LEUKOCYTES AND INFLAMMATION IN THE PATHOGENESIS OF THE EARLY STAGES OF DIABETIC RETINOPATHY

by

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*We also certify that written approval has been obtained for any proprietary material contained therein
Dedication

To my children Frances, Erick, and Daniel and my mother Anna and her husband Stuart for providing support, navigating those piles of papers, and listening to my rants while I was toiling on this body of work.
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### Abbreviations

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<th>Definition</th>
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<tr>
<td>7/4</td>
<td>Myeloid 7/4 marker, also known as Ly-6B, a marker of neutrophils and monocytes.</td>
</tr>
<tr>
<td>αLβ2</td>
<td>CD11a/CD18, LFA1 integrin</td>
</tr>
<tr>
<td>αMβ2</td>
<td>CD11b/CD18, MAC1 integrin</td>
</tr>
<tr>
<td>ABCD</td>
<td>Appropriate Blood pressure Control in Diabetes</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>ASM</td>
<td>Acid sphingomyelinase</td>
</tr>
<tr>
<td>C57Bl/6J</td>
<td>C57 black 6 stain of mice from Jackson Labs (Jax)</td>
</tr>
<tr>
<td>CD115</td>
<td>Colony stimulating factor 1 receptor (CSF1R), also know an macrophage colony stimulating factor receptor (M-CSFR), a marker of monocytes.</td>
</tr>
<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase receptor type C, a marker present on bone marrow derived cells.</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for disease control and prevention</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>COS</td>
<td>Immortalized cell line derived from monkey kidney fibroblasts</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3, CD11b/CD18 integrin, αMβ2, MAC1</td>
</tr>
<tr>
<td>CR4</td>
<td>Complement receptor 4, CD11c/CD18 integrin, αXβ2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetic Control and Complications Trial</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>DRCRN</td>
<td>Diabetic Retinopathy Clinical Research Network</td>
</tr>
<tr>
<td>DRS</td>
<td>Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>EDIC</td>
<td>Epidemiology of Diabetes Interventions and Complications study</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>Fas</td>
<td>CD95, receptor mediating apoptosis</td>
</tr>
<tr>
<td>FasL</td>
<td>CD95L, ligand mediating apoptosis</td>
</tr>
<tr>
<td>FIELD</td>
<td>Fenofibrate Intervention and Event Lowering in Diabetes study</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formyl-Methionyl-Leucyl-Phenylalanine, a peptide which mimics degraded bacterial or mitochondrial proteins which cause leukocyte activation</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>G-CSFR</td>
<td>Granulocyte colony stimulating factor receptor (CD114)</td>
</tr>
<tr>
<td>GHb</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
</tr>
<tr>
<td>GR1</td>
<td>Epitope consisting of Ly6C and Ly6G</td>
</tr>
<tr>
<td>HeLa</td>
<td>Immortalized cell line derived from human cervix cancer cells</td>
</tr>
<tr>
<td>HEK293</td>
<td>Immortalized cell line derived from human kidney fibroblasts</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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IC50  Half maximal inhibitory concentration, concentration at which effect is inhibited by 50%.
ICAM1  Intercellular adhesion molecule 1
IDDM  Insulin dependent diabetes mellitus, Type I diabetes
IL1β  Interleukin 1 beta
INL  Inner nuclear layer
IVFA  Intravenous fluorescein angiography
LFA-1  Lymphocyte function-associated antigen 1 (integrin αLβ2, CD11a/CD18)
iNOS  Inducible nitric oxide synthase
IPL  Inner plexiform layer
LPS  Lipopolysaccharide, bacterial protein
MAC1  Macrophage-1 antigen (integrin αMβ2, CD11b/CD18)
MCP1  Macrophage chemotactic protein 1 (CCL2)
M-CSFR  Monocyte colony stimulating factor receptor (CD115)
MHCII  Major histocompatibility complex 2
MIDAS  Divalent metal ion-dependent adhesion site
MIF  Macrophage migration inhibitory factor
mREC  Mouse retinal endothelial cells
NAD⁺  nicotinamide adenine dinucleotide
NFκB  Nuclear factor kappa light chain enhancer of activated B cells
NIDDM  Non-insulin dependent diabetes mellitus, Type II diabetes
NIF  Neutrophil Inhibitory Factor
<table>
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<th>Definition</th>
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<tr>
<td>NIH3T3</td>
<td>Immortalized cell line derived from human kidney fibroblast cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform later</td>
</tr>
<tr>
<td>PAR</td>
<td>Poly(ADP –ribose)</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelial derived factor, serpinf1</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Protein Lipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Protein tyrosine kinase 2 beta</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor of advanced glycation end products</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROP</td>
<td>Retinopathy of prematurity model</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDF1</td>
<td>Stromal cell derived factor 1</td>
</tr>
<tr>
<td>SOCS2</td>
<td>Suppressor of cytokine signaling proteins Jak/Stat pathway</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand, CD253</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay, a marker of fragmented DNA due to apoptosis</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLA4</td>
<td>Very late antigen 4, CD49d/CD29, integrin α4β1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type (usually C57Bl6/J)</td>
</tr>
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</table>
Leukocytes and Inflammation in the Pathogenesis of the
Early Stages of Diabetic Retinopathy

Abstract

by

ALEXANDER ALI VEENSTRA

Retinal vascular loss, a critical precursor to visual impairment in diabetic retinopathy, is generally attributed to diabetes-induced aberrant biochemical processes intrinsic to the cells of the retinal vasculature. The role of leukocytes and inflammation in the pathogenesis of diabetic retinopathy was investigated. Abnormalities characteristic of early stages of the retinopathy were measured in chimeric mice lacking iNOS (inducible nitric oxide synthase) or PARP1 (Poly(ADP-ribose) polymerase 1), proteins which play a role in inflammation and diabetes-induced retinal capillary loss. Diabetes-induced capillary degeneration, leukostasis and, and superoxide production in the retina, were inhibited in animals lacking iNOS or PARP1 in only their marrow-derived cells. Neutrophils (and monocytes) play a major role in the retinopathy development, because retinal capillary degeneration likewise was significantly inhibited in diabetic chimeric mice lacking Granulocyte Colony Stimulating Factor receptor in only their marrow-derived cells. Immunodepletion of neutrophils or monocytes inhibited endothelial death otherwise observed when co-culturing leukocytes from wild type diabetic animals with retinal endothelium.
Leukocyte integrin αmβ2 has been shown to mediate adhesion dependent leukocyte mediated damage. The ability of the selective antagonist of αmβ2, Neutrophil Inhibitory Factor (NIF) to prevent diabetes-induced retinal vascular abnormalities and leukocyte activation was investigated in transgenic mice expressing NIF. Diabetes-induced superoxide production, leukostasis and capillary loss in the retina were significantly increased in wild type mice. In contrast, mice expressing NIF did not develop any of these abnormalities. Importantly, NIF did not significantly impair the ability of mice to clear an opportunistic bacterial challenge.

The inflammation associated transcription factor (NFκB) p50 (a product of p105) is increased in retinal vascular endothelial cells of diabetic animals. Diabetes-induced increases in retinal capillary loss and superoxide generation were significantly increased in p105−/− mice. Likewise, leukocytes from diabetic animals lacking p105 killed more endothelial cells in co-culture than diabetic controls.

We conclude that neutrophils (and monocytes) mediate diabetes-induced loss of capillaries in the retina and that inhibition of leukocyte binding integrin αmβ2 may have clinical benefits in the prevention of the early stages of diabetic retinopathy. However, loss of p105/p50 exacerbated diabetes-induced inflammation and vascular pathology of the retina.
1.1 Diabetes

Definition and epidemiology. Diabetes mellitus is a chronic metabolic disease in which improper regulation of glucose leads to abnormal elevated levels of blood glucose or hyperglycemia. In the United States alone, the Center for Disease Control and Prevention estimated that in 2011, 25.8 million children and adults (8.3% of the population) had diabetes, of which 2.1 million were new cases (CDC 2011). An additional 79 million Americans (35%) were estimated to have pre-diabetes (at risk to develop diabetes), in which hemoglobin A1c (HbA1c) or fasted blood glucose was elevated beyond the normal range (5.6% and 100mg/dL respectively) but less than the limit at which diabetes is formally diagnosed (6.5% and 126 mg/dL respectively). Diabetes is the seventh leading cause of death in the US and adults with diabetes are twice as likely to die as similar age non-diabetic adults. The cost of treating diabetes in 2007 was estimated to be 116 billion dollars for health care costs alone. The prevalence of diabetes (and therefore diabetes associated complications) is increasing; diabetes is expected to afflict three hundred and sixty six million people worldwide by 2030 (Wild et al. 2004; Zhang et al. 2010; Frank 2011; Ding and Wong 2012).

Diabetes is divided clinically into two categories. Type I diabetes or insulin dependent diabetes mellitus (IDDM) is often called juvenile diabetes due to a general onset of the
disease in children prior to puberty. Type I accounts for only about 5% of all cases of diabetes. It is generally thought to occur due to an autoimmune reaction, which destroys the insulin producing beta islets of the pancreas. Type I diabetes is treated with injections of exogenous insulin.

Type II diabetes or non-insulin dependent diabetes mellitus (NIDDM) is defined by a resistance of cells to transport glucose into the cell in response to insulin. Type II diabetes historically was diagnosed in adults at least 30 years of age, however the number of new cases of Type II diabetes in adolescents (10-20 years of age) and young adults (20-44 years of age) is increasing rapidly; the CDC reported an estimated 31% of new cases in adolescents are Type II diabetes ((CDC) 2011). Type II diabetes is often associated with obesity, a high fat diet and a sedentary lifestyle. It is generally treated with oral medications, which decrease blood glucose by inhibiting liver glucose production (biguanides) or increasing insulin production (sulfonylureas).

**Complications of Diabetes.** Acute complications of diabetes include insulin-induced hypoglycemia. Long-term complications of diabetes include macrovascular complications (cardiovascular disease, stroke, atherosclerosis, and amputation of extremities due to vascular loss), microvascular complications (nephropathy and retinopathy), and neuronal complications such as tactile allodynia. It is not clear if diabetes-induced macrovascular, microvascular, and neuronal complications share a common pathogenesis other than hyperglycemia, however leukocytes and inflammation in general have been suspected in the pathology of many of the long-term complications.
1.2 Diabetic Retinopathy

Definition and Epidemiology. Diabetic Retinopathy (DR) is a complication of diabetes that leads to a progressive loss of vision. It is the leading cause of blindness in adults under 65 years of age in the United States (CDC 2011). DR is currently defined clinically by vascular lesions (microaneurysms, increased vascular permeability, retinal edema, and neovascularization), whereas impaired visual function (which undoubtedly involves neural cells of the retina) is a major reason for clinical interest in retinopathy.

Clinically, DR is divided into two general stages; an initial non-proliferative stage (non-proliferative diabetic retinopathy, NPDR), in which blood vessels of the retina are progressively lost or occluded, and a proliferative stage (proliferative diabetic retinopathy, PDR), which is marked by the emergence of new blood vessels in the vitreous. The proliferative stage is clinically significant because it is associated with the majority of the cases of vision loss. Furthermore, diabetic edema of the macula, a severe threat to vision, can develop in either stage but is more prevalent in the proliferative stage. The morphology of vascular lesions in DR is independent of the type of diabetes (Type I or II) with the exception of the time of onset and risk factors for the development.

Historically the percentage of diabetic patients developing NPDR in the United States was 95% and 75% for Type I and Type II diabetes in individuals at least 15 years diabetic (Klein et al. 1984a; Klein et al. 1984b). The rate of progression into the proliferative phase of the retinopathy was 11% and 7% for Type I and Type II diabetes over 4 years.
(Klein et al. 1989a; Klein et al. 1989b). According to a recent report by the CDC, the rate of developing retinopathy by the age of 40 in the US has decreased to 28.8% and progression into the advanced stages of the retinopathy has decreased to 4.4% ((CDC) 2011). The observed decrease in the rate of incidence (in the US) is most likely due to changes in the last several decades, which led to better glucose control. These changes include improvements in diet, lifestyle, and glucose lowering medication such as insulin and metformin as outlined by several key studies including the Diabetic Control and Complications Study and the United Kingdom Prospective Diabetes Study (reviewed in the Current Therapies of section 1.2, p. 30).

**Clinical Pathology.**

*Non-proliferative diabetic retinopathy.* Clinically NPDR is assessed during a dilated eye exam or in photos of the fundus by the presence of vascular abnormalities in the retina including yellow exudates (patches of deposited protein), small dots indicative of focal point hemorrhages (leakage of blood from vessels or microaneurysms), and cotton wool spots (focal ischemia in nerve fiber layer). Additional retinal abnormalities, including increased blood vessel tortuosity and swelling, venous beading (consecutive small bulges or dilations in the veins), and blood vessel leakage can be assessed by intravenous fluorescein angiography (IVFA). Blood vessel leakage is defined (during an IVFA) by fluorescein build up outside of structured blood vessels, whereas blocked capillaries can be inferred by a lack of fluorescein in an area of the retina.
The observed retinal abnormalities are consistent with damaged or dysfunctional retinal vasculature. Blood vessel dilation and tortuosity suggest nearby blood vessel occlusion, while cotton wool spots, exudate build up, and retinal edema are thought to be byproducts of increased blood vessel permeability or hemorrhage. Saccular microaneurysms are thought to be unsuccessful attempts to form new blood vessels in the retina. The spectrum of lesions induced in the retina is not unique to diabetes. The same lesions develop also in non-diabetic patients especially with age, although much fewer in number than in persons with diabetes.

The frequency of retinal lesions, especially the microaneurysms and focal hemorrhages, dictate the severity (mild, moderate, or severe) of the NPDR. Factors predicting increased frequency of vascular lesions include high blood glucose (as measured by glycated hemoglobin A1c) and the duration of diabetes (Klein et al. 1984a; Klein et al. 1984b; Klein et al. 1988). An increase in the frequency of the vascular lesions is a risk factor for edema and progression into the clinically significant proliferative stage.

*Proliferative diabetic retinopathy.* PDR is defined by new blood vessel outgrowth (neoangiogenesis) from the retina into the vitreous. In addition to blocking light directly, the new vessels are particularly vulnerable to shear forces imposed by moving vitreous fluid, resulting in hemorrhage into the vitreous, which obstructs vision. Leakage of additional blood can come from focal hemorrhages of the existing vasculature in the retina as well.
In addition to the obstruction of light entering the retina, loss of vision can occur due to disruption or damage of the photoreceptors and ganglion cells caused by swelling of the retina due to edema, focal ischemia (cotton wool spots located above focal hemorrhages) and detachment of the photoreceptors from the retinal pigmented epithelial cells (traction retinal detachment). The edema and cotton wool spots are thought to be caused by leaky blood vessels where as the pulling of the retina by the vitreous is a result of scar tissue formation at the surface of the retina caused by damaged blood vessels.

Immuno histochemical features of early changes in the retina. In post mortem preparations of retina from diabetic humans and animals, capillarieslackin vascular cell nuclei are also devoid of red blood cells and therefore are non-perfused (Cogan et al. 1961; Bresnick et al. 1976b). These acellular capillaries are thought to be tubes of residual basement membrane, which remain after the loss of endothelial cells and pericytes (Figure 1-1, p. 106). In addition, present on some capillaries are pericyte ghosts, which are defined as hump shaped spaces in the basement membrane devoid of the pericyte nucleus and other cellular organelles.

Capillary loss. Acellular capillaries are more common in the superior temporal region of the retina (Kern and Engerman 1995) and also in an annular ring of blood vessels surrounding the blood vessel free zone encircling the macula (Cogan et al. 1961). They increase with the duration of diabetes, and to a lesser extent with age, in a patchwork of clusters. In some of these acellular capillaries, endothelial cell processes continue to line the vessels and are presumably extensions of neighboring endothelial cells, while other
capillaries are completely filled with processes of glial cells (Engerman 1989). It is unclear however if the glial extensions cause occlusion or occur after the loss of the vascular cells.

The mechanism, by which the vascular cells of the capillary are lost, remains unclear, and the dogma of the field has been that the cells die due to intrinsic defects in the biochemical pathways of the cells due to altered metabolism resulting from hyperglycemia. However occlusion of the retinal blood vessels as seen in other retinal vascular diseases such as branched vein occlusion causes pathology similar to that seen in DR (Kuwabara et al. 1961). The closure of one capillary is associated with the dilation of nearby blood vessels, increased vascular permeability, and additional progressive occlusion of adjacent capillaries. The progressive loss of perfused vessels in the retina leads to ischemia and loss of nutrients resulting in the upregulation of VEGF through HIF1α and subsequent neovascular angiogenesis. The cause of capillary blockage in DR is unclear, and may be due to leukocyte adhesion, fibrin clots, or invading cellular processes.

*Loss of Pericytes.* Hyperproliferation of endothelial cells and the loss of pericytes from retinal capillaries have been reported in histological preparations of retinal vasculature from diabetic patients (Cogan et al. 1961; Kuwabara and Cogan 1963; Speiser et al. 1968). This observation led to the postulate that pericyte loss preceded other lesions of DR. In addition to regulating capillary blood flow, pericytes have been shown to inhibit endothelial cell proliferation (Orlidge and D'Amore 1987). However the role of pericytes
in DR is unclear, as pericyte loss has been reported to occur at the same rate as the appearance of acellular capillaries (Mizutani et al. 1996).

*Changes in basement membrane, leukostasis, and ganglion cells.* Other observations made from histological preparations of retina in diabetes include a thickening of the basement membrane, increased leukostasis, and ganglion cell loss. Diabetes-induced thickening of the basement membrane may impede the exchange of nutrients and oxygen between blood and the neural retina (reviewed in thickened basement membranes subsection of section 1.3). The number of leukocytes adherent to the microvasculature in the retina is also increased in diabetes, and the adherent leukocytes may cause occlusion of the retinal capillaries and therefore local patches of hypoxia (leukostasis, reviewed in the leukocyte subsection of section 1.3). Decreased retinal thickness due to loss of cells in the nuclear and ganglion cell layers have also been reported in the neural retina of diabetic animals (reviewed in the neuronal changes in the retina section below).

**Neuronal changes in the retina.** Although DR is defined by vascular changes that occur in the retina, several visual impairments due to diabetes have been documented.

*Measures of vision and electrical signaling.* Decreases in the sensitivity of blue light sensing S-cones (short wavelength) have been reported to precede retinopathy in patients with Type I diabetes (Daley et al. 1987) and worsen with the development of retinopathy (Terasaki et al. 1996). Patients with Type II diabetes exhibit decreases in S-cone sensitivity after the onset of retinopathy. Although the mechanism of the reduced S-cone
sensitivity in diabetes is unknown, the sensitivity has been shown to decrease with increasing levels of glycated hemoglobin A1c (McFarlane et al. 2012). Decreases in contrast sensitivity have also been reported in both Type I and II diabetics prior to the onset of retinopathy (Sokol et al. 1985; Lopes de Faria et al. 2001), while impairment of dark-adapted sensitivity in patients has been reported after the onset of NPDR (Jackson et al. 2012).

In addition to visual sensitivity impairment, gross electrical signaling in the retina is also abnormal in diabetes. The amplitude and latency of the oscillatory potential of the electroretinogram (ERG) (Caputo et al. 1990; Holopigian et al. 1992), as well as defects in the multifocal ERG (Lakhani et al. 2010) also precede the onset of retinopathy in diabetics, and in the case of multifocal ERG, predict the occurrence of future vascular lesions (Harrison et al. 2011). In summary, while diabetes clearly causes a number of impaired neurological responses prior to observable vascular changes in the retina, the mechanisms of the various visual impairments remains undefined and warrants further investigation.

Loss of retinal neural cells. Diabetes may also induce the loss of retinal neurons and ganglion cells, as increases in TUNEL positive ganglion cells and decreases in retinal thickness have been observed in retinal preparations from rodents with diabetes in some studies however contradictory data, especially in mice, has been presented in other studies (Hammes et al. 1995; Barber et al. 1998; Asnaghi et al. 2003; Martin et al. 2004).
Thinner ganglion cell and nerve fiber layers have also been measured in diabetic patients with optical coherence tomography (van Dijk et al. 2010).

a) Summary of reports in rats. In a study by Barber, an increase in TUNEL positive cells was reported in retinal whole mounts from insulin-treated Sprague-Dawley rats, diabetic for one month, and in post mortem retina from a diabetic patient. However, in the absence of cellular markers, it is difficult to discern, which type of cells were TUNEL positive. Nevertheless, roughly 60-80 TUNEL positive cells were present per retina in rats diabetic for one to twelve months compared to 10-20 TUNEL positive cells per retina in non-diabetic age matched controls. The administration of insulin decreased the number of TUNEL positive cells in the GCL compared to diabetic controls not treated with insulin demonstrating the insulin is neuroprotective. In a study by Asnaghi, insulin treated Sprague-Dawley rats diabetic for six weeks developed 150 TUNEL positive cells compared to 50 TUNEL positive cells in non-diabetic controls (Asnaghi et al. 2003). It is not clear why the number of TUNEL positive cells increased in this study, especially in the non-diabetic controls, compared to the study by Barber except that the duration of the experiment was different.

b) Summary of conflicting reports in mice. In the Asnaghi study, insulin treated C57BL/6J mice, diabetic for six weeks, developed 10-20 TUNEL positive cells in the GCL, which was not significantly different from non-diabetic controls. In contrast, a related study by Martin demonstrated that non-insulin treated
C57BL/6J mice, diabetic for twelve weeks, developed a 30% decrease in the number of ganglion cells, 10 caspase-3 positive cells (1/550 in non-diabetic controls) and an undisclosed but significantly higher number of TUNEL positive cells in the GCL per cross section compared to non-diabetic controls (Martin et al. 2004). The difference between the two studies in mice may be due to treatment with insulin in the Asnaghi study, which was noted in the Barber study to be neuroprotective. Furthermore in a study by Mohr, caspase-3 activity retinal lysate from diabetic insulin treated mice was not increased compared to non-diabetic controls at 2 months duration of diabetes, however caspase-3 activity was elevated by roughly 5 and 8% over control in mice 4 and 8 months diabetic respectively and 5% in samples from diabetic patients(Mohr et al. 2002). Mice fed a high galactose diet, (a model of diabetes that results in vascular lesions of the retina) in the Mohr study did not develop the increase in caspase-3 activity. Despite the lack of caspase-3 activity in mice diabetic for 10-12 weeks, the number of cells in the GCL was significantly reduced by 20-25% per cross section (500-550 total), suggesting that the observed GCL loss is independent of caspase-3.

In summary, diabetic rats and non-insulin treated mice appear to develop diabetes-induced loss of retinal ganglion cells a few weeks after the induction of diabetes, while insulin treated mice appear to be more resistant to the loss of retinal ganglion cells. The relationship between the loss of ganglion cells and the diabetes-induced vascular lesions
of the retina has not been explored. The loss of the ganglion cells on vision also remains to be defined.

**Current Therapies.**

*Laser photocoagulation and anti-VEGF.* Treatment for PDR currently consists of ocular injections of anti-vascular endothelial growth factor (anti-VEGF) or destruction of the outer retina with photocoagulation laser therapy in an attempt to slow down the progression of neovascularization, alleviate edema, and preserve central vision. Although about 50% of patients respond completely to a limited number of anti-VEGF injections, some continue to produce new blood vessels and experience retinal edema despite the anti VEGF therapy, suggesting that other factors besides VEGF are involved (Rajendram et al. 2012). Laser photocoagulation can be used in focal areas to cauterize leaky blood vessels or across the periphery of the retina to ablate larger areas of tissue, which may inhibit neovascularization by reducing the consumption of oxygen and nutrients. Similar to anti-VEGF therapy only about half of patients respond adequately to laser therapy (DRS 1981; ETDRS 1991a; Ooi and Hardy 1999). Laser therapy has also been recommended to treat severe NPDR and edema in NPDR (ETDRS 1991a).

*Anti-inflammatory steroids.* Intravitreal injections of triamcinolone, a strong anti-inflammatory corticosteroid, have been shown to reduce retinal thickness (edema), and intra-retinal hemorrhage resulting in an improvement of visual acuity in about 50% of patients with refractory diabetic macular edema over a five year interval (Jonas and Sofker 2001; DRCRnetwork 2008; Gillies et al. 2009). Use of this drug to treat diabetic
edema has declined, as clinical trials of corticoid steroids have been shown to be less effective than laser photo coagulation or anti VEGF therapy, and are associated with the risk of developing glaucoma and cataracts (Beck et al. 2009; Elman et al. 2010). In summary, while many patients do regain or preserve some of their vision with the current treatments for PDR, there remains a need for additional therapies, which inhibit or delay PDR.

**Control of blood glucose concentrations.** Good glucose control has been shown to be beneficial in preventing diabetic retinopathy. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive glycemic control with insulin delayed the onset of the development by 76% in Type I diabetic patients with no retinopathy or minimal to moderate NPDR, and it inhibited the progression of preexisting diabetic retinopathy by 54% (DCCT 1993). After the DCCT trial had ended, the patients were monitored over an additional 10+ year period to see if any lasting benefit persisted in the Epidemiology of Diabetes Interventions and Complications (EDIC) study (White et al. 2010). Adults in the previous intensive control group continued to show slower progression of diabetic retinopathy than those in the conventional therapy group (24%) despite no significant differences in HbA1c (after the DCCT study) between the two groups. Adolescents in the intensive control group of the DCCT study, who had higher HbA1c values than adults in the same group, did not have a persistent benefit in the EDIC study. The follow up study demonstrates that long-term benefits can be achieved by tight control of blood glucose but that good glucose control can be difficult to achieve. In the United Kingdom Prospective Diabetes Study (UKPDS), intensive glycemic control with insulin and
sulfonylureas in Type II diabetic patients reduced the need for laser photocoagulation intervention by 29% and reduced visual acuity loss by 47% (UKPDS 1998a).

**Blood pressure and fenofibrate.** Therapies targeting hypertension and dyslipidemia, two of the risk factors associated with DR in Type II diabetic patients, have had mixed results. The PPARα antagonist, fenofibrate, was originally used in Type II diabetic patients to lower lipids. However, treatment with fenofibrate was shown in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study to lower the risk of progression of diabetic retinopathy (first laser treatment) compared to the control group (rate of progression of 3.4% and 4.9% respectively), over 5 years, independent of lipid lowering effects (Keech et al. 2007). In patients with preexisting retinopathy, the reduction of visual acuity by two lines was also reduced compared to the control group (rate of loss of 3.1% and 14.6%). In the ACCORD study, Type II diabetics receiving fenofibrate had a lower rate of progression of diabetic retinopathy compared to the control group (rate of progression of 6.5% and 10.4% respectively) over 4 years.

In the ACCORD study, the rate of progression of diabetic retinopathy in an intense blood pressure treatment group with various blood pressure medications was not reduced compared to the control group receiving standard treatment for high blood pressure (rate of progression of 10.8% and 8.8% respectively). In contrast, intensive control of blood pressure in hypertensive Type II diabetic patients (target blood pressure <150/85 mm Hg), with an angiotensin converting enzyme inhibitor or a beta blocker, compared to standard blood pressure treatment in a control group (target blood pressure <180/105),
reduced the risk of progression of diabetic retinopathy (from 51.3% to 34%), over a period of eight years (UKPDS 1998b). In addition, a reduction in the loss of visual acuity was also achieved (rate of loss of 10.2% and 19.2% respectively). In a third study on intensive blood pressure treatment in Type II diabetics, the Appropriate Blood Pressure Control in Diabetes study (ABCD), the difference between patients receiving intensive blood pressure treatment (<75 mm Hg diastolic pressure) and a control group receiving moderate blood pressure treatment (<90 mmHg) with regard to the progression of diabetic retinopathy was not statistically significant (Estacio et al. 2000).

The overall rate of progression of diabetic retinopathy, especially the control groups, in the UKPDS study was much higher than that seen in the ACCORD study, suggesting that blood pressure control may be more important in patients with preexisting severe retinopathy. The converse was true in the FIELD study, in which the rate of overall progression was very low compared to the ACCORD study. The reasons why some control groups appear to have lower rates of retinopathy progression remains unanswered and is under investigation with respect to patient histories including previous illnesses, over the counter medications, and genetic markers (personal correspondence Lloyd Aeillo). In addition the use of ACE inhibitors for blood pressure control by the UKPDS study may have additional effects unrelated to blood pressure alone as the renin-angiotensin system has been implicated in the development of diabetic retinopathy in Type II diabetics (Wright and Dodson 2010).

**Animal models.**
Models of diabetes. Diabetes can be induced in fasting animals with injections of streptozotocin or alloxan, which destroy the beta islets of the pancreas and thereby induce Type I diabetes. The two drugs (reviewed in Szkudelski 2001(Szkudelski 2001)) are thought to be taken up by the GLUT2 (Glucose 2) transporter on the beta islets of the pancreas and also by cells in the liver where they induce the generation of superoxide, DNA damage, and eventual cell death. Tumors of the liver are occasionally seen in streptozotocin or alloxan treated animals. Reports of streptozotocin induced neural dysfunction two to four weeks after injection in animals that did not develop hyperglycemia suggest that this model may be inappropriate to study complications of diabetes for several weeks after injection (Pabbidi et al. 2008). Experiments conducted in the first several weeks after the injection of streptozotocin would benefit greatly from similar controls (streptozotocin-injected animals that do not develop diabetes) in addition to animals, which have not been injected with the drug.

Several strains of rodents spontaneously develop diabetes. The Akita and IL2 deficient non-obese diabetic (NOD) mice are models of Type I diabetes. Leptin deficient ob/ob mouse, leptin receptor deficient db/db mouse, and leptin receptor deficient fa/fa Zucker rats are models of Type II diabetes. Mice developing spontaneous Type I diabetes tend to be more diabetic (fewer remaining insulin producing beta cells), in a very short period of time, than mice treated with streptozotocin and thus require immediate insulin intervention to prevent death after the onset of diabetes.
Models of diabetic retinopathy. Early vascular lesions of DR morphologically similar to those induced with streptozotocin or alloxan have also been documented in dogs, rats and mice fed a high galactose diet (30-50%) (reviewed in subsection the Role of sugar, lipids, and oxygen in section 1.3) (Engerman and Kern 1984; Engerman and Kern 1987; Engerman 1993).

Many of the early lesions of diabetic retinopathy occur in monkeys, cats, and dogs. However, research in these animals, especially monkeys, is cost prohibitive and limited by ethical considerations. Furthermore, while the eye is larger, which accommodates the collection of tissue samples and photography of the retina, the number of antibodies commercially available to study protein expression and differentiate cell types is limited. Genetic manipulation of these species is hindered by a lack of in-vitro fertilization drugs and techniques, which work in these species.

Diabetic rhesus monkeys with hypertension (but not without) develop vascular lesions morphologically similar to those seen in diabetic patients with early or advanced NPDR. These lesions include basement membrane thickening, cotton wool spots, intraretinal hemorrhages, hard exudates, areas of capillary non-perfusion, acellular capillaries, vascular leakage, and small microaneurysms but no preretinal neovascularization in streptozotocin induced Type I diabetes (Tso et al. 1988; Buchi et al. 1996) or obesity associated Type II diabetes (Kim et al. 2004). The observed requirement that the diabetic monkeys must have hypertension prior to the development retinal lesions is not mirrored in all diabetic patients, as patients with diabetes but without hypertension can develop...
diabetic retinopathy. However, hypertension has been identified as a strong risk factor for developing diabetic retinopathy especially in Type II diabetes (UKPDS 1998b).

Diabetic cats, which have had a pancreatectomy, develop microaneurysms, vascular leakage, and progressive areas of non-perfusion in their retina (Hatchell et al. 1995; Linsenmeier et al. 1998). In addition, hypoxia has been reported in the retina of the diabetic cat (Linsenmeier et al. 1998).

Lesions of DR morphologically similar to those in diabetic humans have also been documented in dogs made diabetic with alloxan. These lesions include basement membrane thickening, progressive areas of non-perfusion, inter retinal hemorrhages and microaneurisms, but do not include pre-retinal neo-angiogenesis (Engerman and Kern 1984; Engerman and Kern 1987; Engerman 1993).

The retinopathy that develops in mice and rats is more limited, as rodents do not develop cotton wool spots or microaneurysms. Diabetes-induced acellular capillaries and pericyte ghosts, although present, are much less frequent than in dogs, which may be due to a comparatively short lifespan. Vascular leakage in diabetic mice is also less severe than in other animal models. The advantage of mice in particular is the relative ease of making genetic manipulations and the number of commercially available antibodies to mouse proteins. However, careful selection of the background stain of mice is important as many strains of mice develop spontaneous retinal degeneration, especially with age.
1.3 Potential mechanisms in the development of DR.

Sections of this subchapter were previously published in a report in which I summarized discussions at a meeting by the Lasker Foundation on diabetic retinopathy (Lasker 2012).

Role of sugars, lipids, and oxygen.

Glucose and glycemic control. Clinical trials and animal studies have established that hyperglycemia is strongly associated with the severity of retinopathy, and that improved metabolic control of diabetes significantly inhibits the development and progression of diabetic retinopathy (at least in the background stages of the retinopathy) (Klein et al. 1988; DCCT 1993; UKPDS 1998a), suggesting a role for these abnormalities in the pathogenesis of the non-proliferative stages of the retinopathy.

