macrophage cells, called hyalocytes, occupy the periphery of the vitreous cavity, and it was presumed that these are the cells that are present and causal in ERM formation [47]. More recently, Abu El-Asrar and colleagues studied ERM from patients with active and inactive PDR and concluded that vessel-associated cells included bone marrow-derived CD133-positive endothelial precursor cells as well as CD14-positive monocytes that may have also originated from the bone marrow [48]. These cells were more prevalent in samples from patients with active PDR. Thus, ERM formation may involve hyalocytes, whereas additional monocytes/macrophage cells may be recruited from the bone marrow during neovascularization into the ERM.

Zeng et al. used IHC to compare monocyte numbers and morphologies in 21 retinas from diabetic patients, with and without retinopathy, and ten retinas from control subjects [49]. They observed that diabetic retinal sections showed monocytes near pathologic anomalies, including dilated veins, microaneurysms, hemorrhages, cotton wool spots, and retinal and vitreal neovascularization. The prevalence of cells staining with microglial markers near the vasculature led these authors to coin the phrase “microvascular perivasculitis.” This is not unlike the perivascular CD14-positive monocyte/macrophage staining observed in ERM removed from patients with active PDR [48], in that there is no direct evidence that the cells observed by Zeng and coworkers were actually retinal microglia. Thus, we can only conclude that monocytes/macrophages are coincident with the focal pathologies that occur in DR. These cells may originate from the retina (microglia), the vitreous (hyalocytes) or the circulation (macrophages). Another important topic is whether these cells represent a causal agent in the pathologies or are responding to the pathology, and thus, their presence is, in fact, an effect. Monocytes located in the periphery of leaky vessels could represent microglia serving the useful function of phagocytosis of extravasated plasma proteins. On the other hand, these cells could be inflammatory macrophages that have invaded by virtue of adherence and diapedesis through an activated endothelial cell layer.

Phagocytic monocytes in regions of hemorrhages or cotton wool spots would seem necessary if the deposits are ever to be cleared away. Such phagocytic clearance is necessary for the resolution of inflammation, for when it is blocked, inflammation is prolonged and amplified [50].

Microglia are obviously altered in ways suggesting their activation in experimental models of DR, but the precise nature of the activation has not been defined. Like macrophages, microglia can assume a spectrum of inflammatory and anti-inflammatory phenotypes, and phagocytosis can force them toward an anti-inflammatory state. A number of studies have utilized IHC to examine microglial numbers and shapes in diabetic versus control retinas. In rats made diabetic with STZ, there are increased numbers of microglia, or to be more precise, cells reactive with markers to monocytic proteins [39, 51–55]. Many of these cells are less ramified than normal retinal microglia or are even amoeboid in shape [39, 54, 55]. Shortening of dendrites in retinal microglia have also been observed in mice made diabetic by alloxan injection [56], as well as Ins2Akita genetically diabetic mice [57]. There seems no doubt that there are increased number of cells that contain monocyte-specific markers in the retinas of diabetic rodents and that some of these cells present morphologies expected of activated microglia. However, it should be noted that these cells have been identified by IHC using antibodies to markers that are shared by microglia and other monocytes (Iba-1, ED1 antigen, Isolectin B4, CD11b, and CD68), and it is virtually impossible to distinguish activated resident microglia from extravasated plasma monocytes (i.e., macrophages) by virtue of these markers. The most reliably differentiating characteristic of microglia is their highly ramified dendritic morphology, which is lost to various extents during activation. Thus, it is unclear to what extent the increased numbers of monocytes resembling activated microglia are due to invasion of the diabetic retina by circulating macrophage cells. Further, unless markers that are specific for dendritic cells are examined, these monocytes can also be mistaken for microglia. Clearly, further studies are needed to differentiate the activation and proliferation of resident microglia from the invasion of circulating macrophages in DR.