Beneficial effects of improved metabolic control (also called improved glycemic control) in diabetes, however, are not adequate to establish that hyperglycemia is the sole cause of the retinopathy. In addition to the well-recognized hyperglycemia present in poorly controlled diabetes, there are also other alterations that occur, for example, in lipid metabolism and amino acid levels. Reports indicate that variations in glucose levels may cause more abnormalities of cellular structure or function than chronic hyperglycemia, and there is speculation that such variability (which is common in many diabetic patients) might accelerate development of diabetic complications including retinopathy (Monnier 2007).
The evidence that most clearly implicates sugar in initiating the pathogenesis of the retinopathy is the demonstration that non-diabetic animals (dogs, rats and mice) made experimentally hyperhexosemic by feeding a galactose-rich diet develop retinal vascular lesions that are morphologically identical to those seen in diabetes, yet do so in the absence of the other metabolic sequelae that are characteristic of diabetes (Engerman and Kern 1984). It remains unknown, however, whether the experimental galactosemia reproduces all of the early changes seen in the retina in diabetes (biochemical abnormalities inducing upregulation of VEGF and inflammatory mediators, alterations in retinal blood flow and oxygen tension, etc.).

It has been demonstrated in diabetic patients and animals that vascular lesions occur more frequently in the superior and temporal regions of the retina than in other quadrants of the same retina (Taylor and Dobree 1970; Cunha-Vaz 1972; Kern and Engerman 1995). Since the entire retina presumably is exposed to the same blood glucose concentration, this finding suggests that elevated glucose is not by itself adequate to account for the severity of vascular pathology. It is unclear what accounts for the variation among regions of the retina.

*Metabolic memory.* In both patient and animal studies, DR can be better inhibited with improved glycemic control if started from the onset of diabetes (prevention) as opposed to after prolonged hyperglycemia (intervention) (Engerman and Kern 1987; DCCT 1993; Hammes et al. 1993; Holman et al. 2008). This resistance to intervention has been referred to as “metabolic memory”. Intervention with improved glycemic control in early
DR does eventually have beneficial effects to slow down progression of the retinal disease, but such intervention has not been found to inhibit the proliferative phase of the retinopathy. Whether retinal edema is influenced by metabolic memory has not been established. Besides the retina, metabolic memory occurs in other tissues developing pathology in diabetes. The molecular mechanism for metabolic memory and the types of cells mediating metabolic memory are under investigation by several labs (El-Osta et al. 2008; Madsen-Bouterse et al. 2010).

*Molecular sequelae of diabetes or hyperglycemia.* Numerous biochemical pathways activated in hyperglycemia have been linked to the development of at least some lesions characteristic of DR. Taken together, these findings suggest that hyperglycemia produces deleterious changes to retinal cells, leading to vascular and neuroglial dysfunction. Molecular steps by which diabetes or hyperglycemia cause many of these abnormalities, however, needs additional research. Molecular sequelae of diabetes and hyperglycemia that have been postulated to contribute to DR include:

a) *Advanced Glycation End product (AGE) formation.* AGEs are formed by non-enzymatic reactions between aldehydes (like glucose) or reactive dicarboxyls (methylglyoxal, glyoxal) and free amino groups on proteins, lipids, and nucleic acids. Methylglyoxal is generated intracellularly in all mammalian cells via enzymatic and non-enzymatic pathways, with the majority of MG production occurring via the glycolytic pathway. AGEs develop extracellularly and intracellularly. Extracellular AGEs interact with AGE receptors (notably RAGE),
and induce vascular inflammation, macrophage activation, and prothrombotic endothelial activation. AGE-RAGE activation also has been linked to reduced survival signals and oxidative stress. Inhibition of RAGE signaling blocks diabetes-induced defects in electroretinogram function and capillary degeneration (Barile et al. 2005; Li et al. 2011). Work in retinal Mueller cells suggested that inhibition of AGEs with pyridoxamine reduced oxidative stress and glial fibrillary acidic protein levels (Curtis et al. 2011).

b) Polyol pathway It has been observed that elevated glucose levels increase the conversion of glucose to sorbitol and fructose through the polyol pathway via the enzyme aldose reductase. It was originally proposed that increases in sorbitol caused osmotic damage to retinal vascular cells (Gabbay 1975), however this proposal was abandoned by many after clinical trials of aldose reductase inhibitors failed to show a reduction in diabetic retinopathy (Arauz-Pacheco et al. 1992) or neuropathy (SRTRG 1993). It has been since postulated that glucose competes with diabetes-induced aldehydes for aldose reductase. The result is postulated to be a decrease in the co-enzyme NADPH and a decrease in the ability of the cell to neutralize oxidative stress. Deletion of aldose reductase in animals has been shown to inhibit diabetes-induced retinal capillary degeneration and superoxide production in some animal studies, suggesting that more selective inhibitors of aldose reductase warrant further study (Tang et al. 2013).
c) Oxidative stress Diabetes is known to cause oxidative stress in the retina. This abnormality results from increased generation of reactive oxygen species (via mitochondria and NADPH oxidase) and decreased activity of antioxidant enzymes. Mitochondrial-generated superoxide can impair glucose metabolism in a variety of cell types, potentially leading to activation of several molecular pathways that have been linked to diabetic complications (Nishikawa et al. 2000; Du et al. 2003). Recent studies suggest that the oxidative stress in hyperglycemia might come also from uncoupled nitric oxide synthases or arginase I or NADPH oxidase (Caldwell et al. 2009). Inhibition of the oxidative stress with oral antioxidants or over expression of antioxidant enzymes in vivo has been shown to inhibit early stages of the retinopathy in animals (Kowluru et al. 2001; Kanwar et al. 2007) Although not studied specifically for effects on diabetic retinopathy, antioxidants have not been found clinically to have strong effects on the retinopathy. The ability of newer or stronger antioxidants to inhibit diabetic retinopathy merits future study.

d) Diacylglycerol and Protein Kinase C Hyperglycemia increases the synthesis of diacylglycerol which activates Protein Kinase C, initiating signaling pathways in the retina (Ways and Sheetz 2000; Fong et al. 2004) (This topic will be reviewed in the subsection on Role of growth factors and protein kinases).

Role of lipids. Previously, studies of the pathogenesis of DR focused almost solely on hyperglycemia. Recent studies suggest that lipids can contribute to the retinopathy and
therapeutic manipulation of lipids might offer effective therapies to inhibit the retinopathy. The severity of retinopathy in individuals with Type 1 diabetes has been found to correlate with serum lipids, even within the normal range (Lyons et al. 2004). Treatment of individuals having Type 2 diabetes mellitus with the lipid-lowering agent, fenofibrate, reduced the need for laser treatment for DR, although the mechanism of this effect seemed not to be related to plasma concentrations of lipids (Keech et al. 2007).

Diabetes has been shown to decrease key retinal fatty acids including docosahexaenoic acid (DHA), very-long-chain polyunsaturated fatty acids (PUFAs), and retinal fatty acid elongases while increasing the retinal plasma membrane lipid degradation enzyme, acid sphingomyelinase (ASMase). These changes in the fatty acid status of the diabetic retina were associated with increased levels of inflammatory markers, IL6, VEGF, and ICAM-1 (Tikhonenko et al. 2010). Dietary supplementation with DHA inhibited diabetes-induced abnormalities in the retina (including vascular inflammation and capillary degeneration in Type I diabetic animals (Opreanu et al. 2011)), but a higher dose accelerated it (Hammes et al. 1996). Much work needs to be done to better address the contribution of lipids to the development of diabetic retinopathy.

Role of oxygen. A prevalent belief among clinicians and researchers has been that progressive vascular disease results in decreased oxygen tension in the inner retina, leading to local hypoxia, and subsequent induction of VEGF with its vasoproliferative and vasopermeability effects. Laser photocoagulation has been shown to inhibit significantly the progression of advanced retinopathy to neovascularization and vision
loss. The mechanism by which it inhibits the progression of the retinopathy may be due in part to an increase in oxygen availability (due to killing of large numbers of photoreceptors and pigment epithelium). Diabetes-induced decreases in retinal oxygen tension and dilation of retinal blood vessels are reversed following laser photocoagulation (Stefansson 2006). Oxygen therapy has been demonstrated in a small number of patients to have a beneficial effect on diabetes-induced retinal edema, suggesting that hypoxia contributes to retinal edema in diabetes (Nguyen et al. 2004). Low levels of light during sleeping to prevent complete dark adaptation (thereby reducing oxygen consumption by rods) have been reported to improve visual acuity (Arden and Sivaprasad 2012). Evidence in diabetic patients and animals suggest that retinal photoreceptors might be contributing to the development of retinal hypoxia in diabetes. Photoreceptors utilize large amounts of oxygen to maintain their normal function, and loss of some or all of those photoreceptors in rhodopsin-deficient mice (de Gooyer et al. 2006) or in patients with retinitis pigmentosa (Arden 2001) inhibited the diabetes-induced loss of retinal capillaries.

There has been disagreement relating to how soon in DR hypoxia develops in the retina. Tools have been under development to measure retinal oxygen tension noninvasively, but those methods are not yet capable of focusing on the small vessels that deliver the bulk of oxygen to tissues.
Role of vascular abnormalities.

Blood flow. An early event in the retina of diabetics is the inability to regulate capillary pressure by reducing the flow of blood into the capillary with the constriction of arterial pericytes and possibly Mueller cells. Vascular permeability in capillaries is a function of the resistance to fluid flow across the across the vessel wall (mediated by the tight junction breakdown and trans-endothelial channels), and the difference in pressure between the pressure in the capillary and the osmotic pressure in the tissue surrounding the vessel. The permeability of retinal vasculature is increased in diabetes and therefore an increase in blood flow, such as occurs with exercise, should increase the volume of plasma moving into the neural retina. While it is tempting to speculate that this may explain why high blood pressure is a risk factor for edema, the effect of unregulated capillary blood flow in diabetic retinopathy remains unknown.

Basement membrane thickening. Basement membrane thickening due to overproduction of extracellular matrix proteins has been observed in immunohistological preparations of the retinal vascular bed from patients with diabetes, experimental diabetic animals and animals fed a diet of high galactose (which develop diabetes-induced vascular lesions of the retina) (Kern and Engerman 1994; Stitt et al. 1994; Roy et al. 1996). Thickening of the basement membrane may inhibit the exchange of oxygen and nutrients between blood and retinal tissue and increase the risk of occlusion (Cai and Boulton 2002). Treatment of rats fed a high galactose diet with antisense fibronectin RNA oligonucleotides inhibited the thickening of the basement membrane, the loss of pericytes, and the increase of acellular capillaries otherwise observed (Roy et al. 2003).
Role of growth factors and protein kinases.

Vascular endothelial growth factor. Vascular endothelial growth factor (VEGF) induces increased permeability and angiogenesis. Exogenous administration of VEGF in the eye of primates induces blood-retinal barrier break down and neovascularization (Ozaki et al. 1997; Tolentino et al. 2002). It is upregulated in the Mueller cells, glial cells surrounding axons, and some vascular endothelial cells in the retina of humans with NPDR (Amin et al. 1997) and has been found in the vitreous of patients with PDR. The mechanism of VEGF in DR is, however, complex as VEGF is present in the retina of diabetic animals prior to development of vascular lesions, and while anti-VEGF inhibits vascular endothelial expression of ICAM1 and eNOS (endothelial nitric oxide synthase) (Joussen et al. 2002b), anti-ICAM1 antibodies inhibit VEGF induced blood vessel permeability (Miyamoto et al. 1999). Clinical trials of anti-VEGF therapy have resulted in a reduction of severe vision loss. However, about half of the patients do not respond satisfactorily.

Pigment epithelial derived factor. Pigment epithelial derived factor (PEDF), also known as serpinf1, is produced by the retinal pigmented epithelium. Exogenous PEDF has been shown to inhibit angiogenesis in the retina of the mouse retinopathy of prematurity (ROP) model, countering the effects of VEGF (Stellmach et al. 2001). The contribution of other growth factors to DR is still unclear.

Protein Kinase C. Protein Kinase C (PKC) is a family of enzymes which, when activated by increases in diacylglycerol (DAG) or calcium ions, initiate a signal transduction
cascade by phosphorylating serine and threonine amino acid residues of downstream proteins. Ligation of many cytokine receptors (G-protein coupled receptors) releases the Gα subunit, which activates protein lipase C (PLC). PLC subsequently cleaves phosphatidylinositol 3 (PIP3) releasing DAG which in turn activates PKC. In diabetes, hyperglycemia increases the synthesis of diacylglycerol which activates PKC (Ways and Sheetz 2000; Fong et al. 2004).

Increased DAG and PKC activity (especially the PKC β isoform) have been reported in a number of animal and human tissues stimulated by high glucose (Das Evcimen and King 2007) and in the retina of diabetic animals (Shiba et al. 1993). Inhibitors of PKC have been shown to reduce diabetes-induced basement membrane thickening, vascular permeability, and leukostasis of diabetic animals (Nonaka et al. 2000; Xu et al. 2004). Activation of PKC leads to increased VEGF and NADPH oxidases in the retina, upregulation of IL1β in monocytes (Dasu et al. 2007) and oxidative burst in neutrophils (Hand et al. 2007). Clinical studies of PKC inhibitors in diabetic patients have shown the beneficial effects of PKC inhibition on visual acuity, but it did not reduce edema (Sheetz et al. 2013).

Role of inflammation.

Because the early hallmarks of DR (leukostasis, vascular permeability, dilated blood vessels, increased inflammatory cytokines, etc.), are consistent with subclinical chronic inflammation, it has been proposed that DR may be an inflammatory disease (Adamis 2002; Joussen et al. 2004; Tang and Kern 2011). If diabetic retinopathy is a disease
mediated by inflammation and leukocytes, then anti-inflammatory treatments should reduce the severity and prolong the onset of the retinopathy. Evidence to support this theory has been demonstrated in experimental diabetic animals and a few clinical trials (with aspirin, silicates, and corticosteroids).

*Effects of anti-inflammatory therapy.*

a) *Aspirin.* Acetylsalicylic acid (aspirin) is a general anti-inflammatory which inhibits a number of targets including cyclooxygenase 1 and 2 (COX1 and COX2), and transcription factor NFκB nuclear translocation and promotes resolution of inflammation by upregulating lipoxins and SOCS2 (McBerry et al. 2012). The beneficial effect of aspirin in lessening the severity of diabetic retinopathy was initially observed in diabetic patients taking dose aspirin to treat rheumatoid arthritis (Powell and Field 1964). Since then, there have been two clinical studies of the effects of aspirin on diabetic retinopathy with conflicting results. In the DAMAD study aspirin, given at a dose of 4.7mg/kg/three times a day (987 mg/day) reduced the frequency of microaneurysm formation in patients with NPDR (DAMAD 1989). However in the Early Treatment of Diabetic Retinopathy Study (ETDRS) aspirin given at 9.3 mg/kg/day (650 mg/day) did not prevent vision loss, vitreous hemorrhages, or progression from NPDR into PDR in patients with early PDR and mild to severe NPDR (ETDRS 1991b). There are several differences between the two studies, which may explain the disparity in the results. The dose of aspirin given in the DAMAD study was cumulatively higher than that in the ETDRS, and the schedule of giving the aspirin was more
frequent in the DAMAD study. The severity of the retinopathy in patients enrolled in ETDRS study was worse than the severity of the patients enrolled in the DAMAD study. The ETDRS study included patients with PDR and severe NPDR, which may not respond to preventative therapies for the progression of NPDR into PDR. Nevertheless, aspirin appears to be most effective as a preventative therapy prior to the observation of numerous vascular lesions in the retina (severe NPDR).

High dose aspirin, administered shortly after the onset of diabetes, inhibited the early vascular lesions of diabetic retinopathy in diabetic animals. Diabetic dogs, treated with 25mg/kg of aspirin, developed significantly fewer acellular capillaries and hemorrhages in the retina than diabetic controls over a five-year period (Kern and Engerman 2001). In diabetic rodents, high dose aspirin has been shown to inhibit the diabetes-induced increase in retinal acellular capillary formation (Zheng et al. 2007c), blood vessel permeability, eNOS, ICAM1, and leukocyte CD18/CD11b expression (Joussen et al. 2002a).

b) Meloxicam and non-aspirin salicylates. Cyclooxygenase 2 (COX2), one of the numerous targets of aspirin, is a pro-inflammatory enzyme that converts arachidonic acid to inflammatory prostaglandins. Inhibition of COX2 in endothelial cells cultured in high glucose prevents hyperglycemia induced Prostaglandin E2 (PGE2) upregulation (Du et al. 2004). Treatment of rats, diabetic for 1 week, with meloxicam, a COX2 inhibitor, reduced diabetes-induced
blood vessel permeability, eNOS, and ICAM1 in the retina, and leukocyte CD18/CD11b expression (Joussen et al. 2002a). Treatment of diabetic rats for several months with sulfasalazine, a salicylate similar to aspirin that does not inhibit COX2, inhibited the increase of retinal acellular capillaries (Zheng et al. 2007c). Treatment of diabetic rats for 9 months with Nepafenac, an inhibitor of COX1 and COX2, also inhibited diabetes-induced acellular capillaries in the retina (Kern et al. 2007). In summary, it remains unclear if COX2 is important in the pathology of DR.

c) Corticosteroids. Intravitreal injections of triamcinolone, a strong anti-inflammatory corticosteroid, reduces retinal thickness (edema), and intra-retinal hemorrhage and improves visual acuity in diabetic patients with persistent edema as previously discussed (section 1.2 current therapies p. 30). In streptozotocin diabetic rats, intravitreal injections of triamcinolone have been shown to reduce diabetes-induced leukostasis, ICAM1 expression, and vascular leakage in the retina (Tamura et al. 2005).

Cytokines. Cytokines reported to be elevated in the serum, vitreous, or aqueous humor of diabetic patients include MIF, MCP1, SDF-1, IL1β, and TNFα (Elner et al. 1995; Doganay et al. 2002; Dong et al. 2013). The leukocyte attracting chemokines macrophage chemotactic protein 1 (MCP1), and stromal cell derived factor-1 (SDF-1) and leukocyte inhibitory factor macrophage migration inhibitory factor (MIF) are elevated in the vitreous and aqueous humor of diabetic patients (Meleth et al. 2005). Furthermore, levels
of MCP1 and MIF in the aqueous humor increase with progression of the retinopathy (Tashimo et al. 2004). Elevated levels of MCP1 are also present in the retina of diabetic rats, as early as four weeks after the induction of diabetes (Dong et al. 2012). Elevated levels of MCP1 should attract and activate leukocytes in the blood vessels of the retina (Allavena et al. 1994; Carr et al. 1994); however, the elevated levels of MIF may inhibit them from leaving the vasculature and entering the neural retina. High glucose alone is sufficient to upregulate the secretion of MCP1 in endothelial cells. However cytokines and growth factors present in the retina in the early stages of retinopathy including VEGF, IL1β and TNFα also induce endothelial cell secretion of MCP1 (Marumo et al. 1999; Harkness et al. 2003; Takaishi et al. 2003). In addition, macrophages stimulated with MCP1 secrete IL1β and TNFα (Biswas and Sodhi 2002). Thus, leukocytes and retinal endothelial cell have a reciprocal role in activating each other.

Proinflammatory cytokine IL1β is also increased in the aqueous humor of diabetic patients and also increases with progression of the retinopathy (Dong et al. 2013). IL-10, a negative regulator of inflammation is decreased in the aqueous humor and decreases with the progression of the retinopathy. IL1β and TNFα are also increased in the retina of diabetic animals (Carmo et al. 1999; Joussen et al. 2002a). Intravitreal injection of IL1β or TNFα results in a breakdown of the blood-retinal barrier and subsequent vascular leakage (Saishin et al. 2003). Thus adherent macrophages stimulated by high glucose and MCP1 in the retinal microvasculature may contribute to vascular permeability by releasing IL1β and TNFα as outlined in the previous paragraph. Inhibition of TNFα in rats reduces diabetes-induced retinal ICAM1 expression and leukostasis but not VEGF or
leukocyte CD11b/CD18 expression (Joussen et al. 2002a). IL1β may also be important in microvascular endothelial cell death, as injection of IL1β into a normal rat retina results in an increase in acellular capillaries (Kowluru and Odenbach 2004). Strong evidence linking IL1β to diabetes-induced retinal lesions was demonstrated in a study by Vincent and Mohr, in which the number of acellular capillaries in the retinas of mice lacking the IL1β receptor (diabetic for seven months) was not statistically different from non-diabetic controls. However, the number of acellular capillaries in the retina of WT mice, of the same duration of diabetes, was significantly increased (2.5 fold over non-diabetic control) (Vincent and Mohr 2007). However, it is not clear which cells in the retina might be involved (such as leukocytes, endothelial cells, or microglia) or what affect the lack of IL1βR might have on diabetes-induced vascular permeability, and leukocyte and retinal superoxide.

*Role of Leukostasis.* Increased numbers of leukocytes adherent to the endothelium of the vasculature (leukostasis) have been observed in histological preparations of retina from diabetic patients and animals. Leukocytes adherent to the microvasculature of the retina have been postulated to contribute to the diabetes-induced loss of capillaries by occlusion-induced hypoxia, the release of toxic products such as lytic enzymes, inflammatory cytokines, and reactive oxygen and nitrogen products or by induced cell death using the Fas/FasL pathway. The role of leukocytes in the pathology of diabetic retinopathy is reviewed in depth in the following chapter (Chapter 1.4 the role of leukocytes p. 58).
Role of iNOS. Levels of inducible nitric oxide synthase (iNOS) and reactive nitrogen species such as nitric oxide (NO), and peroxynitrite, are increased in many inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and asthma (see review by Zamora) (Zamora et al. 2000; Forstermann and Munzel 2006). The nitric oxide synthases (NOS) are a family of enzyme that catalyzes the conversion of L-arginine to citrulline and nitric oxide. The family includes eNOS (endothelium), nNOS (neuronal), and iNOS (inducible). iNOS is the primary source of nitric oxide (NO) in activated leukocytes, and NO is involved in subsequent modification of protein function (such as via nitration or nitrosylation) and generation of reactive peroxynitrite (Kim et al. 2005a; Zhao et al. 2010). Nitrosylation of COX2 and Arginase-I by iNOS results in an activation of these enzymes (Kim et al. 2005a; Kim et al. 2009). Arginase-I competes with iNOS for L-arginine limiting its ability to produce NO, however it also uncouples the cofactor tetrahydro-biopterin4 (BH4) from NOS causing the uncoupled enzyme to produce superoxide instead of NO. Increased reactive nitrogen species and reactive oxygen species generated by such a process are thought to contribute to the damage observed in chronic inflammation.

Nitrates and nitrites (stable products of nitric oxide) are upregulated in the retina of diabetic patients and diabetic animals, suggesting that dysregulation of iNOS may be important in the pathology of diabetic retinopathy (Kowluru 2002; Mabley and Soriano 2005). In a study by Kern and Engerman, treatment of dogs, diabetic for five years, with aminoguanidine, an inhibitor of iNOS and AGE production, inhibited the diabetes-induced development of microaneurysms, acellular capillaries, and pericyte ghosts
otherwise observed in untreated diabetic controls (Kern and Engerman 2001). Aminoguanidine therapy did not reduce the diabetes-induced formation of advanced glycation end products but it did reduce nitration of protein. Similarly, treatment of diabetic rats with aminoguanidine, for 2 months significantly reduced diabetes-induced increase in iNOS, nitrates, nitrites, and nitrotyrosine otherwise observed in the retina in untreated diabetic controls (Du et al. 2002).

Mice lacking iNOS have an impaired ability to clear a bacterial challenge (due in part to the lack of reactive nitrogen species in leukocytes required for microbial elimination). However, neutrophil rolling, adhesion, and migration and neutrophil integrin CD18 upregulation are not impaired in iNOS deficient mice challenged with bacteria (Hickey et al. 2001; Benjamim et al. 2002). Furthermore, iNOS deficient mice produce normal serum levels of TNFα and IL1β in response to LPS challenge (MacMicking et al. 1995; Chakravortty and Hensel 2003). Thus, diabetes-induced leukostasis and capillary occlusion, as well as TNFα and IL1β mediated inflammation might be expected to be unaffected in mice lacking iNOS.

In a study by Leal and Forrester, upregulation of ICAM1 expression (1.5 fold), leukostasis (2 fold), and permeability of the non-perfused vasculature to Evans blue dye and down regulation of tight junction protein (25%) were observed in the retina of WT mice, two weeks diabetic, compared to non-diabetic controls (Leal et al. 2007). Mice of the same duration of diabetes, but lacking iNOS did not develop any of these diabetes-induced abnormalities in the retina. The results of the study suggest that iNOS regulates
the diabetes-induced upregulation of endothelial cell ICAM1 and leukocyte adhesion however it is unclear if this is due to a lack of iNOS in the endothelium or in the leukocytes or both. It is also unclear if the observed permeability is directly inhibited by the lack of iNOS or if the inhibition of leukocyte binding is also involved.

iNOS deficient mice also do not develop longer-term (2-8 months) diabetes-induced retinal vascular lesions. In a study by Zheng and Kern, mice deficient in iNOS did not develop the diabetes-induced loss of pericytes or increase in acellular capillaries otherwise seen in the retina of WT mice nine months diabetic (Zheng et al. 2007b). Furthermore diabetes-induced increases of nitrates and nitrites (stable metabolites of nitric oxide), eNOS, prostaglandin E2, and superoxide in the retina of diabetic WT mice were inhibited to near normal levels in diabetic mice deficient in iNOS at 2 months duration of diabetes. Diabetes-induced elevation of nitrotyrosine in the retina was increased two fold in non-diabetic controls in WT animals and significantly reduced (1.5 fold non-diabetic) in diabetic animals lacking iNOS, suggesting that eNOS or nNOS may be responsible for the remaining diabetes induced elevation in nitrotyrosine. Diabetes-induced reduction of the amplitude of the electroretinogram b wave and the number of PAR modified proteins in western blots were not corrected in mice lacking iNOS after two months of diabetes. The results suggest that PARP1 is upstream from iNOS, PAR-modification of proteins does not cause acellular capillary formation independent of cell stress due to iNOS, and iNOS is not required for changes in the electroretinogram.
iNOS regulation. The promoter for mouse iNOS contains several transcription factor response elements including two NFκB sites, one AP1 site and ten interferon γ (INFγ) sites (Xie et al. 1993). INFγ is not upregulated in the retina of diabetic animals or in the aqueous or vitreous humor of diabetic patients, therefore NFκB and AP1 are more likely to mediate the diabetes-induced increase in iNOS. In a study by Hwang and Han, mouse microglia stimulated with LPS displayed two phases of iNOS upregulation. Stimulation with LPS for 15 minutes induced nuclear translocation and binding of p50, c-Rel, and p65 and a small amount of RelB to a probe consisting of one of the two iNOS NFκB (Rel) promoter sites (-979 from initiation site) (Hwang et al. 2013). Sustained stimulation for 24 hours resulted in an increase in RelB binding to the probe and a decrease in the amount of RelA but not p50 or c-Rel binding to the probe. In a similar study by Wu and Cieslik, stimulation of mouse macrophages (RAW 264.7 cells) with LPS and INFγ also initiated RelA and C-Rel binding (at the same NFκB binding site) at four hours and at eight hours (Cieslik et al. 2006). Treatment with siRNA for c-Rel inhibited the majority of iNOS mRNA and protein production otherwise present by 4 hours, indicating that c-Rel initiates the majority of the iNOS upregulation in this system. Treatment of the stimulated cells with apocynin or superoxide dismutase (superoxide inhibitors) but not catalase significantly reduced ROS production, C-Rel binding, iNOS mRNA, and iNOS protein production at four hours but not at eight hours consistent with previous studies indicating that early phase iNOS production is enhanced by superoxide (Kuo et al. 2000). Pretreatment with sodium salicylate did not inhibit c-Rel binding at either time point but it did inhibit upregulation of iNOS at four hours, suggesting that other factors besides c-Rel might also be important in early iNOS production. c-Rel binding and subsequent
iNOS production was inhibited at the eight-hour time point by adding additional salicylate every few hours. However, production of iNOS at 8 hours was not inhibited with a corresponding higher initial dose, suggesting that the salicylate is inactivated by something in the cell, presumably superoxide. If superoxide, which is upregulated in the leukocytes of diabetic animals, does inactivate salicylates then high dose aspirin given in a bolus once a day might be expected to be less effective than the same cumulative dosage given several times a day similar to the results of the clinical trials of high dose aspirin in section 1.3.

Incubation of mouse monocytes (THP-1 cells) in high glucose is sufficient to upregulate iNOS (Shanmugam et al. 2003). However it is unclear which of the Rel proteins, if any, are responsible for the diabetes-induced upregulation. Cytokines IL1β, and TNFα, and AGEs, which are increased in the retina in diabetes, should initiate the NFκB canonical pathway and induce iNOS through a mechanism similar to LPS stimulation however, the mechanism of iNOS upregulation by high glucose remains undefined.

Role of PARP1. Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear enzyme which has important roles in both DNA repair and NFκB mediated transcription of pro-inflammatory genes. In ischemia reperfusion, the formation of reactive oxygen and nitrogen species, such as peroxynitrite, can cause DNA damage resulting in activation of PARP1 (Narasimhan et al. 2003). PARP1 cleaves nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide and ADP-ribose, and then transfers the ADP-ribose to nuclear proteins, creating a polymer of ADP-ribose subunits (PAR) on the protein. Transfer of
PAR to the nuclear proteins inhibits replication allowing the cell to repair the DNA damage. Once activated PARP1 rapidly depletes NAD\(^+\), which is also an important cofactor in glycolysis and electron transport, and in doing so can induce necrosis of the cell (Szabo and Dawson 1998; Ha and Snyder 1999). PARP1 binds both p50 and p65 units of NFkB in the nucleus, and blocks the nuclear export signal for these proteins; PARP1 is required to retain the complex in the nucleus prior to transcription of proinflammatory genes (Hassa et al. 2001; Zerfaoui et al. 2010). Nuclear transcription factor AP-1 likewise requires PARP1 as a cofactor for transcription of proinflammatory genes (Andreone et al. 2003).

In a study by Zheng and Kern, inhibition of PARP1 inhibited the diabetes-induced leukostasis and loss of microvascular cells in the retina (Zheng et al. 2004). Polymers of ADP-ribose (PAR) were increased on numerous proteins in retinal lysate from diabetic rats, compared to non-diabetic controls, and were present in the nuclei of pericytes and endothelial cells in vascular beds isolated from diabetic rats, suggesting that diabetes induces DNA damage in the retina. Diabetes-induced increases in leukostasis (4 fold), acellular capillary loss (2 fold), and pericyte loss (2 fold), occurred in the retina of WT rats, nine months diabetic, compared to non-diabetic controls. These increases were significantly inhibited by administration of a PARP1 inhibitor to near normal levels except for leukostasis, which was significantly reduced by one-half. Diabetes-induced increases in retinal ICAM1 (2.5 fold of non-diabetic) were also significantly reduced by the PARP1 inhibitor (1.8 fold of non-diabetic remaining) in rats diabetic for 12 weeks compared to controls.
1.4 Role of leukocytes.

**Classic model of inflammation and leukocyte recruitment.** Leukocytes, which are important in the inflammatory process throughout the body, have been postulated to play a role in the occlusion and loss of retinal capillaries in DR, but the exact mechanism and experimental proof has remained elusive (Chibber et al. 2007). In the classic model of inflammation and leukocyte recruitment, an initiating event such as tissue damage causes the release of proinflammatory chemokines resulting in decrease in the velocity of blood flow due to dilation of local blood vessels. Exposure of leukocytes in the blood to cytokines (or antigen) initiates a G-protein coupled receptor response (cytokine receptor) resulting in a transition from an unstimulated non-adherent state to a transient primed state in which surface integrins rearrange to allow ligand binding and the superoxide producing NADPH oxidase complex assembles on the membrane. Endothelial cells lining the blood vessel are also primed by exposure to the cytokines, and quickly increase the surface concentration of cell adhesion molecules and selectins, including ICAM1 and p-selectin stored in Weibel Palade bodies. The endothelial cells will also begin transcribing additional adhesion molecules including ICAM1 and VCAM1, and inflammatory cytokines including MCP1, which is chemotactic for leukocytes. Sialyl-Lewis X carbohydrates on the surface of the leukocyte bind loosely to lectin-like selectin molecules expressed on the surface of the endothelium, causing the leukocyte to slow down and roll across the surface of the blood vessel. Binding of β2 integrins on the surface of the leukocyte to the adhesion molecules on the endothelium causes the leukocyte to stop rolling and bind tightly to the endothelium. Signaling inside both the
endothelial cell and the leukocyte allows disruption of the endothelial cell tight junction through which the leukocytes migrate out of the blood vessel into the tissue.