Even in experimental DR, the cause of microglial activation is not known; the microglial activation state(s) exhibited in DR has not been characterized and the cause and effect relationships between DR pathologies and microglial activation have not been established. It is feasible that retinal microglial activation is a normal adaptive response to retinal neurodegeneration or triggered by plasma proteins extravasating by virtue of blood–retinal barrier (BRB) breakdown. Microglial activation might be linked to hyperglycemia by the formation of advanced glycation end products (AGE) or other protein glycation products within the retina [58]. It is also possible that microglial

activation is a response to systemic low-grade inflammation caused by diabetes. Chronic low-grade inflammation is a central theme in many diabetic complications, including retinopathy [59, 60]. Markers of systemic inflammation have been found to be associated with diabetic complications [61]. However, studies have not consistently demonstrated an association between DR and systemic inflammatory markers [62, 63]. The HOORN study found a positive association between DR and plasma C-reactive protein (CRP) levels, a sensitive measure of the presence of inflammation [64]. However, other studies have not found CRP to be increased in DR patients [65, 66]. In one study, CRP levels were found to correlate with lower DR prevalence [67]. In fact, inflammatory markers in DR patients seem to be associated with the presence of diabetic nephropathy rather than the severity of DR [63].

Ibrahim and coworkers used IHC with phospho-specific antibodies to demonstrate that phosphorylated forms of the mitogen-activated protein kinase (MAPK) proteins ERK1/2 and p38 co-localize with microglial cells in STZ rat retinal sections, thus suggesting activation of MEK1/2 and MKK3/6 in these cells [68]. Such activation of MAPK cascades is consistent with inflammatory activation of microglia [69]. Ibrahim and coworkers also used IHC staining with retinal microglia marker antibody and with an antibody to Almadori-glycated albumin (AGA) to show that both were localized in the ganglion cell layer [68]. This study also demonstrated that AGA treatment caused microglial activation in vitro and in vivo [68]. AGA treatment of retinal microglia in vitro leads to ERK1/2 and p38 phosphorylation as well as elevated TNF expression [70]. These results suggest that microglial activation in diabetic retinas could be the direct result of hyperglycemia-induced protein glycation products. It should be noted that AGA does not represent an AGE, but rather is an early glycation product [71]. Furthermore, published studies demonstrating activation of microglia by AGA have all been performed using relatively high concentrations (up to 0.5 mg/mL) of AGA produced by a single commercial vendor that was not characterized or evaluated for purity [71]. Given that microglia are activated by minute (less than nanogram per milliliter) concentrations of many TLR ligands, even parts per million impurities in AGA preparations could be responsible for microglial reactions. Regardless of these caveats concerning AGA, the observation that microglia in the diabetic retina tend to exhibit MAPK activation represents an advance beyond simple observation of morphology to suggest a phenotypic change in these cells. What is now needed is a more definitive description of the state of activation of microglia during the progression of DR. Such a description would help to guide therapeutic strategies to maintain microglia in a beneficial activation state.

Understanding the role of microglia and other monocytes in retinal adaptation to diabetes and the progression of DR may allow therapeutic manipulation of their activation states promoting their beneficial aspects and preventing damaging inflammation. For example, the therapeutic effect of glucocorticoid treatment could work through increasing anti-inflammatory microglial apoptosis. Glucocorticoid treatment is a mainstay of DR treatment, specifically targeting DME. Although these steroids can directly promote stability of the BRB, their anti-inflammatory actions surely contribute to their effectiveness. Glucocorticoids blunt the inflammatory response of cells, including microglia, by a number of mechanisms [9]. Glucocorticoid treatment greatly increases the phagocytic activity of monocytes and macrophages [72, 73]. Enhanced clearance of apoptotic eosinophils has been suggested to be a mechanism by which glucocorticoids help to bring about a resolution of airway inflammation in asthma [72, 74]. Phagocytosis of invading neutrophils by tissue-resident macrophages is a major mechanism for resolution of inflammation [75]. There are no studies examining the effects of glucocorticoid treatment on the phagocytic activity of retinal microglia, but one might hypothesize that treatment would accelerate the clearance of both apoptotic neurons and inflammatory plasma proteins caused by neurodegeneration and vascular dysfunction during DR. The phagocytic function of microglia may thus be beneficial by preventing and/or resolving focal inflammation caused by neuronal death or other DR pathological events, such as microvascular hemorrhage and cotton wool spot deposition.

Contributions of other immune cells to retinal inflammation during DR

The study of retinal microglia in DR is confounded by the fact that microglia cannot be easily distinguished from other monocytes, such as invading macrophages. Low-grade inflammation in the retina could include a subtle increase in the infiltration of circulating immune cells, including inflammatory macrophages, which may then affect microglial activation [76]. In 1991, Schröder and colleagues published observations of leukostasis in retinas of rats made diabetic by alloxan treatment [77]. Through esterase staining, these authors concluded that these adherent cells were granulocytes and monocytes, with monocytes being more in number. They also noted that these cells were causing microvascular occlusion and capillary damage. During the next 10 years, studies by Adamis's group and others quantitatively documented leukostasis in retinas of rats made diabetic with STZ and examined the mechanism of cell adhesion. Popularization of the hypothesis that DR is an inflammatory disease is attributed to these studies [78].