**Leukocyte adhesion, vascular permeability, and capillary loss.**

*Leukostasis and leukocyte activation.* In a study of long term (2-9 months) diabetic rats, Schroder and Schmid-Schoenbein (Schroder et al. 1991), documented diabetes-induced leukocyte activation, retinal leukostasis, leukocyte mediated retinal capillary occlusion. In the study, blood isolated from diabetic, (but not non-diabetic) rats reduced tetrazolium salts indicating the presence of an oxidative species. The diabetes induced production of reactive oxygen species by leukocytes has also been observed by our lab in diabetic mice, suggesting that diabetes causes some degree of leukocyte activation. About a third of the monocytes and about an eighth of the granulocytes contained reduced crystals in smears of the incubated blood, demonstrating that a percentage but not all cells in both leukocyte populations were “activated”. The observation that both neutrophils and monocytes isolated from the peripheral blood of diabetic animals are generating superoxide suggests that both subsets of cells are activated and therefore, if leukocytes mediate the diabetes induced vascular damage then either or both subsets may contribute to diabetes induced capillary loss. Incubation of blood from non-diabetic rats with relevant concentrations of high glucose produced similar results demonstrating high glucose can induce at least temporary leukocyte “activation”. The induction of leukocyte activity by short-term exposure of leucocytes to high glucose has been subsequently shown, in a number of studies, to induce a pro-inflammatory state (Shanmugam et al. 2003; Dasu et al. 2007; El-Osta et al. 2008). However, other substances, which are increased in diabetes such as
cytokines and AGEs, also induce and or reinforce leukocyte priming (Barile et al. 2005; Kaji et al. 2007).

The number of monocytes was significantly increased in the peripheral blood at 2 months of diabetes (4 fold) which decreased with the duration of diabetes but remained elevated at 9 months (3 fold) compared to non-diabetic controls. An increase in total blood monocytes has not been observed in diabetic mice in our lab, nor is it referenced in the paper with respect to diabetic patients, the observed increase therefore may be a side effect of the experimental model. The Schroder study did not include any insulin therapy to maintain body weight and wasting of the rats due to diabetes was reported. The incorporation of insulin therapy more closely models diabetic patients and therefore has become the preferred model of experimental diabetes.

In studies of flat mounted retina in the Schroder study, the animals were divided into a non-perfused group and a group perfused with Ringers solution followed by paraformaldehyde. The eyes of the perfused group (and presumably the first group) were fixed in additional paraformaldehyde for 48 hours. Granulocytes and macrophages/monocytes in the isolated retina were stained with pigment containing substrates selective for esterases in each leukocyte subtype. Increased numbers of monocytes and neutrophils were present in the retinal vasculature of diabetic animals in both the non-perfused group and the perfused group compared to appropriate non-diabetic controls demonstrating a diabetes-induced increase in leukostasis. Some of the retinal blood vessels in the perfused diabetic (but not the non-diabetic animals), which
contained adherent leukocytes, also contained red blood cells demonstrating that the adherent leukocytes occluded the vasculature to the extent that the perfusion solution, which contained both the anticoagulant heparin and the vasodilator sodium nitroprusside, could not wash away the red blood cells. The leukocyte mediated capillary occlusion of the retinal vessels of diabetic animals (but not non-diabetic controls) suggests that diabetes induces leukocyte activation and adhesion. However, the eye, brain, and testis are immune privileged organs, and inhibit normal leukocyte diapedesis and subsequent patrolling even in cases of inflammation (Mo and Streilein 2001).

I disagree with the interpretation of data in the Schroder study with regard to diabetes-induced retinal extra vascular leukocytes, capillary loss and neovascularization. In the Schroder study, numerous extravascular leukocytes were documented in the non-perfused eyes of diabetic (but not non-diabetic) animals, which increased in frequency with distance from the retinal vitreal interface, which the authors suggested demonstrated diabetes-induced leukocyte infiltration of the neural retina. If leukocytes were migrating, out of the vasculature into the retina of diabetic animals as the paper suggests then a similar number of extravascular leukocytes should be present in both the perfused and the non-perfused eyes, however documentation of extravascular leukocytes in the perfusion fixed retina of diabetic animals was limited to one granulocyte in the periphery of the retina. The methods detailing the fixation of non-perfused eyes did not include prompt fixation. It therefore seems likely that the extracellular leukocytes may have migrated out of the vasculature and into the neural retina after the eye was enucleated but before the fixative could permeate the tissue. A control for post mortem diapedesis by injection of
leukocyte staining dye into the blood stream a few minutes prior to sacrifice might help to clarify the results.

Diabetes-induced retinal capillary loss was inferred by an inability to follow the outline of capillaries in brightfield microscopy, however without the use of a vascular contrast agent it is difficult to conclude that the methods were sufficient to detect capillary loss. A more robust method such as isolation of the vascular bed would have been useful in examining the capillaries. The images of neovascularization and microaneurysms in the retina of diabetic animals were inconsistent with those seen in other studies. It is unclear what these vascular abnormalities represent.

**Leukocyte adhesion molecules.** A study by McLeod and Lutty demonstrated that neutrophils and leukocyte adhesion molecule ICAM1 is increased on the vascular endothelium of the retina and choroid in eyes from diabetic human donors (McLeod et al. 1995). The study is important in that it correlates the diabetes-induced increase in leukocytes and leukocyte binding molecules in the retina of experimental animal models with that seen in humans. Donor eyes from diabetic and non-diabetic cadavers (1 to 4 hours postmortem) were fixed, and stained with antibodies to ICAM1, a ligand for leukocyte firm attachment, p-selectin and e-selectin, ligands for leukocyte weak attachment mediated rolling, and an esterase specific substrate for granulocytes. ICAM1 expression was upregulated on capillary endothelial cells of the choroid and irregular patches of endothelial cells of the retinal vasculature in retina from diabetic (but not non-diabetic) donors. The diabetes-induced patchy expression of ICAM1 in the retina is
interesting in that, although the entire retina is bathed in high glucose, endothelial activation like capillary loss seems to occur in random patches. Diabetes also upregulated ICAM1 on retinal pigmented epithelium cells, Mueller cell processes at the external limiting membrane and in irregular patches in the nerve fiber layer of the retina. Diabetes upregulated the expression of p-selectin but not e-selectin on the venular endothelium of the choroid, but neither selectin was upregulated in the retina. Neutrophils were elevated in the choroid and retinal vasculature of diabetic patients compared to non-diabetic controls. The number of neutrophils in the vasculature of the retina was positively correlated with higher levels of ICAM1 in the retina.

*Leukocyte adhesion molecules, vascular occlusion, and vascular permeability.* Several key papers by Adamis and colleagues demonstrate that inhibition of diabetes-induced leukostasis also inhibited the diabetes-induced increases of vascular leakage, endothelial cell damage, and pro-inflammatory cytokines in the retina. In a study by Miyamoto and Adamis, diabetes-induced increases in ICAM1, leukostasis, leukocyte occlusion of capillaries, and vascular leakage, in the retinas of rats were studied over the course of 28 days post induction of diabetes with streptozotocin (Miyamoto et al. 1999). ICAM1 (mRNA), a ligand for leukocyte firm attachment, was progressively upregulated in the retina of diabetic animals compared to non-diabetic controls as early as three days after the injection of streptozotocin. The number of acridine orange (non-specific dye) stained leukocytes (in vivo) adherent to the blood vessels in the retina also progressively increased from day three post-injection until day seven and then remained relatively constant through the remainder of the twenty-eight day experiment. Adherent leukocytes
in the retina were observed occasionally to occlude the flow of fluorescein labeled blood over the 28 days of the experiment. Blood flow was not always restored in the previously blocked vessel even in the absence of the stained leukocyte demonstrating that leukostasis can result in putative permanent occlusion. The data suggests that leukocytes, within days of the onset of hyperglycemia, are capable of not only of acute occlusion of the blood vessels in the retina but can lead to permanent occlusion by an unknown mechanism. It is note-worthy that the majority of acridine orange stained leukocytes were not observed to migrate out of the blood vessels and into the neural retina (as Miyamoto subsequently demonstrated for non-diabetic mice injected with LPS (Miyahara et al. 2004)).

Vascular leakage like leukostasis (retention of radiolabeled albumin $^{125}\text{I} / ^{131}\text{I}$ method) also increased by the third day but unlike leukostasis it continued to increase throughout the twenty-eight day experiment. The simultaneous increase in leukostasis and vascular leakage at day three may suggest that the permeability is mediated by leukostasis. Leukocyte disruption of endothelial adherens junctions has been shown to be mediated by CD18 integrin binding (Del Maschio et al. 1996). While entrapment of fluid in the vasculature behind an occluding leukocyte is also possible, the time during which the radiolabeled albumin circulated in the animal was short (10 and 2 minutes) making entrapment less likely. Vascular leakage of fluorescein from the retinal vasculature during the angiogram procedure was not reported and presumably not observed however, this may be due in large part to difficulty in observing a diffuse amount of leaking fluorescein against the background of acridine orange taken up by the retina.
Nevertheless, pictures of dye leaking from the vascular bed would at least help confirm that leakage does occur. The increase in vascular leakage, after seven days without a corresponding increase in leukostasis, may suggest a second mechanism independent of leukocytes or perhaps the effect of the leukocytes on the endothelial cells may simply be cumulative over time.

Treatment of the diabetic animals for seven days with an anti-ICAM1 antibody (IP injection) every other day after inducing diabetes decreased leukostasis and vascular leakage (49% and 86% respectively). The 49% inhibition in leukostasis by anti ICAM1 therapy suggests that leukocyte integrins, which bind ICAM1, the β2 integrins, are involved in diabetes induced-leukostasis. It is unclear, which receptors and ligands account for the remaining 51% of leukocyte binding as only one dose of the antibody was administered and therefore it is possible that ICAM1 was only partially inhibited. The level of soluble ICAM1 in serum is significantly elevated in diabetic patients (Ai and Song 2012) and therefore may complicate the effect of anti-ICAM1 therapy by binding the antibody and by reducing the inhibitory effect of free soluble ICAM1 binding to leukocytes CD18/CD11 integrin.

In a follow up paper, Adamis and Barouch inhibited leukostasis (62%) in rats (diabetic for one week) with anti-CD18 antibodies (Barouch et al. 2000). They report an increase of CD11a (62%) CD11b (54%), and CD18 (38%) expression by flow cytometry on unfixed neutrophils isolated by hypertonic lysis from whole blood of diabetic animals (compared to non-diabetic controls), but do not show the data. ICAM1 is a ligand for the
CD18 integrins. In the study by Barouch, 62% of leukostasis was inhibited in diabetic rats with anti CD18 antibodies while in the previous study only 49% of leukostasis was inhibited with anti ICAM1 therapy, suggesting that either ICAM1 was only partially inhibited (due to insufficient antibody concentration) or a second ligand may be binding the CD18 integrin such as ICAM2.

In a co-culture binding assay of neutrophils to TNFα stimulated rat prostrate endothelial cells, 75% more neutrophils bound to the endothelial cells (after washing off non-bound cells) when they were isolated from a diabetic animal than neutrophils isolated from non-diabetic animals. The observed increase in neutrophil adhesion was inhibited with anti-CD11b (41%) or CD18 (41%) but not CD11a antibodies. The pretreatment of the neutrophils with a “saturating concentration” of anti-CD18 prior to co-culture with endothelial cells might be expected to inhibit the binding of neutrophils by 62% or more as observed with anti CD18 in vivo, but instead the inhibition of binding decreased to 41%. Furthermore, antibodies to CD11b (but not CD11a) inhibited binding by the same percentage, suggesting that CD11b/CD18 accounts for half of the binding, and CD11a/CD18 is not involved. Since TNFα is known to up-regulate VCAM1 (vascular cell adhesion molecule 1) and ICAM1 on the surface of endothelial cells (Galea et al. 1992; Mackay et al. 1993), the observed change in adhesion in the co-culture assay is likely due to ligation of VLA-4 (α4β1, very late antigen 4) on the neutrophils (Davenpeck et al. 1998) to VCAM1 on the endothelial cell. This hypothesis could be easily tested with blocking antibodies to either α4 or β1 by itself and in combination with anti-CD18 antibodies to see if the inhibition by the antibodies is additive. The activation of the
endothelial cells with exogenous TNFα may not be an appropriate model of diabetes-induced leukostasis. It is also unclear if rat prostrate endothelial cells are substantially different from rat retinal endothelial cells in their expression of surface markers. A second possibility is that the concentration of the blocking antibodies was too low in the co-culture experiment, however the reported concentration that was used (30ug/mL per an unknown number of cells) is similar to concentrations (10-20ug/mL per million cells) used in other studies (Prieto et al. 1988; Lo et al. 1989). This could be easily tested by increasing the concentration by 10 fold (200ug/mL per million cells) as other investigators have done (Ohno and Malik 1997). Although CD11a and therefore integrin CD11a/CD18 was upregulated on the surface of the neutrophils, the failure of antibodies to CD11a to inhibit neutrophil adhesion and the similar percentage of inhibition of adhesion for antibodies to CD11b and CD18 suggests that the CD18/CD11a integrin is not activated (not in the extended confirmation) and cannot bind its ICAM1 ligand. This theory could be tested with fluorescent probes, which bind selectively to the open conformation of the integrin (Chigaev et al. 2011). Alternatively, an aliquot of the neutrophils activated with PMA could be used in the co-culture assay with non-stimulated endothelial cells to see if the CD11a antibody inhibits the expected 30% of neutrophil inhibition seen in similar experiments (Prieto et al. 1988; Lo et al. 1989). A third possibility is that neutrophils were inhibited in vivo by 41% or less and an additional CD18 positive cell, monocytes for example, accounted for the additional observed inhibition of leukostasis. The effect of the anti-CD18 therapy on vascular leakage was unfortunately not reported and therefore a comparison to the results of the previous study cannot be made. In summary the results suggest that leukostasis in the
retina of diabetic rats is mediated in part by CD18/CD11b integrins but may also involve another mechanism.

In a related study, Gragoudas and Iliaki (Iliaki et al. 2009) repeated the experiments in the Barouch study using anti-CD49d (α4 of the VLA4 integrin) therapy for ten days in rats two weeks diabetic. Rats injected with streptozotocin that did not develop elevated glucose levels were used as non-diabetic controls. Diabetes induced a 3-4 fold increase in leukostasis and a 2-fold increase in vascular permeability (as measured by Evans blue dye retention) in the retina. Diabetes-induced leukostasis and vascular leakage in the retina was inhibited to near normal levels by doses of 1-5 mg/Kg of anti-CD49d antibody given IP every five days (but not IgG controls). Monocytes isolated from the peripheral blood of diabetic animals adhered 3 fold more than non-diabetic controls to rat prostate endothelial cells stimulated with TNFα in co-culture. Pretreatment of monocytes with anti-CD49d inhibited the diabetes-induced adhesion of monocytes in co-culture. VEGF and TNFα in retinal lysate (measured by elisa) were increased (1-2 fold) in diabetic animals and p65 (NFκB) was also elevated in nuclear extracts of retina from diabetic animals. Diabetes induced levels of VEGF and nuclear p65 retention were inhibited to near normal levels in the retina of diabetic rats treated with the anti-CD49d antibody. Inhibition of diabetes-induced TNFα levels in the retina (62%) was also significantly reduced with the therapy.

The results suggest that VCAM1 / VLA-4 strongly mediate the diabetes-induced binding of leukocytes and vascular permeability in the retina of early diabetic animals. Although
the study did not measure VCAM1 expression in the retina it has been shown to be elevated in the retina of diabetic rats (Zheng et al. 2007c). This study also demonstrated that monocytes, in addition to neutrophils in previous studies, isolated from diabetic animals have increased adherence to endothelial cells in co-culture compared to non-diabetic controls. The elevated levels of p65 in the nuclear extracts of retina from diabetic animals suggest an inflammatory response in the retina; however, the promoters with which p65 is associated, and the cells in which p65 is retained, remain unclear.

*Leukocyte adhesion molecules and cell death.* In a third study, Joussen and Adamis (Joussen et al. 2001) observed an increase in CD18 positive leukocytes adhering to the retinal blood vessel wall (leukostasis) and propidium iodine staining of 90 vascular endothelial cells in the retina of rats diabetic for two months, and similar results in animals diabetic for nine days. The staining of the vascular endothelial cells in the retina with propidium iodine is interesting in that PI, which binds to chromatin in cells with leaky membranes, is often considered a marker of cell death. However, the number of endothelial cells at nine days and at two months staining with PI suggests a high rate of cumulative endothelial loss, and therefore capillary loss, which is not observed in the retinae of rats diabetic for two months compared to non-diabetic controls, nor was it reported in the study. Alternatively, the membrane of the endothelial cells could have an increased permeability or the initial onset of diabetes due to streptozotocin may result in the death of endothelial cells after which the rate of endothelial cell death is relatively low until about two months of diabetes.
IP injections of Anti-ICAM1 antibody every other day after the induction of diabetes inhibited propidium iodine staining of vascular endothelial cells by 90% and leukostasis by 40% in the retina of one-week diabetic rats. A similar therapy with anti-CD18 antibodies reduced leukostasis by 40% but did not reduce the number of retinal endothelial vascular cells staining with propidium iodine in one-week diabetic rats. Since ICAM1 is the ligand for CD18 integrins, and anti-CD18 reduced leukostasis by 20% more than anti-ICAM1, it is also unclear why anti-ICAM1 but not anti-CD18 antibody therapy reduced propidium iodine staining if the endothelial cell damage is indeed mediated by leukocytes.

In another study, Joussen and Adamis (Joussen et al. 2003) found an increase in retinal vascular endothelial cell expression of Fas and an increase in neutrophil expression of FasL in rats, diabetic for one week or six months. Co-culture of endothelial cells with neutrophils from diabetic animals (diabetic for 1, 6 and 24 weeks) resulted in a two-fold increase in the number of TUNEL positive endothelial cells after overnight incubation. The number of leukocyte-induced TUNEL positive cells was inhibited (81%) by pre-treating the neutrophils with anti-FasL but not anti-TRAIL antibody. Forty-eight hours after injection of anti-FasL antibody in two-week diabetic animals propidium iodine staining of vascular endothelial cells in the retina was reduced (91%). The number of TUNEL staining retinal ganglia and vascular cells in retinal cross sections were increased in two-week diabetic animals, compared to controls, which was inhibited in anti-FasL treated animals. Vascular permeability measured by Evans blue dye retention in the retina was increased three fold in diabetic animals, which was inhibited (50%) by FasL
antibody therapy. However, leukostasis, which was increased by two fold in the retina of diabetic animals, was not significantly altered by the anti-FasL therapy.

The results suggest that in diabetes adherent leukocytes expressing FasL induce retinal endothelial cell death (or increased membrane permeability) through the Fas/FasL pathway. It is however, not clear how retinal ganglia cells are influenced by FasL on leukocytes in the absence of data demonstrating leukocyte invasion of the ganglion cell layer. The absence of a reduction in leukocyte adhesion with anti FasL is not unexpected, as it does not prevent ligation of the adhesion molecules on the endothelial cell to the integrin receptors on the leukocytes. The reduction in vascular leakage by 50% and the reduction propidium staining of endothelial cells by 90% suggests that leukostasis may only be necessary to facilitate Fas/FasL interaction. However, in the previous study anti-CD18 therapy reduced leukostasis (40%) and presumably Fas/FasL interaction but the therapy did not reduce propidium iodine staining. In addition clustering of ICAM1 on endothelial cells with antibodies or adherent leukocytes have been shown to induce vascular permeability. The difference in the reduction of vascular permeability by anti-ICAM1 (86% in previous study) and anti-FasL (50%) may be due to additive effects as clustering of ICAM1 with antibodies has been shown to increase the permeability of endothelial cell monolayers (Sumagin et al. 2011).

**Summary.** Diabetes induces an increase in leukocyte adherence to retinal vascular endothelium through an upregulation of cell adhesion molecules on the surface of the endothelium, and by the priming of leukocytes to produce superoxide and a
rearrangement of their integrins to a ligand binding conformation similar to a classical model of inflammation. Inhibition of leukostasis and permeability by antibodies ICAM1, CD18, CD11b and CD49d (VCAM receptor) as well as increased ICAM1 and VCAM1 in western blots suggest that the observed adhesion and subsequent effects are mediated by both the β1 and β2 integrins. It is unclear why the anti-CD49d treatment resulted in a near complete inhibition of diabetes-induced vascular leakage and leukostasis in vivo when anti-ICAM1 and especially anti-CD18 were only partially effective. It is possible that the anti-CD18 and anti-ICAM1 antibody therapy was of insufficient concentration (1 mg/kg 3 times per week) or the timings of the injections with respect to the sacrifice of the animals (48 hours compared to 24 hours in the CD49d study) may account for the discrepancy. A second possibility is that one or more of the anti-integrin antibodies sterically hindered association of the leukocyte with the endothelium. A third possibility is that antibody blockade of one of the integrins inhibited cross talk between the integrins that mediate the diabetes-induced effects in the retina.

Diabetes-induced vascular leakage and propidium iodine staining of endothelial cells in the retina are mediated at least in part by Fas/FasL. The observed up-regulation of Fas by endothelial cells and FasL in diabetic animals with the observation that inhibition of Fas/FasL inhibited endothelial cell death as measured by both TUNEL and PI staining strongly suggests that leukocytes, neutrophils in particular, are killing endothelial cells in the diabetic animal. However, the number of endothelial cells stained with propidium iodine (which presumably have leaky membranes or are dying) does not seem to correlate with the number of acellular or degenerate capillaries observed in our lab at two months.
It is unclear if the endothelial cells may have been proliferating. The lack of any effect of anti-CD18 but the almost complete reduction with anti-FasL antibodies or anti-ICAM antibodies of the number of diabetes-induced PI staining retinal endothelial cells is confusing, as anti-CD18 antibodies decreased leukostasis and leukostasis is presumably required for Fas/FasL interaction. The reduction of diabetes-induced VEGF and TNFα concentrations and nuclear p65 retention in the retina with anti-integrin therapy suggests that leukocytes mediate a general state of diabetes-induced inflammation in the retina.

In addition to Fas FasL, leukocytes have other mechanisms through which they might induce damage in the retina. Following activation, leukocytes undergo respiratory burst and increase production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can combine in the case of superoxide and nitric oxide to form reactive peroxynitrite. In addition activated leukocytes have been shown to damage endothelial cells in culture by the release of hydrogen peroxide (Harlan et al. 1984). Activated leukocytes can also release lytic enzymes and secrete proinflammatory cytokines such as IL1β the receptor for which was required for diabetes-induced retinal capillary loss in the Vincent paper of the previous section.

**Leukocyte subpopulations.** The leukocyte subtypes, which contribute to diabetes-induced retinal capillary degeneration, are unclear, as neutrophils, macrophages, and microglia have been postulated to contribute to the capillary degeneration process. Neutrophils have been implicated in the blockage and non-perfusion of capillaries, endothelial cell apoptosis, and increased permeability of the blood retinal barrier (Smith
et al. 1994; Lutty et al. 1997; Joussen et al. 2001; Joussen et al. 2003; Hirata et al. 2006). Macrophages are also implicated in the blockage and non-perfusion of capillaries in diabetic mice (Schroder et al. 1991; Rungger-Brandle et al. 2000; Iliaki et al. 2009; Serra et al. 2012). In addition it has been suggested that there may be an increased number of T cells in the diabetic retina (Zhaboedov et al. 2001) however I have as of yet been unable to obtain an English translation and thus cannot comment on the content.

In a study by Serra and Forrester, transfer of labeled leukocytes into diabetic mice resulted in leukostasis and capillary occlusion in the retina by CD11b⁺ and CCR5⁺ leukocytes but not extravascular leukocytes. Leukocytes from the spleen were harvested from non-diabetic and two week diabetic mice stained with Calcein AM and injected into the blood of non-diabetic or two week diabetic recipient mice for 1 hour. Leukocyte adhesion was measured by SLO, after which a vascular contrast dye (Evans blue dye) was then injected into the blood and the retinae were harvested, fixed, stained with antibodies to CD3(T cells), GR1(granulocytes and monocytes/macrophages) and CD11b (most leukocytes) CD45(all marrow derived cells) prior to examination of whole mounts with fluorescent microscopy. The number of labeled leukocytes, and leukocyte-mediated occlusions in the retina, was significantly elevated when leukocytes from diabetic donors were transferred to diabetic recipients compared to leukocytes from non-diabetic donors transferred to non-diabetic recipients. Leukocytes from non-diabetic donors transferred into diabetic recipients were similar to Leukocytes from diabetic donors transferred into non-diabetic hosts, but both were elevated in comparison to the non-diabetic into non-diabetic controls. The numbers of adherent cells, which were CD11b⁺ and CD45⁺ but not
GR1$^+$ or CD3$^+$, were significantly increased in the retinal vasculature in diabetic animals. Flow cytometry analysis of blood and spleen and digested retina indicated that CCR5$^+$ CD11b$^+$ cells were significantly increased in the blood and spleen but not in the retina. CCR2$^+$ CD11b$^+$ cells (often associated with inflammation) were only significantly increased in the spleen and CX3CR1$^+$ CD11b$^+$ cells were significantly increased in the retina and blood of mice diabetic for two weeks compared to non-diabetic controls.

The increase in leukostasis by labeled spleenocytes from diabetic donors injected into non-diabetic mice demonstrates that leukocytes (activated in the diabetic animal) retain their ability (activated state) to adhere to endothelium for at least one hour in the absence of high glucose. The reverse experiment resulted in increased leukostasis of spleenocytes from non-diabetic animals in diabetic animals, likely due to activation of the leukocytes by short-term exposure to high glucose as discussed previously. The absence of extravascular leukocytes in the retina suggests that the leukocytes remain in the vasculature at least for one hour. The observed significant increase in CD11b$^+$ CCR5$^+$ spleenocytes (transferred) in the retina of diabetic mice but the lack of the same cells in digested retina not receiving spleenocytes transfer suggests that the properties or populations of leukocytes in the spleen may not reflect those in the circulating peripheral blood.

**Microglia.** Changes in microglia have been reported in the retina of diabetic mice and rats. The number of microglia in the subretinal space between the choroid and RPE increased in rats 12 months diabetic compared to rats diabetic five months or non-
diabetic controls (Omri et al. 2011). In a study by Rungger-Brandle and Leuenberger, microglia in the inner plexiform layer of the retina and Mueller cells were significantly increased in diabetic mice by week four of diabetes and were 2 fold higher than non-diabetic controls by twelve weeks of diabetes (Rungger-Brandle et al. 2000). Microglia stained with isolectin B4 were counted in 3-4 cross sections and the morphology of the cells was noted to change shape from ramified (small soma with numerous long thin arms) in non-diabetic controls to rounded (large soma with infrequent small thick arms) in diabetic animals. It has been my observation that the number of microglia and the shape of the microglia is inconsistent in consecutive cross sections, and does not reflect the density observed in flat mounts. Furthermore, isolectin B4 is not a specific indicator of microglia as it stains at least macrophages as well. Therefore, the observed increase in microglia may not reflect the actual number of microglia in the retina.

In summary, it was not clear if leukocytes mediate the diabetes-induced loss of retinal capillary cells and other vascular abnormalities, and if they do, which subtypes may be involved. Depletion of leukocytes with antibodies or the blockade of adhesion molecules with antibodies is limited by the amount of time the antibodies can be used (1-2 months) prior to the mouse developing its own antibody defense to the foreign antibody therapy, whereas the development of a discernable number of acellular capillaries in the retina requires 6-8 months of diabetes. Studies of the whole mounted retina are further complicated by the autofluorescence, which develops with the formalin fixative, or degenerate architecture that develops with dehydrating fixatives such as acetone and methanol.
Chimeric mice as a tool to study the effects of leukocytes. Studies, in which antibodies are used to deplete leukocytes subtypes in vivo, are limited by the time that the antibody can be administered before the animal develops its own antibodies against a foreign protein, the lack of available non-foreign antibodies, and the cost of administering a large amount of antibody at least once a week. The development of a reliable difference between the numbers of acellular capillaries in the retina of diabetic mice compared to non-diabetic mice requires about 40 weeks of diabetes. Antibody depletion experiments of leukocytes in mice to provide direct evidence that leukocytes play a role in the diabetes-induced development of acellular retinal capillaries is therefore not currently feasible.

The transplantation of bone marrow from a donor mouse, in which a protein of interest has been genetically deleted, into a recipient wild type mouse creates a chimeric mouse in which only the marrow derived cells contain the genetic deletion. This model allows the role of leukocytes (and other marrow-derived cells) in the development of a particular pathology to be studied. Conversely, transplanting bone marrow from a wild type mouse into a mouse lacking a protein of interest (reverse chimera) allows the effect of the protein in all cells except the bone marrow derived cells to be studied. Therefore, mice, in which inflammatory functions are impaired due to a genetic deletion, iNOS\(^{−/−}\) for example, can be used as donors of bone marrow that is transplanted into a WT host to study the effect of the loss of iNOS in leukocytes.
Mice in which a protein of interest is deleted throughout their body can develop compensation for the loss of that protein by a protein with similar structure or function during development. Unintended defects during development due to the loss of the protein can also complicate the experimental model. Mice deficient in macrophage colony stimulating factor MCSF, a growth factor required for the maturation of monocytes and macrophages, for example develop defects in their bones (osteopetrosis) and teeth (don’t develop) due to absent osteoclasts which help regulate calcium levels in the blood, in addition to the desired effect of decreased numbers of monocytes in the circulating blood. Mice deficient in the receptor for MCSF die prior to birth. However transplantation of fetal liver cells, a precursor of bone marrow, from these M-CSFR/- animals into WT mice, as a bone marrow transplant, results in an adult WT mouse in which monocytes are severely reduced in the circulating blood but have normal teeth and bones. Therefore, the chimeric mouse has advantages similar to an induced conditional knockout.

1.5 Neutrophil Inhibitory Factor and Integrin binding.

In the previous chapters, I have reviewed the evidence that diabetes-induced early vascular lesions may be mediated by activated leukocytes. Briefly, diabetes-induced increases in retinal leukostasis, cellular adhesion molecules, vascular permeability, endothelial cell death (as measured by propidium staining), and occluded capillaries are reduced by therapies that inhibit leukocyte adhesion. Leukocyte functions, such as adhesion, release of lytic enzymes, and respiratory burst are mediated at least in part by integrins in the CD11/CD18 family (Hynes 2002).
**Leukocyte mediated endothelial cell injury.** Neutrophils pre-stimulated with PMA (but not unstimulated neutrophils) in co-culture with an unstimulated monolayer of endothelial cells will adhere to the endothelial cells and cause the tight junctions between the endothelial cells to breakdown, followed by endothelial cell rounding, endothelial cell detachment, and eventual endothelial cell death (Ohno and Malik 1997). Pretreatment of the neutrophils with an anti-CD18/CD11b blocking antibody (but not a non-blocking antibody to CD11b) inhibits adhesion, and endothelial cell permeability, and endothelial cell death but not superoxide released by the activated neutrophils (Ohno and Malik 1997). The anti-CD18/CD11b antibody pretreatment also inhibits myeloperoxidase release (neutrophil primary granules) from opsin stimulated neutrophils by 66% (Lum et al. 1994) and hydrogen peroxide release from fMLP stimulated neutrophils otherwise observed (Moyle et al. 1994).

**Potential for undesirable effects by blocking leukocyte adhesion.** Therapies targeting integrins other than MAC1, including humanized anti-CD11a, anti-ICAM1 and anti-CD18, have been associated with increased incidence of infection in clinical trials, including increased fungal and bacterial infections, (Bowen et al. 1998; EAST 2001; Major 2010; Uettwiller et al. 2011). Immune related complications in anti-integrin therapy are not unexpected since humans deficient in CD18 (leukocyte adhesion deficiency I) also are immunocompromised and prone to infections. Thus, inhibition of leukocyte-mediated damage to endothelial cells without simultaneously compromising the immune system requires careful selection of the target. NIF has previously been
administered to patients as an acute therapy after stroke without adverse effects including incidence of infection (Krams et al. 2003). Furthermore, mice expressing NIF or mice lacking MAC1 when challenged with E.coli in a subdermal pocket on their back have normal neutrophil migration into the pocket (Gao et al. 2005).

**Integrin structure and function.** Integrins are a heterodimer of α and β subunits that bind cell surface proteins and extracellular matrix. Both subunits are single pass membrane spanning proteins oriented with the N terminus outside the cell and a cytoplasmic tail containing the C terminus. The extracellular portion of both subunits consists of several structural domains that form a metaphorical leg, which terminates in a head domain. The α subunits in the β2 family of integrins have a head domain which consists of a beta propeller surrounded by alpha helices on top of which are a series of ligand binding loops that form the I (inserted) domain (also known as the A (Von Willebrand factor A like) domain). In addition to providing ligand specificity, the I-domain contains a divalent metal ion-dependent adhesion site (MIDAS) which requires Mg$^{2+}$ occupancy for integrin binding (Lee et al. 1995). The beta subunit head domain contains an I like domain and a MIDAS region (Goodman and Bajt 1996). When the subunits dimerize, the head domains combine to form a hybrid domain on top of the two legs. The cytoplasmic tails of both subunits contain a conserved GFFKR sequence near the membrane, which binds the two subunits together. Deletion of the sequence from either subunit results in constitutive binding of the integrin to its ligand.
Electron microscopy and crystal structures have shown that the extracellular domain of the dimer can adopt several states of conformation. A compact low affinity state in which the extracellular domains of the dimers are bent over so that the I-domain points at the membrane (Xiong et al. 2001; Beglova et al. 2002; Xiong et al. 2002), and ligand binding extended state in which the legs unfold so that the head domain points into the cytoplasm (Nermut et al. 1988; Takagi et al. 2001). Furthermore, in the extended state, the head and legs can adopt two conformations, a closed conformation in which the legs are close together, and an open conformation in which the legs separate allowing the I-domain to rotate slightly which enhances ligand binding.