Endothelial activation and increased expression of adhesion molecules (i.e., ICAM-1) on their surface is thought to drive leukostasis in DR [79]. Heightened expression of ICAM-1 by endothelial cells could be driven by VEGF exposure or activation of the receptor for AGE (RAGE) [80, 81]. Regardless, ICAM-1 blocking antibodies inhibited leukostasis in the STZ rat model by approximately 30 % [82]. However, CD18 expression on neutrophils was also increased in the STZ diabetic rat model, and a CD18 blocking antibody was found to decrease retinal leukostasis by over 60 % in these animals [83]. In addition, knockout mice lacking ICAM-1 or CD18 both exhibit reduced retinal vascular damage and permeability during STZ-induced diabetes [84]. However, because CD18 is expressed by monocytes and lymphocytes, the effects of CD18 inhibition or deficiency could reflect inhibition of adhesion of leukocytes other than neutrophils. Adhered neutrophils could cause vascular dysfunction in DR. Activated neutrophils produce reactive oxygen species that leads to endothelial cell damage [85]. Adhered neutrophils may also directly stimulate endothelial cell death through secretion of Fas-ligand (FasL) and stimulation of the Fas death receptor on endothelial cells, in that a FasL-neutralizing antibody was able to prevent capillary loss and vascular permeability in the STZ rat model [86].

IL-8/CXCL8 and MCP-1/CCL2 are potent neutrophil and monocyte chemoattractants, respectively [87]. IL-8 causes the transmigration of neutrophils across the BBB, as well as their degranulation, release of myeloperoxidase, and respiratory burst [88, 89]. Exposure of human brain microvascular endothelial cell monolayers to VEGF stimulated IL-8 expression and neutrophil transmigration in an IL-8-dependent manner [90]. MCP-1/CCL2 is thought to be directly responsible for controlling monocyte homing to nervous tissue [91]. MCP-1/CCL2 can induce transmigration of macrophages across the BBB [92]. Surprisingly, this is dependent on expression of the chemokine receptor to which MCP-1 binds, CCR2, by both migrating macrophages and vascular endothelial cells. Transgenic expression of MCP-1 in the CNS using an astrocyte-specific promoter (GFAP) caused encephalopathy characterized by increased monocyte numbers and BBB leakage [93]. In experimental light-induced retinopathy, there is an infiltration of macrophages which cooperate with resident microglia to phagocytose dead photoreceptors and then exit the retina by diapedesis into the vasculature in a debris-laden state [94]. Following light damage, MCP-1/CCL2 expression is increased exclusively in Müller cells and the expression coincides with the recruitment of monocytes [95]. In addition, MCP-1/CCL2 can directly promote BBB permeability [96]. Although the findings of high levels of IL-8 and MCP-1 in the vitreous of DR patients do not prove a role for retinal neutrophil and monocyte leukostasis and infiltration in the pathology, this is a likely outcome of their presence.

It should be noted that the leukostasis theory of retinal inflammation and vascular damage in DR is based on experimental models and there is little data demonstrating leukostasis in clinical DR. However, there is evidence that neutrophils may play a role in clinical DR. In a large retrospective study of almost 31,000 persons, Woo and coworkers found that systemic neutrophil counts were increased by approximately 10 % on average in diabetics and an additional 10 % in patients with moderate NPDR or PDR [97]. The ratio of neutrophils to total white blood cell count was significantly increased in PDR. The neutrophil count was well correlated with disease severity, and this parameter corresponded to a 2.7-fold odds ratio in patients within the highest quartile group. In an independent study, the expression of CD18 β -integrin chain on neutrophils was found to be elevated in diabetics with DR and to correlate with severity of DR [98].

Thus, although classical inflammation does not seem to occur in the diabetic retina, neural inflammation, including the adherence of monocytes and granulocytes to the endothelium and microglial alterations, is apparent. While microglial activation surely occurs in DR, the character, cause, and causal role of microglial activation in the pathology are yet to be determined.

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