Two key papers (Takagi et al. 2002; Nishida et al. 2006) have correlated the conformational state and the function of several integrins including two of the β2 integrins. The association of the cytoplasmic tails was mimicked by fusing peptides to c-terminus of both α and β subunits, which formed a disulfide bridge. In the clasped state, the integrin did not bind its ligand. Electron microscopy was used to show that in the clasped state, the dimers adopted the compact bent state. The addition of agents known to cause outside-in activation (Mn$^{2+}$, ligand mimetic peptides, or activating antibodies) resulted in a transformation of the dimer to the extended state with equal number of dimers in the open or closed head conformation. If the clasp was cleaved, some of the bent dimers also adopted an extended state, which is in agreement with the observation that separation of the cytoplasmic tails in inside–out signaling can initiate the ability of the integrin to bind ligand. Ligation of the unclasped dimer results in the extended open head conformation.
Integrins do not possess enzymatic activity. Rather, ligation of the extended integrin with extra cellular ligand such as matrix proteins induces a conformational change in the integrin that separates the cytoplasmic tails of the two subunits, exposing binding sites on the cytoplasmic tails, which then recruit cytoplasmic adaptor proteins such as talin, kindlins and paxillin. The adaptor proteins link the integrins to actin cytoskeleton and signaling and regulatory molecules such as Syk, Pyk2 and PLCγ (Zaidel-Bar et al. 2007).

Ligation of the CD11/CD18 integrins to cellular adhesion molecules (CAMs) mediates binding, as well as bidirectional signaling in both cells. Humans deficient in CD18 are immunocompromised (leukocyte adhesion deficiency I) and exhibit leukocytosis, an inability to recruit leukocytes to the site of infection, and T-cell proliferation defects resulting in an increase in infections.

There are four members, of the CD11 family, CD11a(αL), CD11b(αM) CD11c(αX) and CD11d(αD), which differ in the sequence of their I-domain and their relative expression on cells (Orchekowski et al. 2000). CD11a is found on most leukocytes where it dimerizes with CD18 to form the lymphocyte function associated antigen 1 (LFA-1). LFA-1 is important in leukocyte migration, adhesion to ICAM1, ICAM2 and ICAM3 and co-stimulation of lymphocytes. Mice deficient in CD11a exhibit impaired leukocyte recruitment to site of infection.
CD11b is expressed by neutrophils, monocytes, natural killer cells, and some lymphocytes. It dimerizes with CD18 to form the complement receptor 3 (CR3) or macrophage-1 antigen (MAC-1) integrin. MAC-1 is important in leukocyte adhesion, spreading and phagocytosis. It is rapidly upregulated in neutrophils upon activation by fusion of internal secretory granules with the plasma membrane. MAC-1 binds a number of ligands including ICAM1, NIF, and fibrinogen. Mice deficient in CD11b exhibit defective phagocytosis, adipose accumulation, and defective apoptosis of neutrophils.

CD11c is expressed at high levels on the surface of dendritic cells and at very low levels on innate immune cells and B cells. CD11c dimerizes with CD18 to form the complement receptor 4 (CR4) integrin. CR4 shares similar binding and function to MAC1 on innate immune cells but does not play a major role in neutrophil or monocyte adhesion due to low levels of expression.

CD11d is expressed at low levels on innate immune cells but is upregulated on monocytes as they transition into macrophages (Van der Vieren et al. 1995). CD11d ligands include the matrix proteins fibrinogen, vitronectin, and fibronectin, the adhesion molecules VCAM1 and ICAM3 (but not ICAM1) and coagulation factor plasminogen (Yakubenko et al. 2006). CD11d is upregulated in response to lipid accumulation.

**Neutrophil Inhibitory Factor.** Neutrophil inhibitory factor (NIF) is a canine hookworm derived protein that selectively binds to integrin CD11b/CD18, and thereby inhibits leukocyte adhesion. Pretreatment of activated neutrophils with NIF prior to co-culture
with unstimulated endothelial cells also inhibits adhesion, and endothelial cell permeability, hydrogen peroxide release and endothelial cell death but not superoxide released by the activated neutrophils (Moyle et al. 1994; Ohno and Malik 1997).

NIF has been used to inhibit leukocyte-induced injury in several models of acute injury and inflammation. Acute lung injury and induced pulmonary edema caused by installation of acid, LPS, TNFα, or E.coli into the lung of animal models are characterized by a rapid influx of neutrophils, which accumulate in the lungs, and cause increased permeability of the vasculature resulting in fluid accumulation. Neutrophils in the lung release myeloperoxidase, which injures the lung tissue, promotes fibrosis, and contributes to general state of inflammation. Pretreatment of animal models or human neutrophils transplanted into the animal model with NIF inhibits the influx of neutrophils into the lung, myeloperoxidase release, and subsequent edema (Barnard et al. 1995; Folkesson and Matthay 1997; Zhou et al. 1998; Gao et al. 2005). NIF has also been used to inhibit neutrophil influx and damage in liver following hemorrhagic shock in rats and leukocyte infiltration, leukotriene B4 and myeloperoxidase release in a formalin induced colitis model in rabbit (Bauer et al. 1995; Meenan et al. 1996).

*NIF in animal models of stroke.* Transient focal ischemia in the brain is characterized by an acute blockage of blood flow resulting in cerebral ischemia followed by reperfusion after removal of the blockage. An influx of neutrophils to the area results in an increase in the infarct volume of the cerebral tissue. Administration of NIF into the circulation of rats at the onset of reperfusion, 4 hours after, or 6 hours after the reperfusion significantly
reduced infarct volume and cerebral edema at 24 hours after an acute blockage of two hours (Mackay et al. 1996). In a similar study NIF was administered after reperfusion or two hours post reperfusion in conjunction with tissue plasminogen activator (tPA) or alone and the animals were evaluated at seven days post occlusion (Zhang et al. 2003). The infarct volume was not significantly reduced with the acute administration of NIF at either time point when evaluated at seven days after the occlusion. A significant decrease in infarct volume was achieved if NIF was administered with tPA at 2 hours after reperfusion compared to tPA alone at the same time point. Administration of NIF with tPA after reperfusion did not provide any significant additional benefit in reduction of infarct volume compared to tPA alone. In a third study, NIF was administered at various time points after a two-hour acute blockage in rats and after a permanent blockage. A bolus administration of NIF 2 hours after a permanent blockage did not reduce cerebral infarct volume or improve motor skills 48 hours after the initiation of the blockage. A bolus of NIF administered to animals receiving acute blockage after reperfusion reduced infarct volume and improved motor skills assessment in a dose dependent manner 2 days after the blockage. A delay in the of the administration of NIF for 2 after reperfusion reduced the beneficial effects by half and a delay of 4 hours was not significantly different from non treated animals even at 5 times the concentration evaluated at 2 days after the blockage (Jiang et al. 1998). In a second group of animals, NIF was administered after reperfusion continuously for two days at two different doses and continuously for seven days at which time all of the animals were evaluated. The infarct volume decreased in a dose dependent manner and with the duration of continuous infusion. The authors concluded that while NIF inhibited the influx of leukocytes into the
damaged brain tissue if given within a 4-hour window after the initial onset of blockage, once NIF was eliminated from the circulation over time, neutrophils migrated into the tissue, which persisted at least four days after the reperfusion. Thus, the administration of NIF given as an acute treatment only delayed the leukocyte mediated increase in infarct size and decrease in motor skills similar to acute treatments with hypothermic intervention and NMDA antagonists.

*NIF in clinical trial of stroke.* NIF has been used in a clinical trial for improving patients’ outcomes after stroke. Although NIF demonstrated improvement in patient recovery following a stroke when administered with tPA, NIF alone failed to demonstrate significant improvement. While it is not possible to discern the reason for the failure of the trial from the published data, the authors noted that patients in the study were not screened for thromboembolism (non-resolved clot) for which NIF was previously shown to be effective only when co-administered with tPA (Zhang et al. 2003). The therapy was subsequently abandoned.

*Isolation and characterization of NIF.* In the paper initially describing the discovery and characterization of neutrophil inhibitory factor (NIF), the authors looking for proteins that parasites might produce to evade the host immune response (Maizels et al. 1993), isolated protein fractions from canine hookworm lysate that inhibited the adhesion of phorbol myristate acetate (PMA) activated neutrophils to unstimulated human umbilical vein endothelial cells (HUVEC) (Moyle et al. 1994). The active fractions inhibited the neutrophil adhesion with an IC50 of 20nM and contained a 40-50 kDa band on silver
stained native gel and 45-55 kDa when denatured. Amino acid sequencing of fragmented protein from the active fractions was used to construct primers, which were used to isolate NIF cDNA. In addition to NIF several other NIF-like cDNA were found, suggesting a family of related proteins were present in the active fraction. However, description of these other proteins was reserved for future publications. cDNA for NIF predicted a protein of 274 amino acids containing 10 cysteines, with a molecular weight of 29 kDa. The predicted molecular weight of the protein was much less than the 41 kDa determined by mass spectrometry, or the observed molecular weight of the protein in native or denaturing gels, indicating that NIF is highly glycosylated. Recombinant NIF produced in yeast had a molecular weight of 50-80 kDa, which was attributed to increase glycosylation, and an IC50 of 10-20 nM in the neutrophil adhesion assay which was comparable to hookworm derived NIF or inhibition of neutrophil adhesion using anti-CD18/CD11 antibodies with an IC50 15nM.

In addition to inhibiting leukocyte binding, NIF also inhibited the release of hydrogen peroxide from neutrophils adherent to serum coated plastic (excludes CD11a binding), stimulated with fMLP, by an order of magnitude less than observed for CD18/CD11 antibody. Unlabeled NIF displaced radiolabeled NIF in binding studies with non-activated human neutrophils but not fMLP stimulated neutrophils, suggesting that NIF binds reversibly to the un-activated conformation of integrin CD11/CD18 but irreversibly once the integrin changes to the extended conformation associated with integrin activation. Specificity of NIF for CD18 integrins was confirmed by immunoprecipitation of NIF from human neutrophil lysate with anti CD11b and anti CD18 and to a much
lesser extent by anti CD11a, suggesting that NIF selectively binds the CD11b/CD18 integrin. Flow cytometry of labeled NIF indicated that it bound to neutrophils, monocytes, and a small population of lymphocytes.

The evidence demonstrating specificity of NIF for binding to CD11b/CD18 is complex, as NIF appears to have different effects depending on the assay used and the source of NIF. Papers arguing against the specificity of NIF for CD11b/CD18 often cite the fact that, in co-culture binding assays of neutrophils (stimulated with phorbol ester or TNFα) and endothelial cells, NIF inhibits neutrophil binding to a greater extent than antibodies to CD11a or CD11b alone. Pretreatment of the stimulated neutrophils with antibodies to CD11a, CD11b and CD18 inhibit 30%, 60% and 90% respectively of the number of neutrophils binding after washing away unbound cells (Prieto et al. 1988; Lo et al. 1989; Lo et al. 1999). Pretreatment of neutrophils with both CD11a and CD11b inhibits 90% of the number of neutrophils binding to the endothelium, demonstrating that integrin CD11a/CD18 accounts for 30% and integrin CD11b/CD18 accounts for 60% of neutrophil binding to endothelial cells. Pretreatment of neutrophils with NIF inhibits greater than 90% of the number of neutrophils binding after washing away unbound cells. The evidence suggests that NIF must therefore inhibit both CD11a and CD11b containing integrins in order to achieve the observed 90% inhibition of neutrophil adhesion.

However, several studies using a multitude of binding assays have shown that NIF binds CD11b with a Kd three orders of magnitude greater than NIF binding to CD11c or CD11a. The adhesion of activated neutrophils to fibrinogen, denatured protein, or serum-
coated plastic is mediated by CD11b/CD18 and CD11c/CD18 since CD11a/CD18 does not bind these substrates (Diamond and Springer 1993; Ugarova and Yakubenko 2001). NIF (half maximum inhibition at 2nM) or antibodies to CD11b but not CD11a inhibit the binding of activated neutrophils to these substrates demonstrating that NIF inhibits the CD11b/CD18 integrin with a Kd in the nM range (Lo et al. 1999). In a similar assay, Chinese hamster ovary (CHO) cells, (which do not express the CD18 family of integrins), transfected with CD18 and CD11b or CD11c bind immobilized fibrinogen. NIF inhibited the adhesion of CD18/CD11b CHO cells with IC50 three orders of magnitude less than NIF inhibition of CD18/CD11c (Ugarova and Yakubenko 2001). In another binding assay, the aggregation of CD11b deficient JY lymphoid cells, which occurs in the presence of PMA, is prevented by uM concentrations of NIF (from transfected human dermal microvascular endothelial cells) or anti-CD11a but not CD11b antibodies. The results suggest that NIF does bind CD11a but at concentrations several orders of magnitude higher than that observed for CD11b/CD18 (Lo et al. 1999).

NIF also binds to the I-domain of the CD11a and CD11b subunits with a Kd similar to that observed with the full CD11/CD18 integrin. Recombinant NIF (from CHO cells) bound the I-domain of subunit CD11b expressed as a fusion protein in E.coli, but not the I-domain from CD11a (at nM concentrations) (Muchowski et al. 1994). In competitive binding assays, radiolabeled NIF (from CHO cells) bound to fMLP stimulated neutrophils, was completely displaced by the CD11b I-domain or unlabeled NIF at 25-30nM, while the I-domain of CD11a displaced NIF at a concentration roughly two orders of magnitude higher. Furthermore, in an assay of adhesion mediated hydrogen peroxide
release from neutrophils stimulated with fMLP in co-culture with HUVEC cells, the I-domain of CD11b inhibited half maximal adhesion and peroxide release at 80nM while the I-domain of CD11a inhibited half of the binding at 10uM and did not inhibit the release of hydrogen peroxide. The majority of the residues to which NIF binds therefore appear to be located in the I-domain of CD11b.

Residues involved in NIF binding of CD11b. There are three studies of the amino acids, which mediate binding between CD11b/CD18 and NIF. Since NIF binds to both human and mouse neutrophils, Rieu and Arnaout mutated the amino acids conserved between the two species in the 11 of 16 loops of the CD11b I-domain (that were determined to be solvent accessible from previously published crystal structures (Lee et al. 1995)). Conserved residues were replaced with alanine or lysine and then the mutated CD11b or WT CD11b subunit was co-expressed with CD18 in COS cells. Both groups of cells were incubated with biotinylated NIF and then washed and lysed. Lysates were slot blotted and screened for the presence of NIF. Mutations in Gly143, Asp149, Glu178/179, and Arg208, which are located near the Mg binding MIDAS site, but not R152, inhibited the binding of NIF. The ability of the mutants to bind radiolabeled manganese was not altered from the WT integrin, suggesting that the MIDAS site was functional. Integrins containing the mutated CD11b subunit did not alter the binding of antibodies to the CD11b I-domain, CD11b non-I-domain, or CD18 from the WT integrin, suggesting that the mutant CD11b formed heterodimers with CD18 and that the conformation of the integrin was not significantly altered.
NIF was originally characterized as binding to CD11b but not CD11a or CD11c. Zang and Plow therefore exchanged each of the 16 loops of the CD11b I-domain (that were determined to be solvent accessible from previously published crystal structures (Lee et al. 1995; Qu and Leahy 1995)) with the corresponding loops of CD11a. They co-expressed CD18 and WT or mutated CD11b in HEK293 cells and conducted competitive binding studies with radiolabeled NIF and unlabeled NIF form yeast. Four loops, Pro147-Arg152, Pro201-Lys217, Asp248-Tyr252, and Glu253-Arg261 were identified that increased the Kd by 8 fold or more. Asp149 was conserved between the CD11b WT and CD11a substitutions. Amino acids 143-146 were not included in the study and exchange of Glu178-Thr185 for the corresponding sequence in CD11a only resulted in a 4-fold increase in the Kd. As proof of principle, all four loops of interest from the CD11b subunit were exchanged with the corresponding loops in the CD11c subunit. Competitive binding assays of NIF, using HEK cells transfected with CD18 and WT CD11c or CD11c with the four loops of CD11b, demonstrated that CD18/CD11c did not bind NIF in the nM range but the mutated CD18/CD11c-11b bound with a Kd of 2nM compared WT CD18/CD11b, which bound NIF with a Kd of 7nM.

In a subsequent paper, Ustinov and Plow exchanged the four loops of interest in the CD11b I-domain with the corresponding sequence in CD11a and expressed the CD11b I-domain (WT and mutants) or CD11a I-domain as a fusion protein in E.coli. In competitive binding experiments, NIF did not bind to the mutated CD11b-CD11a mutant or CD11a WT I-domain (nM range) but it did bind to the WT CD11b I-domain (with and without the fusion tag) with a Kd of 5nM. Groups of three amino acids in the loops of
interest in CD11b I-domain were then exchanged for the corresponding amino acids in CD11a. Triple amino acid substitutions, which resulted in an increase of the Kd in NIF binding assays, were further studied by exchanging only one of the amino acids at a time and repeating the binding studies with NIF. Asp149Glu, Arg151Gln, Gly207Leu, Tyr252Asp, and Glu258Ala but not Arg208Leu were identified as CD11a substitutions in CD11b that resulted in an increase in Kd. A further substitution Phe246Trp was made in the CD11b I-domain to allow conformation of the I-domain to be checked using distance sensitive energy transfer between terbium in the MIDAS domain and Trp246. Of the five single amino acid substitutions, only Glu258Ala resulted in a decrease of intensity in the terbium assay, suggesting that this mutant did alter the conformation of the integrin I-domain. As proof of principal, the five amino acids of interest from the CD11b I-domain were exchanged with their respective counterparts in the CD11a I-domain prior to additional binding studies with NIF. NIF did not bind the WT CD11a I-domain (in nM range) but it did bind to the WT CD11b domain and the CD11a/5AA CD11b mutant I-domain with a Kd of 5 and 7nM respectively.

Of the five amino acids identified by each group, only Asp149 was identified as by both as a required residue to bind NIF. While Gly143 was identified in the Rieu paper as an important amino acid, it was not required for NIF to bind to the CD18/CD11c-CD11b integrin (containing the I-domain of CD11c with the four loops of interest from CD11b) in the Zang paper. Substitution of Glu178Ala and Glu179Ala in the Rieu paper resulted in a dramatic loss in the ability to bind NIF while substitution of Glu178-Thr185 for the corresponding sequence in CD11a including Glu178Thr and Glu179Ser in the Zang
paper resulted in only a 4 fold loss in the ability to bind NIF. While not directly comparable, the results suggest that the polar side chains of threonine and serine more closely resemble the hydrophilic side chain of glutamic acid than the short hydrophobic side chain of alanine. However, in the absence of a crystal structure to which NIF is bound it remains unclear if the alanine substitution would result in a conformational change or cause a patch of hydrophobicity, which inhibits NIF binding. Substitution of Arg208 to Leu resulted in a loss of NIF (derived from human cells) binding to mutated integrin CD18/CD11b on COS cells in the Rieu paper but had no effect on the binding of the I-domain of CD11b expressed in E.coli to NIF (derived from yeast) in the Ustinov paper. Furthermore in the Zang paper exchange of CD11b I-domain Arg208-Lys217 for its counterpart in CD11a (including Arg208Leu) resulted in a dramatic loss in the ability of CD18/CD11b-CD11a to bind NIF, while no single amino acid substitution was found in this segment which resulted in a loss of NIF binding to the I-domain of CD11b in the Ustinov paper. It is not clear if the I-domain expressed in E.coli has different properties than the heterodimer integrin expressed in non-native cells including the extended with the head open, extended with the head tucked in or the bent closed state of the integrin or if NIF glycosylation in yeast is significantly different from glycosylation in human cells. The use of fluorescent probes, which bind selectively to the open conformation of the integrin (Chigaev et al. 2011), might help to resolve the noted differences. Furthermore it is curious that the I-domain of CD11b, mimicking the completely exposed head of the activated and extended integrin does not irreversibly bind to NIF as reported by Moyle in binding experiments of NIF to activated leukocytes, suggesting that CD18 or the remaining (non I-domain) of CD11b may play an important role in binding NIF. The
experiments do however reiterate that NIF binds CD11b by 2-3 orders of magnitude tighter than CD11a or CD11c.

*Estimation of NIF binding of CD11d.* Of the five residues identified by Plow and colleagues, an alignment of human and mouse alpha chains (CD11a-CD11d), using NCBI program COBALT, shows that Gly207 and Tyr 252 are conserved in human CD11c, however the differences between the remaining three amino acids were sufficient to lower binding of NIF by three orders of magnitude. Asp 149, Gly 207, and Tyr 252 are conserved in mouse and human CD11d and mouse CD11c. Substitution of Arg 151 in CD11b for Gln151 (polar side chain) in CD11a decreased NIF binding by 50% in the second plow paper. Substitution of Arg 151 in CD11b for Asn 151 in human CD11d would be predicted to have a similar effect based on similarity of Gln and Asn. Substitution of Thr 151 in mouse CD11d would also be predicted to have a similar effect. Substitution of Glu 258 in CD11b (negative charged carboxylic acid) with Gln258 (uncharged amide side chain) in human CD11d is also likely to have a similar effect. Gln258 is conserved in mouse CD11d. Therefore, it is likely that NIF also binds CD11d, especially in mice, but at a Kd one to two orders of magnitude less than CD11b.

The amino acids in NIF, which bind to the CD11b/CD18 receptor, have been explored in a paper by Madden and Stanton(Madden et al. 1997). Using non-glycosylated non-overlapping synthetic peptides spanning NIF only residues 224-252 (C-terminus) were found to block TNFα stimulated neutrophils from binding to HUVECs with an IC50 of 4uM. NIF (derived from yeast) inhibited adhesion with an IC50 of 1nM. Mutation the
first of the two cysteines to alanine in this peptide but not both resulted in a loss of ability to inhibit neutrophil binding. Truncation of C terminal end by 5 but not 7 amino acids did not increase the IC50 of neutrophil binding, while the addition of 5 amino acids on the N terminal end decreased the IC50 to 1uM. The peptide used for the remainder of the study was comprised of amino acids 224-252 with the exception of the cysteines, which were changed to alanine. The peptide and NIF blocked TNFα stimulated neutrophil adhesion to serum coated plastic with IC50 of 6uM and 5nM respectively. In competitive binding experiments, NIF, but not the peptide (even at 100uM), displaced radiolabeled NIF from the CD11b I-domain expressed as a fusion protein by E.coli or isolated CD11b/CD18, suggesting that the peptide binds to a site different from that to which NIF binds. The high IC50 of the peptide (1 uM) compared to yeast derived NIF (1 nM), in the binding assays suggests that either glycosylation is important or more likely that several amino acids in remaining N-terminus 227 amino acids of NIF also bind to CD11b/CD18. Immunoprecipitation of the peptide incubated in the lysate of activated neutrophils would also be useful in establishing to what receptors the peptide binds. A more classic approach in which NIF is expressed as a truncated protein by removing successive short peptides from either end might yield more clues about which segments of NIF are important for binding CD11b.

1.6 NFκB regulation of inflammation

**Definition and function.** The NFκB /Rel (Nuclear factor kappa light chain enhancer of activated B cells) family of proteins are a group of transcription factors which initiate the production of proinflammatory mediators such as cytokines in response to cell stress or
inflammatory stimulus (Hoffmann and Baltimore 2006; Lawrence 2009). However, the function of these proteins is complex as they are also important in the resolution and inhibition of inflammation. The Rel proteins are associated with a number of chronic inflammatory diseases including rheumatoid arthritis, asthma, Alzheimer’s disease and the induction of Type I diabetes (Tak and Firestein 2001; Granic et al. 2009).

The NFκB/Rel family of proteins is defined by the inclusion of a Rel homology domain near their N terminus which contains three regions that mediate dimerization between family members, binding to inhibitors (IκB) and binding to DNA (Beinke and Ley 2004; Hoffmann et al. 2006). There are five members in the Rel family RelA (p65), RelB, c-Rel, p50, and p52. Slight differences in the sequence of the Rel domain of each of the five members, mediate the binding affinity of any of the 15 possible combinations of the Rel proteins as dimers and the binding affinity of a dimer pair for a particular NFκB response element (Phelps et al. 2000). One exception is RelB, which does not form stable homodimers (Ryseck et al. 1995). The sequence of the NFκB response elements also vary, and NFκB regulated genes often contain more than one non-identical response element (Schreiber et al. 2006). In addition, only RelA, RelB, and c-Rel contain a transactivation domain required for the initiation of transcription. Therefore, homodimers of p50 and p52 and p50/p52 dimers can function as competitive inhibitors of transcription with the other 11 remaining combinations. However a trimer of transcriptional co-regulator Bcl-3 or CBP and p50/p50 homodimer can function as an activator of transcription (Bours et al. 1993).
The Rel dimers are bound and retained (inactive) in the cytoplasm by the IκB proteins (inhibitor of κB proteins) preventing their translocation into the nucleus. There are five members of the IκB family, IκBα, IκBβ, IκBε, IκBδ (p100, NFκB2), and IκBγ (p105, NFκB1). The IκB proteins may influence which Rel dimers form by stabilizing a particular set of dimers. In addition, it is unclear if Rel proteins can only bind as a dimer to IκB proteins or if monomeric binding of a Rel protein occurs and the IκB Rel protein pair influences the selection of the second Rel monomer. The binding of Rel proteins can also differ between types of cells as RelB does not co-precipitate with p105 in NIHT3T (human kidney) or HeLa (human cervix) cells but does so in Raw264.7 cells (mouse macrophage) (Solan et al. 2002; Savinova et al. 2009).

Two of the IκB proteins, p105 and p100, contain Rel domains, and are constitutively cleaved by the proteasome complex to form Rel proteins p50 and p52 respectively (N terminus fraction). In addition p105, may be cotranslationally processed to produce p50 (Lin et al. 1998). p105 and p100 can form large multimers. Besides their Rel domain which allows them to bind monomeric Rel proteins, p105 and p100 also contain an ankyrin domain which allows them to bind Rel dimers as an IκB protein and an alpha helical domain which allows them to form homodimers (Savinova et al. 2009). RelB binds poorly to all IκB proteins except p100.

Inhibition of IκB binding to Rel dimers is mediated through phosphorylation of the IκB proteins by a third group, the IκK (IκB kinase) proteins. The IκK is a trimer composed of
IκKα, IκKβ and IκKγ (NEMO NF-κB essential modulator) subunits or two IκKα subunits and NIK (NFκB inducing kinase).

When a cell is stimulated through chemokine receptors (receptors for IL1β or TNFα) or toll like receptors (TLR), IκK(αβγ) is activated and phosphorylates IκB bound to a Rel dimer (such as IκBα:p50/RelA or IκB:p50/c-Rel). The phosphorylated IκB is subsequently ubiquitinated by an E3 ligase and destroyed by the proteasome. The Rel dimer is then free to translocate into the nucleus. This process mediated by IκK(αβγ) is known as the canonical pathway. p105 multimers are also processed in a similar manner by the canonical pathway resulting in complete degradation of p105 (Salmeron et al. 2001).

Alternatively, if the cell is stimulated by CD40 or RANKL (receptor activator of nuclear factor kappa-B ligand), the IκK(ααNIK) is activated to phosphorylate the IκB similar to the previous pathway. In the special case of RelB/p100, p100 is the IκK, which upon phosphorylation is cleaved to p52, leaving RelB:p52 free to translocate to the nucleus. This second pathway is known as the non-canonical pathway. In the canonical pathway, the phosphorylation of IκB is mediated by IκKβ subunit and happens within minutes but is also quickly resolved, whereas in the non-canonical pathway, IκKα mediates phosphorylation over the course of hours but the effect persists. However, receptors that activate the non-canonical pathway also activate the canonical pathway.
Negative regulation. There are several negative regulators of NFκB mediated gene transcription. As previously discussed cytokine signaling activates the canonical pathway and induces proinflammatory genes however after several hours the canonical pathway also initiates transcription of anti-inflammatory genes including TGFβ1 and PDG2 and it initiates leukocyte apoptosis to resolve inflammation (Lawrence et al. 2001). While IKK(αβγ) initiates the degradation of IκB, it also phosphorylates both RelA and c-Rel initiating their removal by the proteasome as well (Lawrence et al. 2005). The IκB protein genes are targets of NFκB activity so that while IκB is initially ubiquitinated and degraded, new IκB is rapidly made which can bind Rel proteins exiting the nucleus. In addition to competing for response elements, p50:p50 dimers also recruit histone deacetylases (HDAC) which repress transcription by allowing the DNA to bind tightly to the histone (Elsharkawy et al. 2010). Bcl-3 in the nucleus binds to p50 dimers in the nucleus, which increases the half-life of the complex and therefore occupancy of response elements (Carmody et al. 2007).

Terminology. The terminology of the Rel proteins is confusing and sometimes misleading. C-Rel is sometimes referred to as Rel. NFκB1 is often used to describe p105 or p50, and NFκB2 is used to describe p100 or p52. Antibodies to p50 and p52 also label their precursors p105 and p100. NFκB is commonly used to refer to p50/p65 heterodimers. The canonical pathway is used to describe p50/p65, IκBα, and IκK(αβγ) while the non-canonical pathway is often used to describe everything else.
**NFκB in diabetic retinopathy.** The hallmarks of early diabetic retinopathy are consistent with the low-grade chronic inflammation of the retina. As previously reviewed in the section on inflammation, proinflammatory cytokines including IL1β, TNFα, and MCP1, and inflammation-associated proteins such as iNOS are up regulated in the retina and surrounding fluids (aqueous and vitreous humor) of diabetic animals and humans. These inflammatory proteins are targets of NFκB mediated transcription. iNOS deficient mice don’t develop the diabetes-induced lesions of the retina (Zheng et al. 2007b). Inhibition of the diabetes-induced increase of iNOS by blocking NFκB mediated transcription should result in a similar finding. Incubation of glial cells in high glucose has been reported to increase MCP1 in a p50 dependent manner (Harada et al. 2006), suggesting that diabetes-induced elevation of MCP1 in retina may also be p50 dependent.

In addition to inflammation, the early stages of DR are also characterized by the progressive loss of retinal capillary endothelial cells, resulting in the formation of acellular capillaries, which is thought to cause local hypoxia and eventual progression of the retinopathy into the proliferative stage. High glucose alone is sufficient to increase nuclear enrichment of p50/p65 and cell death in cultured endothelial cells and this process is inhibited by blocking nuclear import of p50 (Zheng et al. 2004). Nuclear enrichment of p50 has also been shown to increase in the retinal capillary endothelial cells of diabetic rats. Acetylsalicylic acid (aspirin), a non-specific inhibitor of NFκB mediated proinflammatory gene transcription (McBerry et al. 2012), inhibited-diabetes induced retinal vascular lesions in diabetic dogs and rodents as previously reviewed (Kern and Engerman 2001; Zheng et al. 2007c). PARP1, a protein required for p50/p65
retention in the nucleus, inhibited the diabetes-induced leukostasis and loss of microvascular cells in the retina (Zheng et al. 2004).

**Characterization of mice lacking p105.** The Rel dimer p50:RelA is the predominant dimer in the initial proinflammatory phase of NFκB signaling, followed by c-Rel:p50. RelA deletion is embryonic lethal, however p50−/− mice are viable and p52 does not compensate for the loss of p50 (Hoffmann et al. 2003). Mice deficient in both p50 and p105 have an increased susceptibility to certain infections, but also have a strong inflammatory phenotype due to over production of cytokines (Ishikawa et al. 1998). These mice are unable to fight off streptococcus pneumonia, but clear E.coli normally (Sha et al. 1995). They do not resolve lesions of Leishmania due to a defective TH1 response. Antigen driven proliferation and functional maturation of CD4 T cells to helper cells is impaired (Das et al. 2001; Artis et al. 2003). However, they are more resistant to infections with encephalomyocarditis virus, due to increased production of INFβ. The mice are resistant to allergic airway inflammation and collagen-induced arthritis, but more susceptible to helicobacter induced colitis (Yang et al. 1998; Campbell et al. 2000). Stimulation of macrophages from these mice with LPS results in normal IL1β and TNFα secretion but not IL6 or IL12 (Sha et al. 1995; Lamhamedi-Cherradi et al. 2003). In summary, inflammatory processes, which require p50-mediated transcription, are inhibited, while those that do not initiate a poorly restrained inflammatory response due to the lack of p105 and p50 mediated inhibition.

**1.7 Hypothesis and specific aims**
In summary, the literature supports the concept that a chronic inflammatory state exists in the retina in diabetes that leads to loss of endothelial cells and pericytes resulting in hypoxia and subsequent neovascular outgrowth into the retina. While leukocytes appear to be involved in diabetes-induced vascular lesions of the retina, direct evidence by elimination of leukocyte function or subpopulations of leukocytes in long-term studies (2-8 months of diabetes) have not been reported. I propose to test this hypothesis by transplanting bone marrow between wild type and mice in which the leukocyte inflammatory functions are impaired or in which specific leukocyte subpopulations are impaired or missing. Previous studies by my lab have demonstrated that mice deficient in iNOS or PARP do not develop the diabetes induced vascular lesions of the retina. However, it was not known which cells containing these proteins are important with respect to the development of diabetes induced vascular lesions in the retina. iNOS is the principal enzyme which makes NO in leukocytes, and PARP is required for retention of NFκB transcription factors in the nucleus of leukocytes. Therefore, if leukocytes mediate the loss of capillaries and pericytes in the retina, then mice in which only the leukocytes are missing PARP or iNOS should not develop these lesions. If instead endothelial cells and pericytes die due to defects in altered metabolism in retinal cells, than mice lacking iNOS or PARP1 in only their marrow-derived cells should still develop the diabetes induced retinal lesions. To identify which subsets of leukocytes are involved in the early pathogenesis of DR, I will first examine which leukocytes are increased in the vasculature and tissue of the retina using immunostained whole retina from mice perfused with a marker that will be present on leukocytes if they migrate post mortem. I will also conduct long-term experiments in mice deficient in the receptor required for granulocyte
or monocyte development and maturation during hematopoiesis, which will result in a lack of either cell type in our diabetic models. The use of these particular mutations will have the advantage over mice lacking the respective colony stimulating factors in that marrow from the receptor deficient animals can be transferred into WT mice, which will have developed normally but will not produce the leukocyte subset of interest after adoptive transfer.

The role of leukostasis in early diabetic retinopathy is controversial in that there are reports of therapies that inhibit leukostasis but not diabetes-induced acellular capillaries in the retina, suggesting that leukocyte adhesion is not critical in the diabetes-induced pathology. However, leukocyte mediated damage of endothelium is generally considered to be adhesion dependent and usually involves CD18 integrins. We propose to study the role of leukocyte adhesion dependence in the pathology of early diabetic retinopathy using a novel protein expressed in transgenic mice, which selectively blocks CD18/CD11b. This particular approach will also allow us to evaluate the inhibition of CD18/CD11b as a long-term therapy.

High glucose is sufficient to induce endothelial cell death in vitro in a NFκB p50 dependent manner. The diabetes-induced inflammation in the retina also likely involves NFκB transcription of pro-inflammatory proteins such as iNOS. We will test the hypothesis that p50 is required for diabetes-induced loss of retinal capillary endothelial cells and increase in inflammation using an available mouse model that is deficient in
p105 and p50 in both long term (30 weeks) and short term (10 week) studies to evaluate retinal vascular changes and biomarkers of inflammation respectively.

**Overall hypothesis:** I postulate that leukocytes mediate the majority of the diabetes-induced loss of retinal capillaries. Transplantation of bone marrow from donor mice in which the leukocyte inflammatory functions are impaired or in which specific leukocyte subpopulations are absent will inhibit the retinal capillary cell death and other abnormalities that are postulated to play a role in the development of diabetic retinopathy.
SPECIFIC AIMS

Aim 1: Test the hypothesis that leukocytes mediate diabetes induced retinal vascular lesions by transplanting bone marrow from mice with known leukocyte defects iNOS\(^{-/-}\) and PARP\(^{-/-}\) mice into WT mice and from WT mice into these deficient animals and determine which subpopulations of leukocytes mediate diabetes induced retinal vascular lesions by transplanting bone marrow from G-CSFR\(^{-/-}\) and M-CSFR\(^{-/-}\) mice into WT mice and vice versa, and documenting what subpopulations of cells are present in the retina.

Aim 2: Test the hypothesis that leukocyte dependent diabetes induced retinal vascular lesions are mediated by the binding of integrin CD18/CD11b with the selective inhibitor NIF.

Aim 3: Test the hypothesis that diabetes-induced retinal endothelial cell loss and inflammation require the involvement of NFκB transcription factor p50 using p105 deficient mice.
1.8 Tables and Figures

Figure 1-1.  Histological lesions of diabetic retinopathy

A. Microaneurysms in vascular bed isolated from diabetic patient. Arrows indicate microaneurysms.

B. Vascular bed isolated from diabetic mouse. Arrows indicate acellular capillaries
CHAPTER 2
MARROW-DERIVED CELLS REGULATE THE DEVELOPMENT
OF EARLY DIABETIC RETINOPATHY AND TACTILE
ALLODYNIA IN MICE

Most of the work in this chapter about the role of leukocytes in the pathogenesis of
diabetic retinopathy was previously published in Diabetes, Vol 61, 3294-3303(Li et al.
2012).

2.1 Introduction
Diabetic retinopathy and neuropathy are leading causes of blindness and pain in
industrialized nations. The early stages of diabetic retinopathy (DR) are characterized by
vascular abnormalities (permeability, non-perfusion and degeneration) and degeneration
of some retinal neurons (Davis et al. 1997; Barber et al. 1998). The progressive
degeneration of retinal capillaries in diabetes can cause retinal ischemia, which then
stimulates the subsequent neovascular response characteristic of advanced DR (Bresnick
et al. 1976a; Kern 2007). Inflammatory markers are also increased in retinas from
diabetic patients and animals. Our previous studies have shown that systemic inhibition
of inflammation using germline deletion of iNOS (inducible nitric oxide synthase) or an
inhibitor of PARP1 (poly(ADP-ribose) polymerase 1) significantly inhibited the capillary
degeneration and other early lesions of diabetic retinopathy in animals (Zheng et al. 2004;
Both iNOS and PARP1 are important proteins in inflammation and immunity (MacMicking et al. 1995; Altmeyer et al. 2010). iNOS is the primary source of nitric oxide (NO) in activated leukocytes, and that NO is involved in subsequent modification of protein function (such as via nitration or nitrosylation) and enhanced generation of reactive species (including superoxide and reactive nitrogen species) (Kim et al. 2005a; Leal et al. 2007; Zhao et al. 2010). PARP1 is a nuclear enzyme which ADP-ribosylates proteins, and has important roles in both DNA repair and NF-κB mediated transcription of pro-inflammatory genes (Hassa et al. 2001; Zerfaoui et al. 2010).

Most studies to date have focused on cells of the retina and its vasculature as the main sites for the biochemical abnormalities that cause diabetes-induced retinopathy. Leukocytes become abnormal in diabetes, and adhere to and occlude some blood vessels (Joussen et al. 2001; Kim et al. 2005b). Moreover, whole-body deletion of ICAM1 or CD18 inhibited development of early DR (Joussen et al. 2004). How this effect was mediated, and whether this finding implicated only marrow-derived cells in the development of the retinopathy, remained unclear. To directly test the contribution of inflammation (specifically pro-inflammatory processes within myeloid-derived cells) in development of the early stages of DR, we generated chimeric mice, which lacked either iNOS or PARP1 only in their marrow-derived cells.

Some diabetic patients develop a hypersensitivity to light touch (tactile alldynia) which can result in pain and discomfort (Otto et al. 2003). Germline deletion of iNOS or PARP1
or treatment with PARP1 inhibitor significantly inhibits diabetes-induced tactile allodynia (Obrosova et al. 2008; Vareniuk et al. 2008). Microglia (which are marrow-derived cells) in the spinal cord have been implicated in diabetes-induced allodynia in mice (Tsuda et al. 2008). We therefore used chimeric animals in which iNOS and PARP1 had been deleted from marrow-derived cells to study the role of leukocytes also in the development of a diabetes-induced neural complication.

2.2 Materials and Methods

Animals.

Experiments conformed to the guidelines of ARVO and CWRU. Offspring from breeder pairs of WT (C57Bl/6J), iNOS<sup>−/−</sup> (B6.129P2-Nos2<sup>tm1Lau</sup>/J), GFP (C57BL/6-Tg(ACTB-EGFP)1Osb/J), and PARP1<sup>−/−</sup> (129S-Parp1<sup>tm1Zqw</sup>/J) (Jackson laboratories) and G-CSFR<sup>−/−</sup> (C57BL/6-G-CSFR<sup>PKGNeo</sup>) mice (kindly provided by Dr. D. Link, Washington University, St. Louis, MO) were housed in air filtered units. Diabetes was induced in male mice with streptozotocin (55 mg/kg) and maintained with 0.1-0.2 units of NPH insulin (0.0217±0.0037 units/gram/week/mouse) (Kern et al. 2007; Zheng et al. 2007a; Zheng et al. 2007c). Hyperglycemia was quantified via blood glucose concentrations, and every 2–3 months by glycated hemoglobin levels (Variant II total GHb Program; BioRad).

Generation of chimeric mice

Recipient mice (non-diabetic or 2 weeks diabetic) were irradiated with 2 600 rad doses, 3 hours apart, and injected intravenously with 3-5 million bone marrow cells from donor
mice. Donor mice lacking M-CSFR in their marrow-derived cells were generated by injecting irradiated wild type (WT, C57Bl/6J) mice with MCSF-R⁻/⁻ fetal liver cells (kindly provided by Dr. R Stanley Albert Einstein College, NY). Chimeras lacking iNOS, PARP1, M-CSFR or G-CSFR only in their marrow-derived cells, were generated by transplanting marrow from iNOS⁻/⁻, PARP1⁻/⁻, G-CSFR⁻/⁻ or chimeric M-CSFR⁻/⁻ donors into irradiated wild type (WT, C57Bl/6J) hosts (identified for example as iNOS⁻/⁻ → WT; names to the left and right of arrow refer to marrow donor and recipient, respectively). “Reverse” chimeras lacking iNOS or PARP1 in all cells except marrow-derived cells, were generated by transplanting marrow from WT donors into irradiated iNOS⁻/⁻ or PARP1⁻/⁻ hosts (WT → iNOS⁻/⁻). To control for irradiation and chimeric procedures, we generated chimeras in which marrow from WT or mice expressing green fluorescent protein (GFP) was transplanted into WT recipients (WT → WT and GFP → WT, respectively) and in some cases the head of the recipient was shielded with lead during irradiation.

**Evaluation of bone marrow grafts in chimeric mice**

To verify the extent of the replacement of native bone marrow with donor marrow in iNOS and PARP1 chimeric animals, DNA was extracted from blood using DNeasy kit (Qiagen), and amplified with PCR Supermix (Invitrogen) using the following primers:

CGA-CAT-GGT-GTC-CAA-AAG-TG PARP1-F1,
GGT-GGT-TTT-TCC-CAA-ACC-TT PARP1-R1,
CAG-CTG-GGC-TGT-ACA-AAC-CTT iNOS-F1,
The extent of native bone marrow replacement in G-CSFR, M-CSFR, and GFP chimeric animals, was verified using flow cytometry (LSRII, BD). Leukocytes, isolated from EDTA anti-coagulated peripheral blood with RBC lysis buffer (eBioscience), were stained with anti B220-EF450, CD11b-AF488, CD115-PE, GR1-AF649, CD4/CD8a-APC-750 (eBioscience). Leukocytes from GFP animals were stained with CD11b-PE but not CD11b-AF488 or CD115-PE. B220⁺ and CD4/CD8a⁺ cells were used to identify T and B cell populations which were excluded from the analysis of GR1⁺ and CD11b⁺ cells by electronic gating. Granulocytes (neutrophils) were defined as GR1 hi, CD11b⁺ cells and monocytes were defined as CD11b⁺ cells outside of the granulocyte population. Subpopulations of leukocytes were expressed as a percentage of the total number of leukocytes.

**Leukostasis**

Leukocytes adherent to retinal vessel walls after perfusion were labeled with FITC-Concanavalin A (Vector) and counted (Kern et al. 2007; Zheng et al. 2007a; Zheng et al. 2007c).

**Immunohistochemistry of the retina**

Retinas perfused with Concanavalin-A were fixed (PFA, 0.03% Triton-X100, 4 hours), washed (PBS), and blocked (2x PBS, 0.03% Triton-X100, 20% goat serum, 5% BSA) (Chan-Ling 1997). Flat mounted retina were stained using anti Collagen-IV(Abcam), Concanavalin-A(Vector), CD45 (Calbiochem), IBA-1(Wako), GFP(Rockland), Nimph-
R14 (kindly provided by Dr. E. Pearlman Case Western Reserve University, Cleveland, OH), 7/4(AbD-Serotech), MHCII-IA/IE, CD11b, B220, CD4/CD8a (eBioscience), PE(Rockland), Rabbit (Jackson-ImmunoResearch), rat-IgG2b and rat-IgG2a (AbD-Serotech). Secondary antibodies goat anti-PE, Rat-IgG2b and Rat-IgG2a were labeled with Dylight 594, 649, and 750 dyes respectively (Thermo Scientific) according to manufacturer’s instructions.

**Measurement of cytokine and inflammation association proteins by mRNA**

mRNA was extracted from retina using RNasyPlus Mini kits (Qiagen). cDNA was generated with superscript-III first-strand system (Invitrogen) followed by RT-qPCR (Applied Biosystems) using the following primers:

TCT-TTG-ACG-CTC-GGA-ACT-GTA-GCA iNOS-F1,

TAG-GTC-GAT-GCA-CAA-CTG-GGT-GAA iNOS-R1

CAT-CTT-CTC-AAA-ATT-CGA-GTG-ACA-A TNFα-F1,

TGG-GAG-TAG-ACA-AGG-TAC-CC TNFα-R1,

CAC-AGC-CTA-CCA-AAA-CAG-CCA COX2-F1,

GCT-CAG-TTG-AAC-GCC-TTT-TGA COX2-R1,

ACT-CAA-CAC-GGG-AAA-CCT-CAC-C 18S-F1,

CCA-GAC-AAA-TCG-CTC-CAC-C 18S-R1,

GCC-TTG-GTA-GAG-AGT-GAG ICAM1-F1

GAC-CGG-AGC-TGA-AAA-GTT-GTA ICAM1-R1
In vitro co-culture assay of leukocyte mediated endothelial cell death.

Mouse retinal endothelial cells (mREC; generated from Immortomice) (Su et al. 2003) were grown in DMEM containing 10% FBS and 5.5 or 25mM glucose. The media was changed every other day for 5 days. When mRECs were 80% confluent (500,000 cells), leukocytes (100,000; purified from blood or bone marrow with RBC lysis buffer) were added to the mREC and incubated for 24 hrs. Leukocytes (bone marrow) in some groups were pre-incubated with 1mM alpha lipoic acid or 1μg of anti-FasL (BD) for 1 hr. After 24 hours, mREC were gently rinsed with PBS to remove leukocytes, incubated with trypsin for 2 minutes, and washed twice in PBS. Viability of mREC was measured by trypan blue exclusion with a hemocytometer. Sample identity was masked during counting.

Leukocytes, isolated from EDTA anticoagulated peripheral blood (RBC lysis buffer), were depleted of specific subsets using anti Ly6G or CD115-PE (eBiosciences) and anti-PE magnetic beads with an Automax system (Miltenyi). Aliquots of the leukocyte preparation before and after immunodepletion were used in the co-culture assay described above. Depletion was verified via flow cytometry (LSRII BD) using anti B220-450, CD11b-488, CD115-PE, GR1-649, CD4/CD8a-APC/750 (eBioscience). B220+ and CD4/CD8a+ cells were excluded from the analysis of GR1+ and CD11b+ cells.

Measurement of superoxide generation
Leukocytes, mREC (after co-culture with leukocytes), or retinas from perfused mice were incubated in Krebs-Hepes buffer (with 5 or 25mM glucose) (25 min, 37°C, 5%CO₂). Luminescence was measured 5 minutes after the addition of 0.5mM lucigenin as previously described (Du et al. 2003; Zheng et al. 2007a). mREC luminescence values were normalized to the value of mREC (high glucose, no leukocytes) controls for each assay.

Histopathology of the retina
Degenerate capillaries and pericyte loss (“pericyte ghosts”) were quantified in 6-8 fields in the mid-retina (×400 magnification) of fixed, trypsinized retina as previously reported (Bresnick et al. 1976a; Kern and Mohr 2007).

Retinal thickness and number of cells in the ganglion cell layer were measured in formalin fixed, paraffin-embedded eyes sectioned at the optic nerve with light microscopy (Zheng et al. 2007a).

Leukocyte iNOS and CD18 Expression
Peripheral blood from carbon dioxide anesthetized mice was fixed (20 min, 2% PFA on ice), stained with B220-450, CD11b-PE, GR1-649, CD4/CD8a-APC/750 (eBioscience), rabbit anti-iNOS (M-19/N-20 SantaCruz), anti-rabbit-488 (Jackson-Immunoresearch), and analyzed via flow cytometry (Auffray et al. 2009). CD18 surface expression was similarly evaluated using CD11b-488 and CD18-PE (eBioscience).
Tactile allodynia.

Allodynia was measured using previously reported techniques (Calcutt et al. 1996; Berti-Mattera et al. 2008). Data is reported as the pressure at which the 50% paw withdrawal threshold for each animal in response to Von Frey filaments (Stoelting) of logarithmically increasing stiffness.

Statistics

Groups were compared using ANOVA followed by Fisher post-hoc test to generate p values. Error bars in graphs represent ±1 SD. Sample sizes are indicated in the figure legends or on the figure. Due to differences in donor background strains, PARP1−/−→WT and WT→WT chimeras were not compared.

2.3 Results

Evaluation of diabetes

Experimental induction of diabetes using streptozotocin resulted in a statistically significant (p<0.05) elevation of blood glucose (335±44 mg/dl vs. 124±9 for non-diabetic controls) and glycated hemoglobin (11.4±0.7% vs. 2.9±0.2 for non-diabetic controls). None of the diabetic groups varied significantly from the diabetic controls with respect to these measurements.

Evaluation of marrow grafts in GFP, iNOS, and PARP1 chimeric mice
Ten weeks after the marrow transplantation, >94% of the white blood cells in the chimeric GFP, iNOS, or PARP1 animals came from the marrow donor, based on PCR measurements of iNOS or PARP1 DNA or counts of GFP-positive cells in GFP→WT animals. There were no significant differences among diabetic groups with regard to hematocrit (41%±0.8) or circulating white blood cell count (3.6 ±0.4 million cells/ml). All chimeras were found to be healthy except for the WT→PARP1−/−, which died within 2 months of irradiation. PARP1 is important in DNA repair, and therefore we assume that irradiation of PARP1−/− mice caused severe damage to DNA, resulting in reduced life expectancy.

*Molecular processes regulated by iNOS or PARP1 within marrow-derived cells are major causes of the degeneration of retinal capillaries in diabetic mice*

Thirty weeks after the onset of diabetes, retinas from diabetic animals (both non-irradiated WT and WT→WT) developed the expected significant increases in degenerate (acellular) capillaries (3.1 and 2.5 fold of non-diabetic values respectively), (Figure 2-1A-B, p. 135) and pericyte loss (Figure 2-1C, p. 135). These abnormalities are characteristic lesions of the early stages of DR (Addison et al. 1970; Engerman and Kern 1993; Engerman and Kern 1995; Joussen et al. 2001; Tang et al. 2003; Joussen et al. 2004; Rota et al. 2004; Zheng et al. 2004). In contrast to WT and WT→WT diabetic animals, development of degenerate capillaries was significantly inhibited in diabetic iNOS−/−→WT chimeras (reduced to 1.3 fold of non-diabetic). Vascular pathology in diabetic WT→iNOS−/− (reverse) chimeras was similar to the diabetic WT→WT controls (2.2 fold of non-diabetic), indicating that capillary degeneration was more dependent on iNOS in
marrow-derived cells than in cells of the neural retina (endothelium, pericytes, glia, and neural cells). Capillary degeneration in diabetic PARP1−/−→WT animals likewise was only slightly (although significantly; 1.3 fold) greater than the non-diabetic PARP1−/−→WT animals indicating that a lack of PARP1 in marrow-derived cells is sufficient to inhibit the diabetes-induced capillary degeneration in the retina. This interpretation must be tempered by the recognition that the c57Bl/6 strain is not a perfect control for the 129S strain from which the PARP1 was deleted (see Discussion section). No significant differences in retinal capillary loss were found between non-diabetic chimeras or between non-diabetic irradiated and non-irradiated controls, indicating that the irradiation process did not increase capillary loss.

The number of capillary pericyte ghosts was not significantly different between diabetic controls and diabetic chimeras lacking iNOS or PARP-1 (Figure 2-1C, p. 135), however an unexplained variability in pericyte loss in some groups of non-diabetic chimeras confounded the ability to assess the effect of diabetes in the various groups.

30 weeks of diabetes did not significantly alter either retinal thickness or the number of cells in the Ganglion Cell Layer (Table 2-1, p. 132) in the WT C57Bl/6 mice, so the effect of diabetes on these parameters in the chimeras was not assessed.

**Effect of iNOS and PARP1 in marrow-derived cells on superoxide generation in diabetes**

It has been previously reported (Du et al. 2003; Kern et al. 2007; Gubitosi-Klug et al. 2008) that the generation of superoxide by retina is increased in WT diabetic animals,
and the ability of therapies to inhibit this increase predicted whether or not the therapy would inhibit diabetes-induced capillary degeneration (Kern et al. 2007; Zheng et al. 2007a; Gubitosi-Klug et al. 2008). To differentiate superoxide generation by retinal cells from that of blood cells in the blood vessels, blood was removed by perfusion in chimeric mice (2 months diabetic). The number of residual leukocytes following perfusion (<20 per retina as per Figure 2-4B, p. 141) is not sufficient to account for the increase in retinal superoxide, because ex vivo measurement of superoxide with 100-fold more leukocytes (isolated from diabetic mice) yielded less than 1% of the superoxide measured in the retina. The diabetes-induced increase in superoxide generation observed in retinae collected from perfused non-irradiated WT or WT→WT animals was significantly less than that in retinas collected from perfused iNOS−/−→WT or PARP1−/−→WT chimeras, but not in WT→iNOS−/− chimeras (Figure 2-2A, p. 137). Similarly, diabetes-induced superoxide production by leukocytes (bone marrow) was significantly increased in WT controls (3.5 fold above non-diabetic value) and in WT→iNOS−/− (3.9 fold) (all p<0.05), but was inhibited in iNOS−/−→WT or PARP1−/−→WT chimeras (both 1.2 fold, not significant).

To investigate if marrow-derived cells from diabetic animals might increase superoxide in capillary cells, mREC were co-cultured with peripheral blood leukocytes from non-diabetic or diabetic mice. mREC co-cultured with leukocytes from diabetic WT animals generated more superoxide than either mREC co-cultured with leukocytes from non-diabetic WT animals or mREC grown in either high or low glucose media without leukocytes (Figure 2-2B, p. 137). mREC incubated with leukocytes from diabetic iNOS−/−
WT animals in contrast did not exhibit significant increases in mREC superoxide production compared to mREC incubated with leukocytes from diabetic WT mice. We conclude that much of the diabetes-induced generation of superoxide measured in isolated retinas required involvement of marrow-derived cells.

Effect of iNOS and PARP1 in marrow-derived cells on diabetes-induced retinal markers of inflammation

mRNA for iNOS, cyclooxygenase-2 (COX2), tumor necrosis factor alpha (TNFα), and intercellular adhesion molecule-1 (ICAM1) (Figure 2-3, p. 139) and leukostasis (Figure 2-4A, p. 141) were significantly increased at two months of diabetes in retinas from perfused WT mice, and these increases were significantly inhibited in diabetic iNOS−/− →WT and PARP1−/− →WT chimeras. The diabetes-induced increase in leukostasis was not significantly inhibited in WT →iNOS−/− reverse chimeras, demonstrating that iNOS in marrow-derived cells had more influence on leukostasis than did iNOS in cells of the retina. There was a slight but significant increase in leukostasis caused by irradiation (non-irradiated non-diabetic verses WT →WT non-diabetic animals; Figure 2-4A, p. 141), which was observed in all non-diabetic chimeras (no significant change in leukostasis between irradiated non-diabetic chimeras and the non-diabetic WT →WT control). Shielding the head during irradiation did not alter diabetes-induced retinal leukostasis compared to unshielded diabetic animals (Figure 2-4B, p. 141). Nevertheless, the deletion of iNOS or PARP from marrow-derived cells significantly inhibited diabetes-induced leukostasis compared to the similarly irradiated WT →WT diabetic animals, indicating
that the inhibition of diabetes-induced leukostasis in iNOS- and PARP-deficient leukocytes was not due merely to irradiation.

*Leukocyte infiltration of the retina in diabetes*

Having determined that myeloid-derived cells were involved in the pathogenesis of vascular lesions of the retina in diabetes, we next sought to assess if the retina might be damaged by a classical model of leukocyte-mediated infiltration and inflammation. We investigated if cells migrated out of the vasculature into neural retina using immunohistochemical techniques on flat-mounted retinas from both WT and GFP\(\rightarrow\)WT chimeric mice at 10 and 30 weeks of diabetes. GFP\(^+\) cells were not observed to incorporate into the vasculature (as endothelial cells or pericytes). Outside of the vasculature of diabetic mice, we identified ramified IBA-1\(^+\) microglia and perivascular macrophages (Figure 2-5 A, p. 143) and a small number of CD45\(^+\) cells, the majority of which were co-stained with Concanavalin-A, suggesting emigration post profusion. The apparent absence of leukocytes in the neural retina of diabetic mice was not due to technical problems, as we were able to detect 7/4\(^+\)/MHCII\(^-\) (a putative neutrophil population) and 7/4\(^+\)/MHCII\(^+\) (a putative monocyte population) leukocytes within the lumen of retinal blood vessels (Figure 2-5 B-C, p. 143).

*Effect of microglia in diabetes.*

Myeloid-derived microglia are known to migrate into the retina even in non-diabetic animals (Xu et al. 2007). Microglia populate the inner plexiform layer (INL) and outer plexiform layer (OPL) of the retina in the mouse and are reported to change shape upon
activation from a ramified form with numerous thin extensions to a rounded form with short infrequent extensions. The shape of the microglia in the INL was not obviously different between diabetic and non-diabetic GFP\(\rightarrow\)WT chimeric mice at 30 weeks of diabetes (Figure 2-6 p. 145). Microglia in the OPL of diabetic GFP\(\rightarrow\)WT chimeric mice were perhaps less ramified but did not appear to be rounded compared to non-diabetic controls (Figure 2-7 p. 147). CD45\(^+\) (red) GFP\(^+\) (green) microglia were evident in the IPL and OPL of the retina of both non-diabetic and diabetic GFP\(\rightarrow\)WT chimeric mice at 30 weeks but few CD45\(^+\) (red) GFP\(^+\) (green) microglia (10-20) were present compared to resident CD45\(^+\) GFP\(^-\) (red only) at 10 weeks.

The total number of microglia, the number of resident microglia (GFP\(^-\)), or the number of new microglia (GFP\(^+\)) in the INL was not significantly different between diabetic and non-diabetic GFP\(\rightarrow\)WT chimeric mice (Figure 2-8 A, p. 149). In contrast the number of resident microglia but not the new microglia in the ONL was significantly decreased (45\% p<0.05, excluding values at the periphery) in diabetic GFP\(\rightarrow\)WT chimeric mice compared to non-diabetic controls. The total number of microglia in the ONL was likewise decreased in diabetic mice but was only significantly different from non-diabetic controls in the center half of the retina. We conclude that the majority of microglia lost in the ONL are WT resident microglia, which were present in the retina prior to the bone-marrow transplant.

To differentiate the effect of irradiation on microglia loss from the effect of diabetes on microglia loss we compared the relative number of microglia in non-diabetic and diabetic
GFP→WT chimeric mice with diabetic and non-diabetic WT, non-irradiated mice at 8 weeks of diabetes. Similar to our previous results at 10 weeks of diabetes, there were very few GFP+ microglia present in the retina of the chimeric animals at 8 weeks and therefore they were not counted separately. Irradiation significantly diminished the number of IBA-1+ microglia in the outer (but not inner) plexiform layer by 42% (p<0.05) in both diabetic and non-diabetic GFP→WT mice compared to non-irradiated controls (Figure 2-9, p. 151). No significant difference in the number microglia was present in diabetic mice compared to non-diabetic controls within the respective non-irradiated and GFP→WT (irradiated) groups. Irradiation therefore significantly decreases the number of resident microglia in the OPL, and the resident microglia likely continue to decrease with diabetes despite similar rates of new microglia entering the OPL in both diabetic and non-diabetic mice irradiated mice (Figure 2-10, p. 153).

Diabetic irradiated WT→WT chimeric mice do not develop significantly more or fewer acellular capillaries or pericyte ghosts (Figure 2-1A and C, p. 135) or generate more or less retinal superoxide (Figure 2-2A, p. 137) compared to diabetic non-irradiated WT animals. We interpret this data as indicating that it is unlikely that the microglia in the retina contribute appreciably to the diabetes-induced vascular injury, since a reduction in their number did not similarly reduce or increase vascular pathology. Therefore, the retinal vascular damage in diabetes is likely due to the marrow-derived cells remaining within the blood vessels.

*Mechanism of leukocyte-mediated retinal injury in diabetes*
To investigate how capillary cells might be injured by marrow-derived cells from diabetic animals, mREC were incubated with purified leukocytes (from bone marrow) from non-diabetic or diabetic mice. After 24 hr co-culture, the number of dead mREC was significantly increased by leukocytes from WT diabetic mice compared to non-diabetic WT mice (independent of whether the endothelial cells had been incubated in 5mM or 30mM glucose) (Figure 2-11, p. 155). Since leukocytes might damage vascular endothelium via direct contact (such as via the Fas/FasL system (Joussen et al. 2003; Lee and Ferguson 2003)) or release of soluble mediators such as superoxide (Lentsch and Ward 2000), we examined both possibilities. The increase in endothelial death during co-incubation with leukocytes from diabetic animals was significantly inhibited by addition of the antioxidant, lipoic acid, or a blocking antibody against FasL during the incubation. Co-culture of mREC with leukocytes from diabetic animals lacking iNOS resulted in fewer dead endothelial cells than did leukocytes from WT diabetic animals, and neither lipoic acid nor anti-FasL treatment had any additional benefit with iNOS$^{-/-}$ leukocytes. We conclude that marrow-derived cells in diabetes can kill endothelial cells by release of soluble factors as well as contact-mediated routes, and deletion of iNOS from leukocytes inhibits these processes.

Since leukocyte-mediated death of mREC was reduced in the absence of iNOS, we determined which subsets of peripheral blood leukocytes upregulate iNOS in diabetes. Flow-cytometry demonstrated that 4.02±2.35% of neutrophils (GR-1$^{\text{high}}$, CD11b$^{+}$, b220$^{-}$, CD4$^{-}$,CD8a$^{-}$) and 3.46±2.67% of monocytes (GR-1$^{\text{low}}$, CD11b$^{+}$, b220$^{-}$, CD4$^{-}$, CD8a$^{-}$) cells had elevated levels of iNOS in blood from mice diabetic for 3 months, compared to
0.66±0.47 and 0.58±0.64%, respectively, in non-diabetic mice (p<0.015, and 0.05) (Figure 2-12, p. 157). Thus, iNOS-mediated death of retinal endothelial cells is caused by only a small subset of leukocytes.

**Evaluation of leukocyte subpopulations on vasculature damage in diabetes**

To determine which subpopulation of marrow-derived cells was contributing to endothelial death, Ly6G+ or CD115+ leukocytes were removed by immunodepletion from the peripheral blood of WT diabetic and non-diabetic mice (Niedermeier et al. 2009), and removal was confirmed by flow-cytometry (Figure 2-13, p. 159, and Table 2-2, p. 133). Ly6G and CD115 are regarded as markers of cells from granulocyte and monocytes lineages, respectively. Depletion of either Ly6G+ or CD115+ cells from the leukocytes of diabetic animals reduced endothelial death in co-culture experiments by 44±11% and 50±15%, respectively (Figure 2-14A, p. 161) compared to a non-immunodepleted aliquot of the same sample. This data is consistent with an interpretation that both neutrophils and monocytes contribute to the endothelial death in these co-cultures, but since the CD115 immunodepletion also depleted some granulocytes (Table 2-2), the role of monocytes in the endothelial death cannot be unequivocally established from this data.

The causal role of neutrophils in the vascular histopathology of DR was confirmed in long-term in vivo experiments. Granulocyte Colony Stimulating Factor (G-CSF) controls maturation of myeloid precursor cells into granulocytes, and severe neutropenia develops in its absence (Liu et al. 1996). Chimeric mice, which lacked G-CSFR only in their marrow-derived cells (G-CSFR−/→WT), were generated, and effects on diabetes-induced
degeneration of retinal capillaries assessed at 10 months of diabetes. Granulocytes were reduced from 18% to 1.6% in the G-CSFR<sup>−/−</sup>→WT chimeras (Figure 2-13 p.159 and Table 2-2 p. 133). Diabetes-induced capillary degeneration was significantly inhibited in G-CSFR<sup>−/−</sup>→WT chimeras (Figure 2-14B, p. 161). Co-culture of leukocytes from diabetic G-CSFR<sup>−/−</sup> animals with mREC likewise resulted in significantly less endothelial death than that detected after incubation with leukocytes from WT diabetic animals (Figure 2-14A, p. 161). Efforts to generate chimeras in which marrow cells were deficient in MCSF-R (deficient in cells of the monocyte lineage (Dai et al. 2002)) were not successful in long term studies, because monocytes (CD115<sup>+</sup>, CD11b<sup>+</sup>, GR-1<sup>low</sup> cells) unexpectedly repopulated the marrow in diabetic animals, but not in non-diabetic controls (not shown).

Effect of iNOS and PARP1 on tactile allodynia

As reported previously (Calcutt et al. 1996; Calcutt 2004), WT diabetic rodents develop a hypersensitivity to light touch compared to non-diabetic controls. We show that pro-inflammatory processes in leukocytes are required for the development of diabetes-induced tactile allodynia. Tactile allodynia developed in both naïve WT and WT→WT diabetic animals but not in diabetic iNOS<sup>−/−</sup>→WT or PARP1<sup>−/−</sup>→WT chimeras) (Figure 2-15, p. 163). Thus, myeloid-derived cells are major contributors to the dysfunction of peripheral tactile allodynia in diabetic mice, and these processes are regulated at least in part by iNOS or PARP1 within the myeloid cells.
2.4 Discussion

Our current results demonstrate that molecular processes regulated by iNOS or PARP1 in bone marrow-derived cells are critical to the development of diabetes-induced retinopathy and allodynia. Since iNOS and PARP1 both have pro-inflammatory actions in diabetes and other diseases (Virag and Szabo 2002; Tinker and Wallace 2006; Kern 2007), the present study suggests that pro-inflammatory processes in marrow-derived cells play critical roles in the capillary degeneration and other lesions of the early stages of diabetic retinopathy and allodynia.

While marrow from iNOS−/− animals was transplanted into the recommended background strain, the PARP1−/− bone marrow was obtained from mice derived on a 129S background, so we cannot exclude the possibility that the presence of 129S marrow in a C57Bl/6 host inhibited by a different mechanism the diabetes-induced retinal inflammation otherwise observed in WT→WT diabetic animals. Nevertheless, both 129S and C57Bl/6 are MHC b haplotype, and neither have any known immunodeficiencies, which would confound interpretation of histopathology in the present study. The systemic inhibition of PARP1 with PJ34 (Zheng et al. 2004) has been shown to inhibit diabetes-induced retinal inflammatory-like processes and vascular loss, so we conclude that beneficial effects reported herein for PARP1−/−→WT chimeras are most likely due to the lack of PARP1, and not a genetic difference between C57Bl/6 and 129S strains.

Oxidative stress has been implicated in the development of DR (Du et al. 2003; Kern et al. 2007; Gubitosi-Klug et al. 2008), and systemic inhibition of that stress has inhibited
the early stages of DR (Kern et al. 2007; Zheng et al. 2007a; Gubitosi-Klug et al. 2008). While several different types of cells found in the retina have been found to produce increased superoxide in hyperglycemia or diabetes, we demonstrate that the co-culture of leukocytes from diabetic animals with mREC results in a significantly higher superoxide output from mREC than that observed due to high glucose media alone (no leukocytes), leukocytes from non-diabetic animals or leukocytes from iNOS\textsuperscript{-/-}→WT diabetic animals.

White blood cells are known to provide products to retinal endothelial cells by transcellular delivery (Talahalli et al. 2010), resulting in production of pro-inflammatory/toxic products by retinal cells (which we speculate includes superoxide).

We did not observe an effect of diabetes (at 10 or 30 weeks duration) on leukocyte emigration into the neural retina. Whether emigration occurs earlier in diabetes is unclear. We speculate that classical transmigration of leukocytes from the vasculature into the neural retina is not a necessary part of the mechanism by which leukocytes mediate diabetes-induced degeneration of capillaries.

Adherence of leukocytes to the luminal walls of the retinal vasculature clearly is increased in diabetes unless either iNOS or PARP1 is absent from marrow-derived cells, but it has not been clear what role, if any, adherence might play with respect to damage of the retinal tissue. Leukostasis might contribute to retinal damage through vessel occlusion resulting in hypoxia, activation of endothelial cells through ICAM1 dimerization, or simply as a tether during transcellular substrate delivery. However, some
reports (Gubitosi-Klug et al. 2008; Kern et al. 2010) have dissociated leukostasis from diabetes-induced degeneration of retinal capillaries.

Leukostasis requires “activation” of leukocytes and endothelial cells. It has been previously reported that iNOS−/− leukocytes challenged with LPS exhibit increased binding (Hickey et al. 2001). Our studies show that CD18 is expressed on the surface of leukocytes in the iNOS−/− chimeras (Table 2-3, p. 134). Diabetes-induced increase in ICAM-1 mRNA (marker of endothelial activation) is inhibited in retinas of iNOS→WT or PARP-1→WT chimeras (Figure 2-3, p. 139). Similarly, the diabetes-induced increase in leukocyte superoxide generation (leukocyte activation) is inhibited in leukocytes from iNOS→WT or PARP-1→WT chimeras. We conclude that diabetes is sufficient to activate both endothelial cells and leukocytes in the retina of WT and WT→WT mice but not in iNOS−/−→WT or PARP-1−/−→WT mice, and the result is that leukostasis is inhibited in the chimeras.

Additional mechanisms by which leukocytes might kill retinal endothelial cells include the FasL/Fas system, and the release of reactive oxidant species. We provide evidence that these mechanisms do contribute to leukocyte-mediated endothelial cell death in co-culture, and these processes are inhibited when iNOS is absent from marrow-derived cells only.

Diabetes increased mRNA of pro-inflammatory molecules (TNFα, iNOS, and ICAM1) in the retina of WT mice, but not in the retina of iNOS−/−→WT and PARP1−/−→WT
chimeras. These results, combined with the diabetes-induced increases in retinal superoxide, suggest that marrow-derived cells from diabetic animals induce inflammation in the neural retina without migrating out of the blood vessels into the neural retina. Nevertheless, this inflammation did not cause a reduction in the number of ganglion cells in the neural retina. While co-cultures of mREC incubated with leukocytes demonstrate that leukocytes from diabetic animals can kill endothelial cells and induce endothelial superoxide generation under those in vitro conditions, it is not clear in vivo if the toxic effects of leukocytes on endothelial cells are direct (ie Fas/FasL or transcellular delivery of toxic products such as superoxide) or indirect.

G-CSF controls maturation of myeloid precursor cells into granulocytes, notably neutrophils. Reduced susceptibility of the G-CSFR−/− chimera to develop diabetes-induced vascular lesions of the retina provides strong evidence that neutrophils (and perhaps monocytes) play critical roles in development of the early stages of DR Both neutrophils and monocytes adherent to the perfused retinal vasculature were observed in diabetic WT and GFP→WT mice. Monocyte function has been reported to be impaired in G-CSFR deficient animals, thus leading to ambiguity about the role of cells in the monocyte lineage in the pathogenesis of the retinopathy (Gregory et al. 2010). The conclusion that neutrophils and perhaps monocytes damage the retinal vasculature in diabetes is further supported by in vitro studies in which the ability of leukocyte subtypes from diabetic animals to kill retinal endothelial cells was assessed. Interestingly only a subset of neutrophils and monocytes show the diabetes-induced increase in iNOS expression,
suggesting that the vascular pathology might only be caused by a small fraction of these cells.

Marrow-derived cells apparently also contribute to the development of diabetic complications in other tissues. Microglia have been implicated in diabetes-induced allodynia in diabetic mice (Tsuda et al. 2008). We extend this by showing that deletion of either iNOS or PARP1 only from marrow-derived cells inhibits the development of this diabetes-induced tactile allodynia. Our evidence that the neural dysfunction is secondary to the activity of these enzymes in myeloid-derived cells has been previously unrecognized. It remains unclear if marrow-derived cells other than microglia are involved in diabetes-induced tactile allodynia.

Future investigations on the pathogenesis of diabetic complications need to expand beyond the traditional tissue- or vascular-specific view of complications, to also include bone marrow-derived cells. It is not yet clear what activates the inflammatory-like processes in diabetes, but inflammatory processes in marrow-derived cells offer a new therapeutic target to inhibit the development of diabetic complications.
2.5 Tables and figures

Table 2-1. Effect of marrow-derived cells on diabetes induced changes in retinal thickness and ganglion cell count.

The thickness of retina and number of cells in the ganglion cell layer of WT C57BL/6 mice were not significantly altered after 30 weeks of diabetes in the posterior retina (adjacent to the optic nerve) and at the mid-retina (halfway from the optic nerve to the periphery) on both sides of the disc. Analyses of additional animal groups were therefore not done (n = 6).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>phenotype</th>
<th>Location</th>
<th>Retinal thickness</th>
<th>Ganglion cells / 100 um</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT C57Bl/6</td>
<td>ND</td>
<td>Mid-retina</td>
<td>165.0 ± 8.7 µm</td>
<td>11.2 ± 1.2</td>
</tr>
<tr>
<td>WT C57Bl/6</td>
<td>SD</td>
<td>Mid-retina</td>
<td>157.0 ± 9.1 µm</td>
<td>11.1 ± 1.1</td>
</tr>
<tr>
<td>WT C57Bl/6</td>
<td>ND</td>
<td>Posterior retina</td>
<td>175.0 ± 10.9 µm</td>
<td>10.6 ± 1.1</td>
</tr>
<tr>
<td>WT C57Bl/6</td>
<td>SD</td>
<td>Posterior retina</td>
<td>167.4 ±7.9 µm</td>
<td>11.1 ± 1.4</td>
</tr>
</tbody>
</table>
Table 2-2. Leukocyte subtype population.

Leukocyte subtype population before and after immune-depletion of Ly6G and CD115 positive cells in leukocytes isolated from diabetic and non-diabetic WT and in diabetic and non-diabetic G-CSFR\textsuperscript{−/−} \(\rightarrow\) WT chimeric mice. (# and @ denote cell populations that were significantly different from WT controls p<0.05 in both \textit{in vitro} depletions and \textit{in vivo} chimeric mice) \(n\geq3\).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>depletion</th>
<th>phenotype</th>
<th>granulocytes</th>
<th>monocytes</th>
<th>lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Ly6G</td>
<td>SD</td>
<td>2.3±1.9% #</td>
<td>5.3±1.2%</td>
<td>86.2±7%</td>
</tr>
<tr>
<td>WT</td>
<td>Ly6G</td>
<td>ND</td>
<td>1.5±0.0% #</td>
<td>4.2±0.5%</td>
<td>81.2±8.1%</td>
</tr>
<tr>
<td>WT</td>
<td>CD115</td>
<td>SD</td>
<td>14.3±4.7%</td>
<td>0.1±0.0% @</td>
<td>82.3±6.5%</td>
</tr>
<tr>
<td>WT</td>
<td>CD115</td>
<td>ND</td>
<td>24.0±5.8%</td>
<td>0.1±0.0% @</td>
<td>72.9±7.1%</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>SD</td>
<td>22.3±11.6%</td>
<td>9.3±2%</td>
<td>68.5±13.1%</td>
</tr>
<tr>
<td>WT</td>
<td>none</td>
<td>ND</td>
<td>18.8±9.7%</td>
<td>4.0±1.2%</td>
<td>84.8±1.5%</td>
</tr>
<tr>
<td>G-CSFR\textsuperscript{−/−} (\rightarrow) WT</td>
<td>none</td>
<td>SD</td>
<td>1.7±1% #</td>
<td>4.4±0.7%</td>
<td>87.0±3.5%</td>
</tr>
<tr>
<td>G-CSFR\textsuperscript{−/−} (\rightarrow) WT</td>
<td>none</td>
<td>ND</td>
<td>1.6±1% #</td>
<td>3.7±1.2%</td>
<td>92.2±1.7%</td>
</tr>
</tbody>
</table>
Table 2-3. Leukocyte surface expression of CD18 in fixed whole blood (n=2).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Marker</th>
<th>phenotype</th>
<th>granulocytes</th>
<th>monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>CD18</td>
<td>ND</td>
<td>31±1%</td>
<td>60±1%</td>
</tr>
<tr>
<td>WT</td>
<td>CD18</td>
<td>SD</td>
<td>36±2%</td>
<td>65±1.3%</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>CD18</td>
<td>ND</td>
<td>23±2%</td>
<td>60±1.3%</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>CD18</td>
<td>SD</td>
<td>32±1%</td>
<td>65±1.7%</td>
</tr>
</tbody>
</table>
Figure 2-1. Diabetes-induced retinal vascular pathology is inhibited in chimeric mice lacking either iNOS (iNOS−/−→WT) or PARP1 (PARP1−/−→WT) in bone marrow-derived cells only.

A. Degeneration of retinal capillaries is significantly inhibited in iNOS−/−→WT or PARP1−/−→WT mice, but not in “reverse” chimeras (lacking these enzymes throughout the body except marrow-derived cells; WT→iNOS−/−; WT→PARP1−/− was lethal). No significant differences in retinal capillary loss were found between non-diabetic chimeras or between non-diabetic irradiated and non-irradiated controls. Diabetes duration was 30 weeks. Non-diabetic (white bars); diabetic (black bars) (n≥6)

B. Degenerate capillaries (thick arrow) and rare pericyte “ghosts” (thin arrows) in the retinal vasculature.

C. The number of capillary pericyte ghosts was not significantly different between diabetic controls and diabetic chimeras lacking iNOS or PARP-1, however an unexplained variability in pericyte loss in some groups of non-diabetic chimeras confounded the ability to assess the effect of diabetes in the various groups. non-diabetic (white bars) and diabetic (black bars) (n≥5)
A

Degenerate capillaries (per mm² retina)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degenerate Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-irradiated</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>WT→WT</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>WT→iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>PARP1&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Significance:
- p<0.001
- ns

B

Degenerate capillaries (per mm² retina)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degenerate Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-irradiated</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>WT→WT</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>WT→iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>PARP1&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Significance:
- p<0.01
- ns

C

Degenerate capillaries (per mm² retina)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degenerate Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-irradiated</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>WT→WT</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>WT→iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>PARP1&lt;sup&gt;−/−&lt;/sup&gt;→WT</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Significance:
- p<0.01
- ns
Figure 2-2. The diabetes- or glucose-induced increase in superoxide production is significantly inhibited in chimeric mice lacking either iNOS or PARP1 in marrow-derived cells only (iNOS\(^{-/-}\)→WT) or (PARP1\(^{-/-}\)→WT) but not in chimeric mice lacking iNOS in all cells except bone marrow (reverse chimera, WT→iNOS\(^{-/-}\)).

A. Retinal superoxide generation. Non-diabetic (white bars); diabetic (black bars) Duration of diabetes was 8-10 weeks. n as indicated below the graph.

B. mREC co-cultured with leukocytes from diabetic animals generated more superoxide than mREC co-cultured with leukocytes from non-diabetic mice or mREC in high glucose culture media. This leukocyte-induced enhancement of mREC superoxide generation is inhibited if the leukocytes are isolated from diabetic mice deficient in iNOS. (Data was normalized to superoxide production of mREC in high glucose, n = 5). mREC cultured in normal glucose media (no leukocytes) (white bar with vertical stripes), mREC cultured in high glucose media (no leukocytes) (white bar with horizontal stripes), mREC co-cultured with leukocytes from non-diabetic mice (white bars), mREC co-cultured with leukocytes from diabetic mice (black bars). Duration of diabetes was 3 months.
A

![Graph showing retinal superoxide generation.](image)

- non-irradiated
- WT→WT
- iNOS\(^{-/-}\)→WT
- WT→iNOS\(^{-/-}\)
- PARP1\(^{-/-}\)→WT

- Culture Media: (mM glucose) 5 25
- Leukocyte donor: --- ---
- Mouse genotype: --- ---

B

![Graph showing endothelial superoxide production.](image)

- Culture Media: (mM glucose) 5 25
- Leukocyte donor: ND SD
- Mouse genotype: WT WT iNOS\(^{+/-}\) iNOS\(^{-/-}\)
Figure 2-3. Diabetes-induced increases of mRNA for proteins associated with inflammation are significantly inhibited in chimeric mice lacking iNOS in marrow-derived cells only.

The increase in diabetes-induced retinal mRNAs for iNOS, tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and Intercellular adhesion molecule1 (ICAM1) are inhibited (compared to non-diabetic controls) in animals lacking iNOS in bone marrow-derived cells only. Diabetic chimeras lacking PARP1−/− likewise are only slightly greater than their non-diabetic controls. WT (white bar with vertical stripes), iNOS−/−→WT (white bars with diagonal stripe) mice, and PARP1−/−→WT (white bar with horizontal stripes). Duration of diabetes was 8-10 weeks. n = 4 animals per group. All values were normalized to non-diabetic values of each group. Statistical comparisons indicated by horizontal bars lacking p values are all p<0.05.
Diabetes-induced increase in mRNA (fold of non-diabetic)

iNOS

TNFα

COX-2

ICAM-1

p<0.05

p<0.05

p<0.05

p<0.05
Figure 2-4. Diabetes-induced increase in leukostasis is significantly inhibited in chimeric mice lacking iNOS in marrow-derived cells only.

A. The diabetes-induced increase in leukocytes adherent to the interior lumen of retinal blood vessels is inhibited in animals lacking either iNOS or PARP1 in bone marrow-derived cells only. No significant differences in leukostasis were observed between non-diabetic chimera groups. Non-diabetic (white bars), diabetic (black bars), (Reverse chimera mice WT→PARP1−/− animals died prior to experiment and thus were not included). Duration of diabetes was 8-10 weeks. n as indicated below the graph.

B. Shielding of the head during irradiation in chimeras (SD→SD, and ND→ND) does not change diabetes-induced markers of early pathology, at 2 months diabetes. n≥3. non-diabetic (white bars) and diabetic (black bars).
A

Leukostasis (adherent WBC / retina)

- non-irradiated
- WT → WT
- iNOS⁻/⁻ → WT
- WT → iNOS⁻/⁻
- PARP1⁻/⁻ → WT

(n) (n) (n)
(12) (12) (6)

p<0.001 p<0.01 ns

B

Leukostasis (adherent WBC / retina)

- non-irradiated
- WT → WT
- WT → WT

(p<0.001)

(shielded) (no shield)

(p<0.05 p<0.01)

(n) (n)
(6) (6)
Figure 2-5. Microglia are the only bone marrow-derived (GFP+) cells migrating into the neural retina of GFP→WT chimeras at 10 or 30 weeks of diabetes.

Microglia were defined by shape and CD45$^+$ or IBA-1$^+$ labeling. Scale bar for all pictures = 100um.

A. Inner plexiform layer of flat mounted retina from chimeric mice (GFP→WT) diabetic for 10 weeks. Microglia were stained with IBA-1 (green).

B. Inner plexiform layer of flat mounted retina from chimeric mice (GFP→WT) diabetic for 10 weeks. Neutrophil and monocyte marker 7/4$^+$ (yellow), monocyte microglia marker MHCII (red), Concanavalin A (purple). Arrows indicate neutrophils and monocytes in vasculature. (IBA$^+$ green was removed for clarity).

C. Outer plexiform layer of flat mounted retina from chimeric mice (GFP→WT) diabetic for 30 weeks. GFP$^+$ (green), CD45$^+$ (red), Neutrophil and monocyte marker 7/4$^+$ (yellow), and collagen IV (blue).
Figure 2-6. Diabetes does not change the number or shape of microglia in the inner plexiform layer of GFP→WT chimeric mice at 34 weeks of diabetes.

GFP (green) and CD45 (Red)

A. Microglia in the INL of non-diabetic GFP→WT chimeric mice

B. Microglia in the INL of diabetic GFP→WT chimeric mice
Figure 2-7. Microglia are reduced in the outer plexiform layer of GFP→WT chimeric mice at 34 weeks of diabetes.

GFP (green) and CD45 (Red)

A. Microglia in the OPL of non-diabetic GFP→WT chimeric mice

B. Microglia in the OPL of diabetic GFP→WT chimeric mice
Figure 2-8 Diabetes does not inhibit the migration of new GFP\(^+\) microglia into the retina of GFP\(\rightarrow\)WT chimeric mice at 34 weeks duration.

Microglia were counted in two rows of consecutive optical fields in flat mounted retina beginning at the periphery of the temporal side, passing through the optic nerve and ending at the periphery of the nasal side. Green bars represent (new) GFP\(^+\) CD45\(^+\) microglia which have migrated into the retina after the bone marrow transplant. Red bars represent (resident) GFP\(^-\) CD45\(^+\) microglia which were present in the retina of the WT mouse prior to bone marrow transplant. Hatched light red and light green bars represent microglia in the retina of diabetic animals. Open dark red and dark green bars represent microglia in the retina of non-diabetic animals. n>3 for all samples.

A. The number of new GFP\(^+\) (green) and resident GFP\(^-\) (red) microglia in the IPL of the retina of 34 week diabetic GFP\(\rightarrow\)WT chimeric mice are not significantly different from non-diabetic controls (p>0.1 for all groups compared)

B. The number of new GFP\(^+\) (green) microglia in the OPL of the retina of 34 week diabetic GFP\(\rightarrow\)WT chimeric mice are not significantly different from non-diabetic controls (p>0.1) however the number of resident GFP\(^-\) (red) microglia are reduced in the diabetic GFP\(\rightarrow\)WT chimeric mouse (p<0.05).
A

Temporal distance from optic nerve in um Nasal

B

Temporal distance from optic nerve in um Nasal
Figure 2-9 Irradiation but not diabetes reduces the number of resident microglia in the OPL in the retina of GFP→WT mice at 8 weeks of diabetes.

White and black bars represent microglia in the retina of non-diabetic and diabetic WT mice respectively that were not irradiated. Dark red open bars and light red hatched bars represent microglia in the retina of non-diabetic and diabetic GFP→WT chimeric mice respectively. Very few (10-20) GFP+ microglia in the retina of GFP→WT chimeric mice were present at 8 weeks post transplant and therefore they were not counted.

A. The number of resident microglia in the INL of the retina per field. p>0.1 between any of the four groups of animals.

B. The number of resident microglia in the ONL of the retina per field. Resident microglia are significantly reduced (p<0.05) in irradiated animals compared to non-irradiated animals. All other p values were > 0.1.
A

Temporal distance from optic nerve in um Nasal

Microglia /0.26 mm² retina

-2375 -1900 -1425 -950 -475 475 950 1425 1900 2375

non-diabetic WT non-irradiated
diabetic WT non-irradiated
non-diabetic GFP chimeria
diabetic GFP chimeria

B

Temporal distance from optic nerve in um Nasal

Microglia /0.26 mm² retina

-2375 -1900 -1425 -950 -475 475 950 1425 1900 2375

non-diabetic WT non-irradiated
diabetic WT non-irradiated
non-diabetic GFP chimeria
Diabetic GFP chimeria
Figure 2-10 Irradiation and diabetes reduce the number of resident microglia in the OPL of the retina at 34 weeks of diabetes.

Representative values of the number of microglia in the ONL of the retina a at point midway between the periphery and optic nerve. White and black bars represent microglia in the retina of non-diabetic and diabetic WT mice respectively that were not irradiated. Dark red open bars and light red hatched bars represent GFP− CD45+ microglia in the retina of non-diabetic and diabetic GFP→WT chimeric mice respectively. Dark green open bars and light green hatched bars represent GFP+ CD45+ microglia in the retina of non-diabetic and diabetic GFP→WT chimeric mice respectively.
Microglia /0.26 mm² retina

- Non-irradiated
- Irradiated diabetic
- Irradiated non-diabetic

- New GFP⁺
- Resident

8wks 8wks 34wks
Radiation Diabetes
Figure 2-11. Diabetes enhances the ability of leukocytes to kill mREC *in vitro*.

Leukocytes from diabetic WT animals co-cultured with mREC caused more cell death than leukocytes from non-diabetic WT mice, and this effect was independent of the glucose concentration (high or normal) in the culture media. Leukocyte mediated mREC death was inhibited by lipoic acid (1mM) or a blocking antibody against FasL (1.0μg/mL) in the co-cultures, or by using leukocytes lacking iNOS. mREC co-cultured with leukocytes from non-diabetic mice (white bars), mREC co-cultured with leukocytes from diabetic mice (black bars, n=4). Statistical comparisons indicated by horizontal bars lacking p values are all p<0.001.
<table>
<thead>
<tr>
<th>Leukocyte donor</th>
<th>ND</th>
<th>ND</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
<th>SD</th>
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<td>Mouse genotype</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>iNOS-/-</td>
<td>iNOS-/-</td>
<td>iNOS-/-</td>
</tr>
<tr>
<td>Culture Media:</td>
<td>(mM glucose)</td>
<td>5</td>
<td>25</td>
<td>5</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>in vitro treatment</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Lipoic acid</td>
<td>FasL</td>
<td>Lipoic acid</td>
<td>FasL</td>
</tr>
</tbody>
</table>

Leukocyte mediated endothelial death (% of total endothelial count)

- p<0.001
- ns
Figure 2-12 The number of neutrophils and monocytes staining positive for iNOS expression is increased in blood isolated from diabetic mice (SD) compared to non-diabetic littermates (ND).

Granulocytes and monocytes (panel 1) were selectively gated from CD8<sup>a</sup><sup>-</sup>, CD4<sup>-</sup>, B220<sup>-</sup>, GR1<sup>+</sup> and CD11b<sup>+</sup> leukocytes. 4.02 ± 2.35% of neutrophils (panel 2) and 3.46 ± 2.67% of monocytes (panel 3) have elevated levels of iNOS in leukocytes isolated from the blood of mice diabetic for 3 months, compared to 0.66 ± 0.47 and 0.58 ± 0.64% respectively in the non-diabetic mouse (all p<0.05) n≥3
Figure 2-13. Leukocyte populations in the blood of WT and chimeric mice.

Panel 1. Forward vs Side scatter plots. In figures A-D, putative granulocytes are circled. T and B cells were excluded from further analysis by gating on the population which was negative for B220, CD4, and CD8a staining (plot not shown).

Panel 2. In figures A-D Gr1\textsuperscript{high} CD11b\textsuperscript{+} granulocytes are enclosed in gate p4 and Gr1\textsuperscript{low/-} CD11b\textsuperscript{+} monocytes /macrophages are enclosed in gate p5.

Panel 3. In figures A-D, CD115\textsuperscript{+} monocytes are enclosed in gate p7.

A. Leukocyte population in diabetic (SD) WT mice prior to depletion.
B. Leukocyte population in SD WT mice after CD115\textsuperscript{+} (monocyte) depletion.
C. Leukocyte population in SD WT mice after Ly6G\textsuperscript{+} (granulocyte) depletion.
D. Leukocyte population in diabetic granulocyte colony stimulating factor receptor \textsuperscript{-/-} chimeras (granulocyte deficient) (G-CSFR\textsuperscript{-/-} \rightarrow WT).
Figure 2-14 Neutrophils are a major contributor to endothelial death and vascular degeneration in diabetes.

A. *In vitro* killing of mREC by leukocytes isolated from WT diabetic mice was significantly reduced when the leukocytes were immunodepleted of Ly6G$^+$ or CD115$^+$ cells (white bars, n= 8), or when leukocytes were isolated from diabetic G-CSFR$^{-/-}$→WT chimeras (not immunodepleted) (black bars, n= 3). The diabetes-induced increase in endothelial death due to incubation with leukocytes from each experimental group (i) was set to 100% by the formula (SD$_i$-ND$_i$)/((Avg(SD$_{WT}$)-Avg(ND$_{WT}$)).

B. Chimeric mice lacking the receptor for G-CSFR in marrow-derived cells were protected from diabetes-induced degeneration of retinal capillaries (30 weeks duration of diabetes, n= 4). Non-diabetic white bars; diabetic black bars.
Leukocyte mediated endothelial death (% of control)

A

B

Degenerate capillaries (per mm² retina)
Figure 2-15. Pressure on the hind paw required to elicit a response (tactile allodynia) is altered in diabetic mice, and this effect was inhibited in chimeric mice lacking either iNOS or PARP1 in bone marrow-derived cells only.

Reported as the pressure threshold at which the paw is withdrawn 50% of the time. Non-diabetic (white bars); diabetic (black bars), n ≥6 animals per group.
Tactile Allodynia (gms pressure)

- non-irradiated
- WT → WT
- iNOS\(^{-}\) → WT
- PARP\(^{-}\) → WT

Significance:
- \(p < 0.001\)
- ns
CHAPTER 3

ANTAGONISM OF CD11B WITH NEUTROPHIL INHIBITORY FACTOR (NIF) INHIBITS VASCULAR LESIONS IN DIABETIC RETINOPATHY

The majority of the work in this chapter about the role of NIF in the pathogenesis of diabetic retinopathy has been published in the online journal PLOS One {Veenstra, 2013 #611}.

3.1 Introduction

Diabetic retinopathy (DR) is a leading cause of blindness in working age adults (Zhang et al. 2010; Frank 2011; Ding and Wong 2012). Vascular abnormalities in the retina, including capillary non-perfusion and degeneration, and vascular leakage are detectable in the early non-proliferative stages of the retinopathy. The progressive loss of retinal blood vessels and increased vascular leakage in the non-proliferative phase of DR is believed to cause ischemia and edema, respectively, which lead to progression of the retinopathy into the clinically significant, proliferative (neovascular) phase (Bresnick et al. 1976b). Improved glycemic control has been shown to inhibit the diabetes-induced loss of retinal capillaries in animals (Engerman 1976) and the progression of DR (DCCT 1993; Klein et al. 1994), however good glycemic control has been difficult to achieve, and therefore new therapeutic approaches are needed.
Also detectible in the early stages of diabetic retinopathy in both diabetic animals (Schroder et al. 1991) and patients (McLeod et al. 1995) is an increase in the number of leukocytes adhering to retinal blood vessels (leukostasis), and an increase in the expression of retinal intercellular adhesion molecule-1 (ICAM1, CD54). Leukocytes have been implicated in the loss of retinal capillaries, but the exact mechanism for this degeneration has remained elusive (Chibber et al. 2007). Leukocytes have been shown to occlude retinal capillaries in diabetic animals (Schroder et al. 1991; Miyamoto et al. 1999). Furthermore some but not all of the occluded capillaries remain occluded by unknown mechanisms after the leukocytes leave the capillary demonstrating that temporary occlusion by leukocytes can lead to permanent occlusion and eventual capillary death (Takahashi et al. 1998; Miyamoto et al. 1999). More direct evidence for leukocyte mediated capillary loss has been demonstrated in long term diabetic mice in which only bone marrow derived cells, leukocytes, were genetically altered (Li et al. 2012).

Leukocyte functions, such as adhesion, activation, priming, and respiratory burst are mediated in part by integrins in the CD11/CD18 family. CD11b/CD18 (α₄β₂, MAC-1), an integrin on the surface of leukocytes that binds many ligands including ICAM1 (Kishimoto et al. 1989), has been shown to mediate in vitro endothelial damage and death caused by activated leukocytes (Lum et al. 1994). In vivo antibody therapy against ICAM1 or CD18 has been reported to inhibit diabetes-induced retinal leukostasis and endothelial injury in mice, suggesting that integrin/CAMs might make a good target for therapy (Miyamoto et al. 1999; Barouch et al. 2000; Joussen et al. 2001). Additional anti-
inflammatory therapies have been reported which decrease diabetes-induced retinal leukostasis and permeability or retinal ICAM1 such as corticosteroids (Tamura et al. 2005), RAGE inhibitors (Kaji et al. 2007), and salicylates (Zheng et al. 2007c).

NIF is a selective antagonist of the $\alpha_{m}\beta_2$ integrin (CD11b/CD18 or Mac1) but not other CD18 containing integrins such as CD11a/CD18 LFA-1 (Barnard et al. 1995; Ustinov and Plow 2002). NIF blocks the binding of several $\alpha_{m}\beta_2$ ligands, including ICAM-1, fibronectin, C3bi, and denatured protein, by blocking the pocket where the ligands bind, although the residues to which NIF binds on CD11b are different from the residues, which bind other ligands (Rieu et al. 1994; Zhang and Plow 1996). Thus, NIF-mediated inhibition of the interaction between leukocytes and endothelial cells may offer advantages not seen with other approaches.

We present evidence that antagonism of the $\alpha_{m}\beta_2$ integrin by NIF inhibits diabetes-induced degeneration of retinal capillaries, as well as inhibiting other abnormalities of retina that are believed to contribute to the vascular degeneration. Importantly, NIF did not impair the ability of leukocytes to resolve opportunistic bacterial infections. These findings suggest that the $\alpha_{m}\beta_2$ integrin on leukocytes plays a critical role in diabetes-induced leukostasis, retinal production of superoxide, and degeneration of retinal capillaries, and that these abnormalities can be inhibited by NIF.
3.2 Methods

Ethics Statement

All experiments complied with guidelines established by The Association for Research in Vision and Ophthalmology (ARVO) and Case Western Reserve University (the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health). The protocol was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (Protocol 2010-0156). Mice were terminally anesthetized with CO₂ immediately prior to harvest of blood and tissues. Anesthesia for non-terminal procedures to reduce discomfort consisted of 0.1mL/20g body weight IP of 15mg Ketamine, 3mg Xylazine, and 0.5mg Acepromazine / 1.4mL saline.

Animals

Male offspring from NIF<sup>+/−</sup> CD1 males (Gao et al. 2005) bred with female wild type (WT) mice (CD1, NIF<sup>−/−</sup>, Charles River) were separated by genotype NIF<sup>+/−</sup> or WT and housed in filtered air cages. Diabetes was induced at approximately eight weeks of age in male mice fasted for 6 hours prior to daily IP injection of 200uL of sterile filtered (0.22µm) streptozotocin (60 mg/kg ms) (MP Biomedical) in Citrate buffer (0.53mM Citric acid, 0.47mM Sodium Citrate pH 4.5) over the course of 5 days. Food was provided immediately after injection. The streptozotocin solution was used immediately after dissolving the stock powder in the citrate buffer (the solution is photosensitive and quickly self inactivates). Fasted blood glucose (6 hr fast) was obtained from tail blood at 7, 14, and 20 days after the last injection using EasyMax N blood glucose test strips and meter (Gemco medical, Ohio). Animals with fasted blood glucose greater than 250
mg/dL were assigned to the diabetic group. Diabetic mice were weighed weekly and body weight was maintained as needed (2g / 2 week loss threshold) with 0.1-0.2 units of Humulin N (NPH) insulin diluted 1:10 in sterile diluent (Eli Lilly) 2-3 times per week. Hyperglycemia was quantified via blood glucose concentrations, and every 2–3 months by glycated Hemoglobin A1c (Variant HbA1c; BioRad) (Kern et al. 2007; Zheng et al. 2007b; Zheng et al. 2007c). Mice were harvested at 10 weeks and at 40 weeks duration of diabetes to evaluate retinal physiologic abnormalities and histopathology (capillary degeneration), respectively. NIF expressing mice (Gao et al. 2005) were the generous gift of Dr. A. Malik, Department of Pharmacology, The University of Illinois College of Medicine, Chicago, Illinois. All chemicals and reagents were purchased from Sigma unless otherwise specified.

**Leukostasis**

Anesthetized mice were perfused at 200mmHg (Infusurge 4010 pressure infuser), 18mL/min via the aorta for 2 minutes with warm saline followed by 10mL of PBS containing 200uL of Concanavalin A FITC (Vector 5mg/mL). Residual Concanavalin A was washed away with an additional 2 min of perfusion with saline. The retina was isolated as detailed below and FITC-Concanavalin A-labeled leukocytes adherent to the walls of retinal blood vessels were counted as the number of observable leukocytes per retina using fluorescent microscopy (Kern et al. 2007; Zheng et al. 2007b; Zheng et al. 2007c).

**Leukocyte isolation**
0.5-1mL of whole blood was drawn via cardiac puncture from CO₂ anesthetized mice using a syringe containing 0.05mL of 7.2mg EDTA /mL PBS. Blood was immediately transferred to 7.2mg EDTA vaccutainer tubes (BD) and stored on ice for 2-10 minutes. Whole blood was incubated with 3 mL of RBC lysis buffer (Ebioscience) on ice for 5 minutes, centrifuged at 200xg for 7 minutes, re-suspended in 3 mL of lysis buffer, centrifuged again, and washed twice in Krebs-Hepes buffer (HBSS (Mediatech, 1/10 normal calcium), 0.02M Hepes (Gibco) and 5 or 25mM glucose for leukocytes from non-diabetic or diabetic donor mice, respectively) (Li et al. 2012).

Retina isolation
The anterior section was removed from enucleated eyes of perfused mice with Teflon coated razor blades (Electron Microscopy Sciences). Intact retina was carefully separated from any attachment at the ciliary body and sclera with a micro-spatula, and at the optic nerve with micro dissection scissors. Incisions in a cloverleaf pattern were made to ease mounting on microscope slides and prevent folding during the superoxide assay.

Superoxide
Retinae were incubated in 200uL Krebs-Hepes buffer with 5 or 25 mM glucose for 5 min, at 37°C, and 5% CO₂. Leukocytes (400,000 cells per 400uL buffer) were incubated under identical conditions for 25 minutes. Luminescence indicating the presence of superoxide was measured 5 minutes after the addition of 0.54mM (final concentration) Lucigenin using a luminometer (Monolight 2010) (Du et al. 2003; Zheng et al. 2007a).
Histopathology

Eyes enucleated from mice diabetic for 40 weeks and age-matched non-diabetic controls were fixed in buffered formalin for at least one week. The fixed retina was isolated, rinsed in running water overnight, and then digested with 40 U/mL elastase (Calbiochem), 5mM EDTA 100mM sodium phosphate, and 150mM NaCl pH6.5 at 37°C for 2-3 hours (Laver et al. 1993). Nonvascular cells were brushed away over the ensuing day, and the isolated vascular beds were transferred to charged microscope slides (Superfrost/Plus Fisher Scientific) and allowed to dry prior to PASH (periodic acid, Schiff base, hematoxylin) staining.

The number of capillary junctions (an estimate of the number of capillaries) and nuclei of blood vessels (both pericyte and endothelium) per square mm were counted in non-diabetic WT and NIF+/- expressing mice to determine if the presence of NIF altered normal vascular development in the retina.

Degenerate (acellular) capillaries were quantified in 6-8 fields in the mid-retina across all quadrants of retina as previously reported (Bresnick et al. 1976b; Kern and Mohr 2007). Briefly, acellular capillaries were counted in 0.419 mm² fields centered halfway between the optic nerve and the periphery. Capillaries were considered acellular only if no nuclei were in present in the capillary prior to the capillary junction. Capillaries were counted only if the width of the capillary at any point prior to a junction was at least 20% of normal capillary width and if the capillary length was at least three times normal capillary width.
Co-Culture

A mouse retinal endothelial cell line (mREC; generated from Immortomice), (Su et al. 2003) greater than 99% positive for CD31 by flow cytometry, was cultured on 10 cm gelatin-coated plates in DMEM with 10% FBS and 5.5 or 25mM glucose, which was changed every other day until the cells were 80% confluent. Leukocytes (200,000/ plate) purified from whole blood were added for 24 hours, after which mREC were gently rinsed with PBS to remove leukocytes, incubated with trypsin for 2 minutes, and washed twice in PBS. Viability of mREC was measured by trypan blue exclusion with a hemocytometer (Li et al. 2012). Sample identity was masked during counting. mREC (Su et al. 2003) were the generous gift of Dr. Nader Sheibani, Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin, United States of America

Bacterial clearance in air pouch model

A skin abscess air pouch model (Van Ziffle and Lowell 2009; Kamen et al. 2011) was used to assess the ability of mice to clear a bacterial infection in the presence of NIF. Briefly, an air pouch was created on day 1 by injecting anesthetized mice with 5 mL of sterile air under the skin above the spine. On day 4, the air pouch was re-inflated with 3mL of air. On day 7, Luria broth (LB) was inoculated with Klebsiella pneumoniae (ST258 UHKPC SS7 N1181172 677/1607) from an overnight culture at RT, which was then grown to 0.6 OD at 37°C. Bacteria were washed 3 times in PBS (500xg, 10 minutes) and diluted to 0.2 OD. 300x10^6 bacteria in PBS (=0.5mL of 0.2OD bacteria) were
injected into the air pouch. Twenty-four hours later, the air pouch of anesthetized mice
was lavaged with 3mL of Hank’s Balanced Salt Solution (HBSS, Mediatech) containing
0.15 M sodium citrate, and a hemocytometer was used to count the number of leukocytes
present in the lavage fluid. 200uL of the lavage fluid was immediately mixed with 2uL of
10% Triton-x 100 to lyse leukocytes containing live bacteria, and stored on wet ice until
after leukocytes were counted in the remaining lavage fluid. 1/100 serial dilutions of the
lysed solution were plated onto LB agar. Colony forming units (CFU) on the LB plates
were counted the next day after overnight growth at 37°C. Dilutions of lavage fluid
which resulted in greater than 200 CFU per plate were regarded as too numerous to count
reliably. In such cases, the next serial dilution of plated lavage fluid was used to calculate
the number of CFU in the undiluted lavage fluid. Klebsiella pneumonia was the generous
gift of Dr. R. Bonomo, Louis Stokes Veterans Affairs Medical Center, Cleveland, Ohio,
United States of America.

Statistics

Groups were compared using ANOVA followed by Fisher post-hoc test to generate p
values. Error bars in graphs represent±1 SD. Sample sizes are indicated in the figure
legends or on the figure.

3.3 Results

NIF is a hookworm-derived protein that binds to integrin CD11b/CD18. The effect of
NIF on the development of retinal lesions (capillary loss and leukostasis) and pathogenic
markers (superoxide production) characteristic of the early stages of diabetic retinopathy were measured in experimentally diabetic mice and non-diabetic controls.

**Effect of NIF on normal blood vessel development in the retina.**

No significance differences (p>0.05, n=6) were found between the number of capillaries (junctions) 812±78/ mm² (WT) and 841±68/ mm² (NIF+/−) or the number of nucleated cells in blood vessels 2510±247/ mm² (WT) and 2494±245/ mm² (NIF+/−). In addition, no gross morphological changes were apparent in the isolated retinal vasculature (Figure 3-1, p. 185).

**Evaluation of diabetes**

Experimental induction of diabetes with streptozotocin (SD) resulted in a significant increase of non-fasted blood glucose (p<0.001) and glycated hemoglobin HbA1c (p<0.001) in diabetic WT mice compared to non-diabetic WT controls over the 40 weeks of study (Table 3-1, p. 185). Data at 10 weeks diabetes was similar. The presence of NIF in non-diabetic or diabetic animals did not significantly alter blood glucose values or HbA1c.

**Capillary loss in the retina**

Diabetes-induced retinal vascular capillary loss is an important clinical endpoint in the development and progression of DR Degeneration of retinal capillaries in WT mice diabetic for 40 weeks was significantly greater than in age-matched non-diabetic WT mice (Figure 3-2, p. 187). In contrast, diabetic mice expressing NIF had significantly less
capillary loss than did WT diabetic controls. Thus, diabetes-induced retinal capillary loss can be inhibited by selective $\alpha_m\beta_2$ leukocyte integrin antagonism.

**Leukostasis**

The number of leukocytes adherent to the vascular endothelium of the retina are observed to increase in diabetic mice (Schroder et al. 1991) and humans (McLeod et al. 1995). The adherent leukocytes have been postulated to contribute to occlusion and degeneration of retinal blood vessels in diabetes (Miyamoto et al. 1999). Ten weeks after the onset of diabetes, WT mice showed the expected diabetes-induced significant increase in leukostasis in the retina (Figure 3-3, p. 189), whereas diabetic mice expressing NIF did not develop a significant increase in retinal leukostasis. Since NIF is a selective antagonist for $\alpha_m\beta_2$, the results suggest that diabetes-induced leukostasis in the retina is largely due to leukocyte $\alpha_m\beta_2$ binding to a ligand on the endothelial cell.

**Retinal and leukocyte superoxide**

Superoxide production is increased in the retina of diabetic mice and has been implicated in the pathogenesis of DR. By 10 weeks of diabetes, WT mice developed a significant increase in retinal superoxide production. In contrast, diabetic mice expressing NIF did not produce greater than normal amounts of superoxide. (Figure 3-4A, p. 191)

Leukocyte superoxide production has been previously reported to be increased in mice after several weeks of diabetes, suggesting that the leukocytes are activated in diabetic animals (Li et al. 2012). Consistent with this, WT mice developed a significant increase
in leukocyte superoxide production by 10 weeks after the onset of diabetes. (Figure 3-4B, p. 191) Interestingly, the leukocyte generation of superoxide was not inhibited in mice expressing NIF, and in fact was significantly increased in non-diabetic mice expressing NIF compared to WT controls. Leukocyte superoxide production in diabetic mice expressing NIF appeared to increase but was not significantly different from diabetic WT controls.

**Leukocyte-mediated killing of endothelial cells**

The ability of leukocytes from diabetic animals to induce capillary death was investigated in vitro using co-cultures of endothelial cells and fresh leukocytes. Leukocytes added to plates of mouse retinal endothelial cells in vitro were observed to settle onto the endothelial cells within minutes. Overnight incubation of the endothelial cells with leukocytes from WT diabetic mice resulted in significant increases in the number of dead endothelial cells compared to the number of dead endothelial cells observed when using leukocytes from non-diabetic controls. (Figure 3-5, p. 193) In contrast, leukocytes harvested from either diabetic or non-diabetic animals expressing NIF did not significantly elevate the number of dead endothelial cells. We conclude that NIF inhibits the ability of leukocytes activated by diabetes to kill endothelial cells.

**Bacterial Clearance**

NIF interferes with leukocyte binding and function, and therefore might decrease the ability of the immune system to respond to microbial infections. Mice expressing NIF did not exhibit any increase in incidence of sickness (general observation of activity and
kyphosis) or morbidity compared to WT controls, suggesting that their immune system was not critically impaired (data not shown). A skin pouch abscess model with an opportunistic strain of bacteria was used to more rigorously evaluate the ability of leukocytes to migrate to, and eliminate, a bacterial challenge in control and NIF-expressing mice.

The number of leukocytes present in lavage fluid harvested 24 hours after inoculation with bacteria was not significantly reduced in animals expressing NIF compared to WT controls. (Figure 3-6A, p. 195) Thus, NIF did not significantly impair the ability of the host to recruit leukocytes to the site of infection (including chemokine production, leukocyte chemotaxis, or leukocyte diapedesis).

The number of colony forming units (live bacteria) recovered from the lavage fluid was not significantly increased when the lavage fluid was harvested from NIF expressing mice compared to WT controls (Figure 3-6B, p. 195), indicating that NIF did not significantly impair the ability of the leukocytes to phagocytize or to kill the phagocytized bacteria.

3.4 Discussion

Several pieces of data indicate that leukocytes and leukocyte adhesion to endothelial cells play a critical role in diabetes-induced retinal capillary loss. First, deletion of proteins mediating leukocyte adhesion, ICAM1 or its binding partner on leukocytes integrin subunit β2 (CD18), significantly inhibited the diabetes-induced vascular permeability and
degeneration of retinal capillaries in animals (Joussen et al. 2004). Furthermore, diabetes-induced retinal capillary loss was also inhibited in animals deficient in leukocyte and inflammation associated proteins such as iNOS or PARP1 (Zheng et al. 2007b). Adoptive transplantation of leukocytes (bone marrow) deficient in iNOS or PARP1 was sufficient to inhibit diabetes-induced retinal capillary degeneration in WT animals. In the reverse experiment, adoptive transfer of WT leukocytes (marrow) into animals deficient in iNOS was sufficient to restore the diabetes-induced retinal capillary degeneration demonstrating that leukocytes were required to develop the diabetes-induced retinal vascular pathology (Li et al. 2012). In addition, leukocyte-mediated endothelial injury has been shown in vitro to be greater when the leukocytes are isolated from diabetic mice (Li et al. 2012) or patients (Tian et al. 2013) than when using leukocytes isolated from their non-diabetic counterparts.

The current results provide evidence that NIF-mediated blockade of the leukocyte $\alpha_m\beta_2$ integrin significantly inhibited a number of important diabetes-induced abnormalities in retina. Notable among these abnormalities inhibited by NIF is the important clinical endpoint of diabetes-induced degeneration of retinal capillaries. NIF also inhibited the diabetes-induced increase in leukocytes adhering to retinal blood vessels. Since NIF binds to, and blocks, the $\alpha_m\beta_2$ integrin, this was not unexpected, but it does demonstrate a long-term effect of NIF, for which compensation (adhesion through LFA-1 binding) did not occur. NIF protein expression patterns and serum concentration in NIF+/− CD1 mice could not be independently confirmed due to the current lack of an available anti-NIF
antibody, however NIF has been reported to be found in all organs of NIF\(^{+/+}\) mice (Gao et al. 2005).

Mice expressing NIF (NIF\(^{+/+}\) CD1) have been reported to have increased circulating neutrophils and decreased lymphocytes compared to non-NIF expressing CD1 mice (Gao et al. 2005) and a shift in the subpopulations of leukocytes might be a factor in the observed differences in diabetes-induced retinal lesions. This seems unlikely to explain our results, however, as we have previously reported that neutrophils were a major cause of the retinopathy in diabetic mice (Li et al. 2012), and therefore an increase in neutrophils would be expected to cause more vascular pathology not less as was observed.

NIF also inhibited the diabetes-induced increase in retinal superoxide production. We previously have observed that the ability of a therapy or genetic manipulation to inhibit the diabetes-induced generation of superoxide by the retina strongly predicts the ability of that therapy to inhibit retinal capillary degeneration in long-term studies of diabetic rodents (Kern et al. 2007; Zheng et al. 2007a). The inhibition of diabetes-induced retinal production of superoxide by blocking \(\alpha_m\beta_2\) integrin and associated leukocyte binding to endothelial cells in the present report is consistent with our previous report demonstrating that leukocytes were required for diabetes-induced generation of superoxide by the retina (Li et al. 2012). Our results suggest that retinal superoxide produced by an unidentified cell type in the retina is mediated by leukocyte \(\alpha_m\beta_2\) interaction with capillary endothelial cells.
Leukocytes from animals expressing NIF exhibited increased superoxide production compared to WT controls (Figure 3-4, p. 191) however, the increase in production was only significant in non-diabetic NIF expressing animals. The results suggest that NIF may bind as a partially activating ligand consistent with recent observations that integrin receptors can adopt multiple confirmations to yield different functions (Simon 2011). Despite the ability of NIF to inhibit superoxide generation by the retina, NIF did not inhibit diabetes-induced superoxide release by leukocytes, similar to the reported inability of NIF to inhibit PMA-activated leukocyte superoxide production (Ohno and Malik 1997).

Leukocytes isolated from diabetic or non-diabetic animals expressing NIF did not cause increased death of retinal endothelial cells, even though they generated more superoxide than did leukocytes from animals not expressing NIF. These results are consistent with previous studies demonstrating that anti-α\textsubscript{m}β\textsubscript{2} antibody blocked endothelial cell injury from activated leukocytes without inhibiting superoxide production by those leukocytes (Moyle et al. 1994; Ohno and Malik 1997). We conclude that the superoxide released by leukocytes “activated” by diabetes or by NIF ligation to the α\textsubscript{m}β\textsubscript{2} integrin is not sufficient to cause the diabetes-induced leukocyte mediated endothelial death.

Therapies targeting integrins other than MAC1, including humanized anti-CD11a, anti-ICAM1 and anti-CD18, have been associated with increased incidence of infection in clinical trials, including increased fungal and bacterial infections, (Bowen et al. 1998;
Immune related complications in anti-integrin therapy are not unexpected since humans deficient in CD18 (leukocyte adhesion deficiency I) also are immunocompromised and prone to infections. Thus, inhibition of leukocyte-mediated damage to endothelial cells without simultaneously compromising the immune system requires careful selection of the target. In our hands, NIF blocked the diabetes-induced degeneration of retinal capillaries, yet did not impair immune function, as evidenced by near normal leukocyte recruitment and bacterial elimination when directly challenged with an opportunistic organism. The apparent decrease in residual bacteria in the NIF expressing animals might be related to the increase in superoxide generation by leukocytes in those animals.

NIF has previously been administered to patients as an acute therapy after stroke without adverse effects including incidence of infection (Krams et al. 2003). Although NIF demonstrated improvement in patient recovery following a stroke when administered with tissue plasminogen activator (tPA), NIF alone failed to demonstrate significant improvement. While it is not possible to discern the reason for the failure of the trial from the published data, the authors noted that patients in the study were not screened for thromboembolism (non-resolved clot) for which NIF was previously shown to be effective only when co-administered with tPA (Zhang et al. 2003). Subsequently, this therapeutic approach was abandoned. The present study demonstrating that NIF significantly inhibits development of a slowly developing retinopathy in diabetes suggests that the careful inhibition of adhesion between activated leukocytes and the
vascular wall is still a valid therapeutic target for diabetes and possibly a variety of other chronic inflammatory diseases.

In contrast to our results demonstrating a beneficial effect of inhibiting the interaction between leukocytes and endothelial cells, other investigators have reported that enhancing the binding of leukocytes to endothelial cells inhibited vascular disease such as neointimal thickening following denudation (Faridi et al. 2013). However, this approach may be less efficacious in diabetes where adhesion of activated leukocytes may cause additional occlusion of capillaries and leukocyte-mediated endothelial damage.

Even though NIF had therapeutic value in the present study of diabetic retinopathy, it is unlikely that NIF in its current form will be suitable for chronic administration to inhibit diabetic complications. NIF is a foreign protein, and therefore may lead to generation of anti-NIF antibodies, as has been observed when NIF was used as a vaccine (Ali et al. 2001). To develop a therapy for clinical use in chronic diseases will require development of a small molecule or peptide that reproduces beneficial actions of NIF without inducing an undesirable immunologic response.

We conclude that ligation of the $\alpha_m\beta_2$ integrin on leukocytes with a molecule on the surface of endothelial cells (presumably ICAM-1) is critical to the diabetes-induced degeneration of retinal endothelial cells. NIF blocks this interaction, and therefore inhibits the diabetes-induced retinal capillary degeneration that is characteristic of early
diabetic retinopathy. Importantly, NIF accomplishes this without inhibiting the normal immune response to infection.
3.5 Tables and Figures

Table 3-1. Evaluation of diabetes in NIF expressing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Streptozotocin</th>
<th>Duration (weeks)</th>
<th>non-fasted blood glucose (mg/dL)</th>
<th>H1Ac (%)</th>
<th>Weight (g)</th>
<th>n</th>
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</thead>
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<tr>
<td>WT</td>
<td>no</td>
<td>32</td>
<td>114±16</td>
<td>4.0±0.9</td>
<td>62±9</td>
<td>10</td>
</tr>
<tr>
<td>NIF⁺</td>
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<td>137±27</td>
<td>3.9±0.2</td>
<td>66±16</td>
<td>10</td>
</tr>
<tr>
<td>WT</td>
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<td>410±73</td>
<td>9.3±1.1</td>
<td>72±10</td>
<td>9</td>
</tr>
<tr>
<td>NIF⁺</td>
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<td>451±74</td>
<td>9.6±0.9</td>
<td>70±11</td>
<td>10</td>
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<tr>
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<td>167±22</td>
<td>3.1±0.1</td>
<td>59±5</td>
<td>10</td>
</tr>
<tr>
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<td>58±12</td>
<td>10</td>
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<tr>
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<td>475±60</td>
<td>8.4±0.5</td>
<td>46±3</td>
<td>10</td>
</tr>
<tr>
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<td>10</td>
<td>488±58</td>
<td>8.4±0.4</td>
<td>44±8</td>
<td>12</td>
</tr>
</tbody>
</table>

p values are listed in the results section
Figure 3-1. Neutrophil inhibitory factor (NIF) does not inhibit normal vascular development in the retina.

No differences in capillary density were apparent in elastase digested vascular preparations of fixed retina isolated from A) non-diabetic WT, B) non-diabetic NIF^{+/−} mice. Images were captured at 40x.
Figure 3-2. NIF inhibits diabetes-induced retinal capillary loss.

A) WT mice diabetic for 40 weeks developed significantly more retinal capillary loss than non-diabetic controls. Mice expressing NIF did not develop the diabetes-induced retinal capillary loss and were not significantly different from non-diabetic controls. Streptozotocin induced diabetic mice (SD) are black bars and non-diabetic (ND) mice are white bars (n=4 for non-diabetic and diabetic WT animals, n= 6 for non-diabetic NIF expressing animals, and n= 5 for diabetic NIF expressing animals).

B) Retinal vasculature isolated from diabetic WT mouse. Black arrows indicate acellular capillaries.
Acellular capillaries (per 2.08 mm^2 retina)

- ND WT
- SD WT
- ND NIF+
- SD NIF+

A

B

[Image of a tissue section showing acellular capillaries, with arrows indicating specific areas]
Figure 3-3. NIF significantly decreases diabetes-induced leukostasis in the retinal vasculature.

Diabetes significantly increased the number of adherent leukocytes in the retinal vasculature in WT mice but not in diabetics expressing NIF. Duration of diabetes was 10 weeks. Streptozotocin induced diabetic mice (SD) are black bars and non-diabetic (ND) mice are white bars (n=5).
Leukostasis (adherent WBC / retina)

WT

NIF⁺

p<0.001

p<0.05

ns

ns
Figure 3-4. NIF inhibits the diabetes-induced up-regulation of superoxide in retina but not circulating leukocytes.

A) Superoxide generation increased in the retina of WT animals diabetic for 10 weeks, but not in diabetics expressing NIF (n=5).

B) Superoxide production is increased also in leukocytes of WT animals diabetic 10 weeks compared to non-diabetic controls. In contrast, the presence of NIF resulted in an elevated leukocyte superoxide production in non-diabetic animals which was further increased in NIF mice diabetic for 10 weeks (n=5).
A

Retinal Superoxide RLU/ug protein

ND WT  SD WT  ND NIF+  SD NIF+

p<0.001  p<0.01  ns

B

Leukocyte Superoxide RLU/400,000 cells

ND WT  SD WT  ND NIF+  SD NIF+

p<0.01  ns  p<0.01  ns
Figure 3-5. NIF inhibits endothelial cell death caused by leukocytes isolated from diabetic animal.

Co-culture of transformed mouse retinal endothelial cells (mREC) with leukocytes resulted in significant increases in the number of dead endothelial cells when leukocytes were isolated from WT diabetic animals compared to leukocytes harvested from WT non-diabetic animals. In contrast, leukocytes isolated from diabetic animals expressing NIF did not significantly increase the number of endothelial cells killed in the co-culture assay compared to non-diabetic animals expressing NIF or diabetic WT animals (n=3).
Leukocyte mediated endothelial cell death (% of total mREC count)

- WT: 10
- NIF*: 5

Significance:
- p<0.01
- p<0.05
- ns
Figure 3-6. NIF does not significantly inhibit immune response to microbial challenge.

K. Pneumonia injected into an air pouch abscess model elicited leukocyte migration into the pouch.

A) The number of leukocytes recovered in 3 mL of lavage fluid 24 hours after bacterial challenge was not significantly different between mice expressing NIF and WT controls. The presence of NIF did not inhibit leukocyte migration (and required chemotaxis) into the site of the inoculation (n=5).

B) The number of colony forming units, representing residual living K. Pneumonia in the lavage fluid, recovered 24 hours after bacterial challenge, was not significantly increased in the presence of NIF. Therefore the presence of NIF did not inhibit the ability of leukocytes to phagocytize and kill K. Pneumonia (n= 4).
CHAPTER 4

DEFICIENCY OF p105 (NFκB1) EXACERBATES DIABETES-INDUCED INFLAMMATION AND VASCULAR DEGENERATION IN THE RETINA

The work in this chapter has only been presented in a poster format at ARVO and represents work done only by myself.

4.1 Introduction

Diabetic retinopathy (DR) is the leading cause of blindness in working age adults. The early stages of DR are characterized by retinal inflammation and progressive degeneration of capillaries. NFκB (a mediator of inflammation) may play a role in diabetes-induced loss of retinal capillaries. Our lab has previously demonstrated that the inflammation associated proteins PARP1 and iNOS are required in diabetes-induced retinal capillary loss (Zheng et al. 2004; Zheng et al. 2007a; Li et al. 2012). PARP1 (in addition to DNA repair) binds both p50 and p65 units of NFκB in the nucleus, and blocks the nuclear export signal for these proteins; PARP1 is required to retain the complex in the nucleus prior to transcription of proinflammatory genes (Hassa et al. 2001; Zerfaoui et al. 2010), whereas iNOS is a target gene of the NFκB mediated transcription. In addition pro-inflammatory cytokines which are also regulated by NFκB transcription have been reported to be increased in the serum, vitreous, or aqueous humor of diabetic patients including MCP1, IL1β and TNFα (Elner et al. 1995; Doganay et al. 2002; Dong
et al. 2013) and also in the retina of diabetic animals (Carmo et al. 1999; Joussen et al. 2002a; Dong et al. 2012).

In addition to inflammation, the early stages of DR are also characterized by the progressive loss of retinal capillary endothelial cells resulting in the formation of acellular capillaries, which is thought to cause local hypoxia and eventual progression of the retinopathy into the proliferative stage. High glucose alone is sufficient to increase nuclear enrichment of p50/p65 and cell death in cultured endothelial cells and this process is inhibited by blocking nuclear import of p50 (Zheng et al. 2004). Nuclear enrichment of p50 has also been shown to increase in the retinal capillary endothelial cells of diabetic rats. Animals treated with non-selective NFκB inhibitors (salicylates) exhibit reduced diabetes-induced retinal vascular lesions and inflammation compared to untreated animals (Kern and Engerman 2001; Kern et al. 2007; Zheng et al. 2007c).

p65/p50 dimers are normally held in the cytoplasm by IkB, which releases the dimer upon phosphorylation by IκK (Hoffmann et al. 2006; Lawrence 2009). The dimer then migrates to the nucleus where it promotes the transcription of iNOS and pro-inflammatory cytokines. p50 is constitutively cleaved from precursor p105 (NFκB1). p50 and p105 also function as negative regulators of NFκB mediated transcription as a competitive inhibitor of transcription factor occupancy of response element and as an IkB respectively. Mice deficient in p105 (and p50) have an increased susceptibility to microbial infections due in part to a resistance to induce inflammation, however once
inflammation is initiated the p105−/− mouse develops a severe chronic inflammatory phenotype (Ishikawa et al. 1998).

We present evidence that the loss of p105 exacerbates the diabetes-induced degeneration of retinal capillaries, as well as other abnormalities of retina that are believed to contribute to the vascular degeneration. These findings suggest that diabetes-induced increases in retinal leukostasis, superoxide production, MCP1 secretion, and acellular capillaries and leukocyte superoxide production are not dependent on p50 mediated transcription. Our findings also suggest that p105 and p50 play a critical role in the inhibition and resolution of inflammation in the retina due to diabetes.

4.2 Materials and methods

*Animals and induction of diabetes*

All experiments conformed to the animal care guidelines of Case Western Reserve University and ARVO. Offspring from breeder pairs of C57Bl/6J, B6129PF2/J and B6;129P-Nfκb1tm1Bal/J (p105−/−) (Jackson laboratories) were housed in filtered air units. Diabetes was induced in male mice with streptozotocin (55 mg/kg BW) and maintained with 0.1-0.2 units of NPH insulin as previously described (Kern et al. 2007; Zheng et al. 2007b; Zheng et al. 2007c). Hyperglycemia was quantified every 2–3 months by measuring total glycated hemoglobin levels (Variant II total GHb Program; BioRad) and by measuring blood glucose concentrations.

*Acellular capillary measurement*
Capillaries lacking nuclei were quantified in six to eight field areas in the mid-retina (×400 magnification) of fixed, trypsinized retina as previously reported (Bresnick et al. 1976a; Kern 2007).

Superoxide assay

Retinae isolated from the enucleated eyes saline perfused mice were incubated in 200uL Krebs-Hepes buffer with 5 or 25 mM glucose for 5 min, at 37°C, and 5% CO₂. Leukocytes isolated from anti-coagulated peripheral blood (RBC lysis buffer eBioscience) (400,000 cells per 400uL buffer) were incubated under identical conditions for 25 minutes. Luminescence indicating the presence of superoxide was measured 5 minutes after the addition of 0.54mM (final concentration) Lucigenin using a luminometer (Monolight 2010) (Du et al. 2003; Zheng et al. 2007a; Li et al. 2012).

In vitro co-culture assay of leukocyte mediated endothelial cell death

Mouse retinal endothelial cells (mREC), (Su et al. 2003) were cultured on 10 cm gelatin-coated plates in DMEM with 10% FBS and 5.5 or 25mM glucose, which was changed every other day until the cells were 80% confluent. Leukocytes (200,000/ plate) purified from whole blood were added for 24 hours, after which mREC were gently rinsed with PBS to remove leukocytes, incubated with trypsin for 2 minutes, and washed twice in PBS. Viability of mREC was measured by trypan blue exclusion with a hemocytometer (Li et al. 2012). Sample identity was masked during counting. mREC (Su et al. 2003) were the generous gift of Dr. Nader Sheibani, Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin.
Measurement of cytokine and inflammation association proteins by mRNA

Retinae were quickly isolated from enucleated eyes of saline perfused mice (15 mL/min) previously anesthetized with CO₂, flash frozen on dry ice and stored at -80°C. Retina was homogenized in 300uL of Trizol (Invitrogen) with 50 uL of 0.5mm RNase free stainless steel beads (Next Advance) in a Bullet blender (Next Advance) for 2x5 minutes at 4°C. mRNA was extracted from Trizol according to manufacturer’s instructions, resuspended in nuclease free water (Ambien), and stored at -80°C until use. cDNA was transcribed from mRNA using iScript Reverse transcription super mix (Biorad) followed by RTqPCR (BioRad) using fast Start Universal SYBR Green master mix (Roche) with the primers listed below (Invitrogen). Products of the PCR reaction were checked for primer dimers on TAE 2.5% agrose gel.

iNOS F1 TCT-TTG-ACG-CTC-GGA-ACT-GTA-GCA,
iNOS R1 TAG-GTC-GAT-GCA-CAA-CTG-GGT-GAA
TNFα F AGC-CCA-CGT-CGT-AGC-AAA-CCAC
TNFα R GCA-GGG-GCT-CTT-GAC-GGC-AG
18S F1 ACT-CAA-CAC-GGG-AAA-CCT-CAC-C
18S R1 CCA-GAC-AAA-TCG-CTC-CAC-CAA-C
ICAM1 F1 ATC-ACC-GTG-TAT-TCG-TAT-CC
ICAM1 R1 CAG-CAC-CGT-GAA-TGT-GAT-CT
VCAM F1 TGT-CAA-CGT-TGC-CCC-CAA-GGA

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Chromatin immunoprecipitation (ChIP)

Peripheral blood in vacutainer tubes with anticoagulant EDTA (BD) was fixed by adding 1% Formaldehyde for 10 minutes on ice and an additional 10 minutes at room temperature. Blood was washed three times in PBS and lysed in lysis buffer (1%SDS, 10mM Tris-HCL, pH 8, 1mM PMSF, pepstatin A and aprotinin) for 10 minutes on ice, and sonicated 5 times for 10 seconds. Debris was removed by centrifugation.

One third of the lysate was retained for DNA input control. The remainder was diluted 10x with 0.01%SDS, 1% triton-100, 1mM EDTA, 10mM Tris-HCL pH 8 and 150 mM NaCl and incubated with rabbit CHiP antibodies to RelA, RelB, c-Rel, p50, and p52 (Santa Cruz Biotechnology) overnight. Immune complexes were collected with goat anti-rabbit antibodies immobilized to magnetic beads (Invitrogen). Beads were washed with elution buffer 1%SDS and 0.1MNaHCO3 at RT for 20 minutes. Crosslinking was reversed by 100ug/mL of proteinase K (invitrogen) for 3 hours at 50C. DNA was extracted with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in TE buffer. DNA was subjected to RTPCR using primers surrounding the two iNOS NFkB binding sites.
4.3 Results

Effect of diabetes and p105 on retinal vasculature.

Diabetes has been previously shown to increase the number of acellular (degenerate) capillaries in the mid retina of mice (Li et al. 2012). As expected, the number of acellular capillaries significantly increased in the retina of mice diabetic for 30 weeks (Figure 4-1, p.209) by 2 fold. This diabetes-induced loss of retinal capillaries was further significantly increased by an additional 2 fold in mice lacking p105 compared to WT controls. The acellular capillaries in the p105−/− mouse were morphologically longer than those in WT mice (data not shown), however the importance of this observation in unknown.

Effect of diabetes and p105 on retinal and leukocyte superoxide production.

Retinal superoxide production has been shown to correlate with the ability of therapies to prevent early lesions of diabetic retinopathy (Kern et al. 2007; Zheng et al. 2007a; Gubitosi-Klug et al. 2008). To differentiate superoxide generation by retinal cells from that of blood cells in the blood vessels, blood was removed by perfusion. Retinal superoxide production was significantly increased in retina from WT mice 10 weeks diabetic (1.4 fold) as has been previously reported (Figure 4-2A, p. 211) (Du et al. 2003; Kern et al. 2007). Diabetes-induced superoxide generation by the retina, like retinal
capillary loss, was further elevated an additional 1.5 fold in animals lacking p105 compared to WT controls.

Leukocyte superoxide generation has also been previously reported to be elevated in leukocytes from diabetic animals (Schroder et al. 1991; Li et al. 2012). Diabetes induced superoxide generation by leukocytes was significantly increased (1.5 fold) as expected in leukocytes from WT mice (Figure 4-2B, p. 211). Leukocyte superoxide generation in non-diabetic mice lacking p105 was significantly elevated and was not statistically different from diabetic WT mice. Superoxide was further elevated by 2 fold in leukocytes from diabetic mice lacking p105 compared to non-diabetic mice lacking p105. We conclude that diabetes induces an increase in superoxide production by leukocytes, and that p105/p50 acts to dampen this effect compared to the level of superoxide generation in the absence of p105.

Effect of diabetes and p105 on leukocyte mediated endothelial cell damage in vitro.

We have previously demonstrated that leukocytes from diabetic animals when cultured in vitro with mouse retinal endothelial cells results in more endothelial cell death than leukocytes from non-diabetic animals or high glucose media(Li et al. 2012). We interpret these data to mean that leukocytes from diabetic animals are activated and therefore kill the endothelial cells. As expected in culture with mREC, leukocytes from diabetic WT animals resulted in more endothelial cell death as measured by trypan blue than leukocytes from non-diabetic mice (Figure 4-3, p. 213). Similarly, in the co-culture assay, leukocytes from diabetic animals lacking p105 also resulted in more endothelial cell
death than non-diabetic controls. The lack of p105 exacerbated the ability of leukocytes from diabetic animals to kill endothelial cells compared to WT controls. Unlike the superoxide generation, leukocytes from the non-diabetic mice lacking p105 did not result in significantly more endothelial cell death in the co-culture assay than leukocytes from non-diabetic WT controls.

**Effect of diabetes and p105 on cytokines and iNOS in the retina.**

Inflammatory cytokines IL1β, TNFα, and MCP1 as well as inflammation associated protein iNOS have been shown to be elevated in the retina of diabetic animals (Carmo et al. 1999; Joussen et al. 2002a; Dong et al. 2012; Li et al. 2012). Diabetes significantly increased mRNA for IL1β, TNFα, MCP1, and iNOS in the retina of WT mice (Figure 4-4, p. 215). In sharp contrast, only mRNA for MCP1 was significantly elevated in diabetic but not non-diabetic mice lacking p105. Furthermore, MCP1 mRNA was 5 fold higher in diabetic mice than WT diabetic mice. mRNA levels for IL1β, TNFα, MCP1, and iNOS in the retina of non-diabetic mice lacking p105 were not significantly different from WT non-diabetic controls.

**The effect of diabetes and p105 on Rel occupancy of the iNOS promoter**

CHiP antibodies to all five members of the Rel family were used to pull down Rel proteins in lysate of whole blood from diabetic and non-diabetic mice lacking p105. The isolated protein was digested and sheared chromatin was analyzed for enrichment of the iNOS promoter. Surprisingly all of the anti-Rel antibodies including anti-p50-chromatin demonstrated some enrichment of the iNOS promoter, however only c-Rel was clearly
elevated. In the absence of data demonstrating that iNOS is elevated in mice lacking p105 the significance of this finding is unknown (data not shown).

4.4 Discussion

Our current results demonstrate that the lack of p105 and p50 does not inhibit the diabetes-induced loss of retinal capillaries. The increase in the number of retinal acellular capillaries and retinal superoxide in diabetic mice lacking p105 and p50 compared to WT mice suggest that diabetes induces inflammation in the retina and the lack of negative regulation due to the loss of p105 and p50 results in unrestrained inflammation consistent with the reported phenotype of the p105⁻/⁻ mouse (Ishikawa et al. 1998).

The increase in leukocyte superoxide generation in the p105 non-diabetic mouse compared to WT controls suggest that these cells have been activated, however, it is unclear if this was due to the lack of p105 present during the isolation of the leukocytes from blood (which would help to resolve any normally transient increases in activation) or an unrestrained activation of the leukocyte due to diabetes. Nevertheless, the generation of superoxide by leukocytes from the diabetic p105⁻/⁻ mouse was significantly higher than the non-diabetic p105⁻/⁻ mouse, suggesting that this parameter is also exacerbated in diabetes.

The ability of the leukocytes from the diabetic p105⁻/⁻ mouse to kill more endothelial cells in the co-culture assay than diabetic controls suggests that leukocyte activation is also increased in the p105 diabetic mouse. However, it is unclear if this is due to increased
activity per leukocyte or more likely an increase in the number of activated leukocytes. Consistent with this concept only a small percentage of granulocytes and monocytes are reported to be activated in diabetic animals (Schroder et al. 1991; Li et al. 2012). In either case the results suggest that the ability of leukocytes (from diabetic p105\(^{-/-}\) animals) to kill endothelial cells is increased by roughly the same fold as the increase in acellular capillaries. The lack of an increase in endothelial cell death in the co-culture assay when leukocytes were isolated from non-diabetic p105\(^{-/-}\) animals despite the increase in observed superoxide reinforces the previous suggestion that the increase in superoxide was a marker of transient activation due to isolation techniques that resolved prior to the addition of these cells in the co-culture assay. However, this theory remains to be tested.

The observed increase in mRNA for IL1\(\beta\), TNF\(\alpha\), MCP1, and iNOS in the retina of WT mice with diabetes in consistent with previous studies. However the lack of increased mRNA for IL1\(\beta\) and TNF\(\alpha\) in the retina of p105\(^{-/-}\) diabetic mice is surprising considering that they appear to have unrestrained inflammation. The results suggest that IL1\(\beta\) and TNF\(\alpha\) mediated cell death do not occur in the diabetic p105\(^{-/-}\) mouse contrary to what has been reported in other studies involving IL1\(\beta\) mediated caspase activation in WT mice (Vincent and Mohr 2007). The 5 fold increase in leukocyte chemokine MCP1 mRNA in the retina of diabetic mice lacking p105 compared to diabetic WT controls is consistent with an increase in retinal leukostasis and putitive subsequent leukocyte mediated enothelial cell death. The degree of diabetes-induced pathology is so different at 30 weeks between the p105\(^{-/-}\) mouse and the C57Bl6/J WT mouse that the profile of retinal cytokines at 10 weeks (of diabetes) may have changed as the progression of the
cumulative capillary loss increased. Alternatively, the initial effect of streptozotocin may have induced retinal endothelial cell death and therefore capillary loss to an extent not previously observed in WT diabetic mice, as increased endothelial cell staining with propidium iodine has been reported shortly after the induction of diabetes by streptozotocin (Jousson et al. 2001). Therefore, a comparison of the p105−/− mouse at earlier time points may help clarify if this mouse model could be used as an accelerated model of diabetic retinopathy.

Regardless of the mechanism, the loss of p105 and p50 do not inhibit the diabetes-induced loss of retinal capillaries. We conclude that their role in regulation of inflammation of the retina in diabetes outweighs any benefit of their inhibition.
4.5 Figure Legends

Figure 4-1 Lack of p105 exacerbates diabetes-induced retinal capillary degeneration.

A Diabetes increases the number of acellular (degenerate) capillaries in mice 40 weeks diabetic. Mice lacking p105 have exacerbated diabetes-induced retinal capillary loss. (n=5) Non-diabetic B29 WT white bars, Diabetic B29 WT black bars, Non-Diabetic p105/- vertical striped bars, Diabetic p105/- diagonal striped bars.
Acellular capillaries (per mm² retina)

- ND WT
- SD WT
- ND p105
- SD p105

Significance levels:
- p<0.05
- p<0.001
Figure 4-2 Lack of p105 exacerbates diabetes-induced retinal and leukocyte superoxide production in mice. Superoxide production was significantly increased in the retina (perfused) and leukocytes of mice 10wks diabetic. Diabetes-induced leukocyte and retinal superoxide production was exacerbated in mice lacking p105. (n=5). Non-diabetic B29 WT white bars, Diabetic B29 WT black bars, Non-Diabetic p105−/− vertical striped bars, Diabetic p105−/− diagonal striped bars.
A

Retinal superoxide RLU/retina

ND WT | SD WT | ND p105^-/- | SD p105^-/-

p<0.05

B

Leukocyte superoxide RLU/400,000 cells

ND WT | SD WT | ND p105 | SD p105

p<0.01
Fig 4-3 Lack of p105 exacerbates diabetes-induced leukocyte ability to kill mouse retinal endothelial cells (mREC) in vitro. Leukocytes isolated from diabetic WT animals co-cultured with mREC for 24 hours killed more mREC than leukocytes isolated from non-diabetic WT mice. The deletion of p105 exacerbated the diabetes-induced killing of mREC by leukocytes. (n=5)
Leukocyte mediated endothelial cell death (% of total mREC count)

- ND WT
- SD WT
- ND p105
- SD p105

Significance levels:
- p<0.01
- p<0.05
- p<0.001
Figure 4-4 Diabetes induces mRNA associated with inflammation in mouse retina and but this effect is limited to MCP1 in mice lacking p105.

A mRNA levels of cytokines (MCP1, IL1β, TNFα) and proteins (iNOS) associated with inflammation in the retina were increased in mice 10wks diabetic. Deletion of p105 exacerbated diabetes-induced mRNA for MCP1 (n = 3).
Increase in Retinal mRNA (fold of non-diabetic WT)
5.1 Summary
A number of studies have demonstrated a relationship between inhibition of leukocyte binding and early biological markers of changes in the retina due to diabetes. These studies implicate leukocytes in the long-term pathogenesis of the retinal vascular capillary bed. To test the role of leukocytes in the development of the retinal vascular pathology, we developed a strategy, which we could use to deplete or inhibit leukocytes in long-term studies of diabetic animals. We used streptozotocin-induced diabetic mice as an in vivo model of Type I diabetes into which we transplanted bone marrow from donors lacking specific proteins to create a chimera in which only the marrow derived cells were lacking the protein of interest in order to demonstrate a relationship between the marrow-derived cells and diabetes-induced lesions of the retinal vasculature. Likewise, we also generated a reverse chimera in which all the cells lacked the protein of interest, except the marrow-derived cells, to demonstrate that the inhibition of diabetes-induced lesions of the retinal vasculature was not dependant on the absence of the protein in cells, which were not marrow-derived. Initially we focused on two proteins, iNOS and PARP1, which are involved in inflammation and leukocyte activation and have been previously shown to inhibit the diabetes-induced vascular lesions of the retina in mice lacking the proteins in all cells of their body. Having determined that leukocytes did mediate the diabetes induced-damage of the retinal vasculature, we evaluated the mechanism that leukocytes
would cause damage in the retina via the classical model of leukocyte infiltration in inflammation using whole mounted retinal vasculature to determine which subpopulations of leukocytes might infiltrate the retina. Upon finding that the leukocytes remained within the vasculature of the retina, we developed an in-vitro model with mouse retinal endothelial cells (mREC) in co-culture with leukocytes isolated from mice to discern the mechanism through which leukocytes might damage the retinal vasculature. We expanded our studies to discern which subtypes of leukocytes might be mediating the retinal vascular loss using G-CSFR and M-CSFR deficient mice, which lack granulocytes and monocytes respectively. We depleted monocytes and neutrophils from the leukocytes used in the co-culture assay to determine if the lack of a particular cell type inhibited diabetes-induced leukocyte mediated endothelial cell death. These studies prove that leukocytes play a casual role in the early stages of diabetic retinopathy.

Damage of endothelium by activated leukocytes, outside the context of diabetes, has been shown to be CD18 integrin adhesion dependent. However, there have been several reports that diabetes-induced loss of the retinal vasculature occurs in the absence of observed leukostasis. In our second study, we tested the hypothesis that CD18 integrin leukocyte adhesion was important in leukocyte-mediated retinal capillary loss in diabetic animals. We used a mouse that expressed a novel protein neutrophil inhibitory factor which selectively inhibits the binding of integrin CD11b/CD18 in a streptozotocin model of diabetes Type I. The use of NIF in our study (as opposed to leukocytes lacking CD11b) allowed us to evaluate the effectiveness and relative safety of long-term anti-
CD11b/CD18 therapy. This finding will allow us to continue work on developing NIF or NIF like compounds as a therapy.

Our lab has collectively identified a putative pathway in murine leukocytes that begins with an undefined action signal or signals due to hyperglycemia which results in the upregulation of iNOS. Chronic inflammation and leukocyte activation undoubtedly involves the NFκB family of transcription factors, of which iNOS is a target. In our third study we sought to test the hypothesis that NFκB1 derived p50 is required for diabetes-induced retinal vascular loss using a mouse lacking the precursor of p50, p105. However current evidence demonstrates that the absence of p105 (and putatively the absence of p50) resulted in a phenotype in which diabetes induced loss of retinal capillaries was exacerbated, suggesting that the lack of p50 and p105 are not only not required for capillary loss in the retina due to diabetes, but also that the diabetes-induced pathology may be accelerated in the absence of these factors.

5.2 Future directions

Expanded studies into subsets of leukocytes. Our initial study demonstrated that marrow-derived cells mediate diabetes-induced retinal capillary loss. We expanded the study to include mouse models in which the leukocyte subtypes that we observed adherent in the retinal vasculature, granulocytes and monocytes, were absent. Although good results were achieved with a G-CSFR→WT chimera, which is a model of neutropenia, our attempts to generate a monocyte deficient chimera using transplanted M-CSFR−/− bone marrow, was unsuccessful in the diabetic mouse. The expansion of the
residual 5% of WT marrow in our recipient irradiated mice receiving M-CSFR cells in the diabetic but not the non-diabetic mice suggests that diabetes strongly induces the maturation of monocytes. The next logical step would be to repeat the experiment using mice lacking specific subsets of cells in the monocyte lineage. The monocyte subset associated with inflammation consists of the cells, which are Ly6C${}^{hi}$, CX3CR1${}^{low}$ and CCR2${}^{+}$. This subset of monocytes is retained in the bone marrow in CCR2${}^{-/-}$ mice. An expansion of the study to include T cells, B cells and NK cells is also warranted as preliminary data with long term diabetic NOD SCID mice indicate a lack of diabetes-induced pathology in retina however the underlying hypotheses for the involvement of T cells is absent as they are not observed to increase in the retina in diabetes.

**NIF binding studies.** Our studies of leukocyte adhesion with the NIF expressing mouse demonstrate that CD11b/CD18 is important in the pathology of DR While NIF itself is unlikely to be a valid therapeutic due to its inherent nature of eliciting anti-NIF antibodies from the host, a study of the residues to which NIF binds or an expanded study of peptides derived from NIF may yield clues about how it binds to CD18/CD11b which could be used to develop small molecule inhibitors or humanized protein inhibitors. These studies are currently being pursued by our lab. The ability of NIF to bind other integrins other than the CD18 family also needs to be explored. While the initial studies of NIF included an immunoprecipitation of NIF bound to cell lysate, the results were limited to the observation of western blot bands consistent with the molecular weight of CD18 and CD11b. In particular, the literature suggests that the β1 integrins, which bind
VCAM1, may mediate leukocyte adhesion in diabetes. To my knowledge, NIF binding studies to VLA-4 (α4β1) have not yet been reported.

**Leukocyte detection studies.** Our studies of inhibiting leukocyte integrin binding with NIF indicate that leukocyte adhesion is required for diabetes-induced retinal capillary loss, however it remains unclear if leukocyte adhesion in the retinal capillaries of the neural retina is required for capillary loss or if the leukocytes binding elsewhere in the retina is required for capillary loss. In our first study, we inhibited the generation of diabetes-induced increases in retinal superoxide by inhibiting the ability of leukocytes to induce inflammation. The exact mechanism of how leukocytes in diabetic animals induced the retina to produce more superoxide remained however unclear. Recently our lab has provided evidence that photoreceptors in the eye generate much of the superoxide which is increased in the retina of diabetic animals (Du et al. 2013). While we examined the neural retina for evidence of extravascular leukocytes, we did not extend our studies into the subretinal space or the choroid. A comparison of the leukocyte populations in these spaces may help to explain how and which leukocytes mediate superoxide generation by the retina in diabetes.

**Further studies with NFκB.** Our study of the role of p50 and p105 in the development of DR suggest that these two factors are more important in inhibiting the inflammation than causing it. However, the pull down of iNOS DNA with anti p50 antibodies needs to be explored further. The most likely reason for the result is that the CHiP antibody is not specifically binding p50 or p105. However, it is also possible that the stress of diabetes
induces the co-translation of p50 not previously seen in the initial testing of non-diabetic mice for p50 and p105. Lysate of the retina, spleen, and blood from diabetic mice needs to be tested for the presence of p50 to exclude this possibility. In addition, the nuclear proteins to which the CHiP antibody is attaching should be confirmed independently on a western blot or by MS/MS of the precipitate separated by a molecular weight resolving gel.

The p105/- mouse may be a good model of accelerated diabetes induced pathology in the retina, which in theory would decrease the time required for the animals to develop acellular capillaries. This could be answered by expanding the current study to include an evaluation of the number of retinal acellular capillaries at 2 and 4 months of diabetes. The duration of diabetes required to reliably discern a difference in retinal acellular capillaries between diabetic and non-diabetic WT mice is about 30 weeks.

However, the p105/- mouse has been reported to develop a severe inflammatory phenotype upon developing inflammation. It is therefore possible that the increase in retinal capillary loss in the diabetic p105/- mouse is mediated a mechanism other than that which occurs in the retina of WT diabetic mice. If the mechanism of capillary loss in the p105/- mouse is the same as that observed in the WT mouse, than therapies, which have, been previously shown to inhibit the diabetes induced retinal pathology, such as the inhibition of RAGE or iNOS should also inhibit development of the pathology in the p105/- mouse. However selection of the therapy should be carefully considered as a
general inhibitor of inflammation such as high dose aspirin may inhibit inflammatory processes in the diabetic p105−/− mouse that are not present in the WT mouse.

Mice deficient in iNOS in only their marrow-derived cells did not develop the diabetes-induced lesions of retina observed otherwise in WT diabetic controls. The increase of the binding of c-Rel to the iNOS promoter in leukocytes from diabetic p105−/− mice compared to non-diabetic controls suggests that c-Rel may be a more appropriate target than p105. c-Rel, unlike p105 or p50, is not associated with the inhibition and resolution of inflammation. The enrichment for c-Rel on the iNOS promoter should however be confirmed in the leukocytes of WT diabetic mice. Our initial observation that each of the 5 Rel proteins were associated with the promoter may reflect that different subtypes of leukocytes may bind different pairs of NFκB dimers to the iNOS promoter. Since the CHiP technique relies on formalin fixed cells it is possible to fix the leukocytes and sort the different populations with flow cytometry. However, subsequent CHiP assays will likely require that leukocytes from several mice be pooled together in order to have sufficient chromatin for the assay especially in the case of monocytes which in the mouse comprise only about 4% of the leukocytes.

Our studies thus far have mainly focused on the diabetes-induced loss of retinal vasculature, whereas evidence is growing that the loss of neural function may also be important. We have studied the effects of some therapies on contrast sensitivity in the mouse. The results although preliminary suggest that therapies that inhibit vascular loss
do not necessarily inhibit decreases in contrast sensitivity. An expansion of these techniques in our future studies should also be considered.
APPENDIX

Detailed Methods

Anesthesia protocols

Blood draw and Leukocyte isolation

Perfusion

Enucleation of eye and retina isolation

RTqPCR of retina and blood

Tail vein Injection

Bone marrow transplant

Superoxide protocol
Anesthesia Protocols (Alex Veenstra V1)

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Procedures

A) CO₂ method

B) Triple cocktail method for mice

A) CO₂ procedure (note this procedure was approved by the Institutional Animal Care and Use Committee IUCAC).

Reagents

Carbon dioxide Tank, CD50, Air gas

Animal gassing chamber, AB-1, Braintree Scientific Inc

Regulator Victor VTS253A set to 3 psi

Procedure

1) For sick animals, give 1-2 mL saline IP 15 min prior to blood draw (dehydration).

2) Turn on carbon dioxide and adjust gas regulator until flow into chamber is a light breeze then turn off regulator.

3) Place animal in chamber and close lid.
4) Turn on carbon dioxide flow into chamber and wait until 10 seconds after animal stops gasping (about 45 seconds).

5) Turn off carbon dioxide flow.

6) Immediately remove animal and harvest tissues of interest (note a delay after animal stops breathing will decrease volume of blood harvested).

7) Clean the chamber when you are done with warm water and soap to remove urine. Do not use alcohol! Invert to dry and line bottom with 2-3 c-fold paper towels.

B) **3xcocktail anesthetic** (note this procedure was approved by the IUCAC)

**Methods section for publication**

Mice were anesthetized with 0.1mL/10g body weight of Triple cocktail (10.7mg/mL Ketamine, 2.4mg/mL Xylazine, and 0.35 mg/mL Acepromazine in saline) given IP.

**Reagents**

- 0.15mL Ketamine HCl (USP, 15 mg of 100 mg/mL, Ketaset, Fort Dodge Animal Health)
- 0.15mL Xylazine HCl (USP, 3 mg (20 mg/mL), AnaSed Lloyd Laboratories)
- 0.05 mL Acepromazine 0.5 mg (10 mg/mL)
- 1.05mL Sterile Saline, 0.9% NaCl (USP, Abbott Veterinary Division (Butler Schein Animal Health))
Procedure

1) Inject 0.1 mL per 10 grams mouse body-weight, IP.

Exceptions
   a) For mice greater than 40 g add an additional 0.1mL per 5 g over 40g to account for absorption of anesthetic by the animal’s fat (example 40 g ms gets 0.4, 50g ms gets 0.5+0.2 = 0.7mL).
   b) Reduce dose given to diabetic animals by one quarter or more. Diabetic animals need less anesthetic because they have less body fat.
   c) Animals that have had LPS or an infection require about half the normal dose for their weight.

2) Allow animal to relax for 10 minutes then proceed.

3) Pinch foot of animal between thumb and forefinger fingernail to check for sedation. If the animal jumps, it is not sedated.
   a) This anesthesia is a vasodilator. It increases NO in blood vessels causing relaxation but also increases iNOS activity/ concentration in leukocytes.
   b) This anesthesia is also a respiratory inhibitor. For long term anesthesia do not continue to give 3x cocktail but instead give only Ketamine (As per IACUC). Do not warm on heating pad after surgery as increased heat will increase the release of anesthetic from adipose tissue and the animal will suffer respiratory collapse.
Blood draw and wbc isolation (Alex Veenstra V1)

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Procedures

A) Blood draw

B) Leukocyte isolation

A) Blood Draw

Reagents

Nanopure sterile water (see comments at end of document on water and tube).

K2 EDTA 7.2 mg vaccutainer (BD 2011-07)

PBS 1x in nanopure water (Sigma P3813)

EDTA fill solution is 7.6 mg EDTA / 1 mL 1x PBS (1ml PBS/EDTA vaccutainer tube)

EDTA wash solution is 4 mL 1x PBS with 50 uL of 0.5M EDTA in nanopure water

1cc syringe with 25gx5/8” needle (sur-vet Thermo 100281) other syringes tend to clot blood

22g 1” needle (BD 305155)

a) Change needle to 22g 1” needle. Discard 25g needle
b) Fill syringes with EDTA wash and incubate for at least 15 min before use. Wash filled syringes may be stored at 4C

c) Just before use, squirt out EDTA wash solution and fill with 0.1-0.2 cc of EDTA fill solution. Tap out and squirt out any bubbles in the syringe and eject fluid until only 0.05 cc remains. Solution good for several hours after which filling solution must be changed.

Stock 0.5M EDTA pH8 (100mL)

a) Combine and stir without heat, 18.612g EDTA, 85 mL nanopure water and 1mL of HCL

b) Add 250uL (up to 15mL total) of HCl every 5 minutes to keep pH in desired range for dissolving EDTA.

c) This solution will take hours to dissolve so prepare ahead of time

d) Once EDTA has dissolved pH to 8.0

**Procedure**


2) Open the chest cavity. Pinch abdomen just above the penis and pull up slightly, cut with scissors near your fingers and extend the cut on both sides to the midline of the mouse. Extend the cut from the abdomen to the bottom of the ribs, carefully cut the diaphragm to deflate the lungs then extend the side cuts up through the rib cage to expose the chest cavity. Any delay will reduce the amount of blood recovered once animal stops breathing.

3) Expel fluid in syringe to 0.05 cc.
4) Insert syringe needle into left side of left ventricle, and continue inserting about 3mm so that the needle passes into the right atria, and eject a small amount of 0.05 cc of the EDTA solution to clear tissue plug from the needle. Turning the syringe and needle slightly while inserting the needle helps the needle to pass through the tissue. Do not puncture outside of the atria by inserting needle too far, or draw any fluid into syringe if by chance you do puncture outside of the right atria. Fluid on the outside of the heart causes clots!

5) Draw blood into syringe at a rate of 0.1 cc per second but not faster than the heart can fill the chamber. The blood should flow easily into syringe. If it does not turn the needle while pulling back on syringe. Drawing blood too fast will lead to collapse of the system and yield only 4 cc of blood. Expect to obtain 8cc. Air in the syringe while drawing blood or a long draw time will also lead to clots.

6) Remove syringe from mouse. Pull air into syringe. Remove needle and note final volume of blood drawn (in cc), remove gloves to eliminate mouse hair and expel blood into an uncapped EDTA vacutainer. Hair from the mouse tends to jump from the syringe and your gloves into the vacutainer and cause clots.

7) Immediately cap and mix vacutainer gently by inversion. Place on wet ice for 2 minutes if adding lysis buffer to blood.

C) Isolate leukocytes
Methods section for publication

Leukocytes were isolated from whole blood with RBC lysis buffer (Ebioscience).

Reagents

Nanopure sterile water (see comments at end of document on water and tube).

PBS 1x in nanopure water (Sigma P3813)

RBC Lysis buffer (ebioscience 00-4333-57)

Trypan blue (Sigma T8154) spin down debris before use / hemocytometer (Reichert Briteline)/ 0.7 mL tube

DMEM 1x with 1g/L or 4.5 g/L glucose, L-glutamine, 110mg/L Na Pyruvate (Gibco 11885)

HBSS 500mL with calcium and magnesium but no phenol red (Cellgrow, 21-023-CV)

HBSS 500mL without calcium, magnesium or phenol red (Cellgrow, 21-022-CV)

D-(+)-Glucose solution - 45% in H2O, tissue culture grade, autoclaved, cell culture tested (Sigma, G8769)

HEPES Buffer Solution (1 M) 238.3 g/L prepared in distilled water (gibco, 15630-106)

a) Krebs-L (Low Glucose Krebs with Ca^{2+}) Combine 500mL of HBSS with Ca, 10.2mL Hepes, 1.02mL of glucose solution.

b) Krebs-LC (Low Glucose Krebs without Ca^{2+}) Combine 500mL of HBSS without Ca, 10.2mL Hepes, 1.02mL of glucose solution.
c) Krebs-HC (High Glucose Krebs without Ca\(^{+2}\)) Combine 500mL of HBSS without Ca, 10.2mL Hepes, 5.1 mL of glucose solution
d) Krebs-LC0.1 Combine 45 mL of Krebs-LC (without Ca\(^{+2}\)) with 5 mL of Krebs-L (with Ca\(^{+2}\))

Stock 0.5M EDTA pH8 (100mL)

Combine and stir without heat

18.612g EDTA

85 mL nanopure water

1mL of HCL

Add 250uL (up to 15mL total) of HCl every 5 minutes to keep pH in desired range for dissolving EDTA.

This solution will take hours to dissolve so prepare ahead of time

Once EDTA has dissolved pH to 8.0

Procedure

a) Lysis I- Allow blood to sit on ice for at least 2 minutes but no more than 10 minutes. Add roughly 3.0 mL of ice cold RBC lysis buffer, cap tube and mix by gentle inversion. Incubate on wet ice for 5 minutes. Look for clots and remove immediately if observed. Centrifuge at 200g for 10 min at 4°C.

b) Proceeded with profusion of mouse if desired at this time.

c) Lysis II- Aspirate to 1 mL (bottom of vaccutainer label), resuspend cells by flicking with your finger (do not pipette or vortex) (flick the end of your nose with your finger. If it hurts you are flicking too hard if you can’t feel it is too
Add 3 mL of ice cold RBC lysis buffer and mix by inversion. Centrifuge at 200g for 10 min at 4 degrees.

d) Wash I- Aspirate to 1 mL then carefully using a 1 mL pipette aspirate to 200uL (where round bottom joins cylinder of the tube). Resuspend cells by flicking with your finger (do not pipette or vortex). Add 3-4 mL of ice cold low (1/10 normal) calcium buffer such as Krebs-LC0.1 (or Krebs-H0.1 for leukocytes from diabetic animal). Centrifuge at 200g for 10 min at 4 degrees.

e) Repeat step 9 (repeat twice if lots of obvious red blood cells in the pellet)

f) Aspirate to 1 mL (bottom of label) then carefully using a 1 mL pipette aspirate to 200uL (where round part joins tube). Resuspend cells by flicking with your finger (do not pipette or vortex). Add 600uL of Krebs-LC0.1 (or Krebs-H0.1 for wbc from diabetic animal). (Or resuspend gradually in Krebs with calcium buffer for Co-culture assay) Flick gently to mix.

g) Keep on ice while counting. Cells are good for several hours.

h) Flick cells to resuspend. Gently remove 15 uL of cells by pipette and add to 15 uL of trypan blue (spin stock solution to remove debris) in a 0.7 mL tube for counting. Count on hemocytometer. Don’t add trypan blue until ready to count that sample.

**Typical values are 10^6 leukocytes recovered from 800uL of blood.**

Notes and guidelines-
Water quality- Leukocytes are extremely sensitive to residual plastic monomer and contaminants in water. Use only sterile nanopure water (in glass bottles, and allowed to sit 24 hours before use or degassed). Don’t store the water in a plastic container. Saline for human irrigation is also acceptable.

Sterile filters- I use a Millipore bottle top filter which I “wash” 3 times with the nanopure water to remove the surfactant by pouring in the Millipore water and allowing it to filter through into the bottle. I autoclave the bottles then swirl the “wash” water from the filter so that I am rinsing the bottle 3 times as well. I use a 1000 mL plastic cylinder to measure the water that I rinse out prior to use.

Plastic tubes- I have found that 50 mL and 15 mL tubes have a lot of residual monomer. A simple sniff test will usually tell you if there is monomer inside, if you can smell it, it is there. Rinse out tubes with sterile water before use.

1.5 mL plastic centrifuge tubes have a tendency to cause the cells to adhere decreasing the leukocyte harvest. The vaccutainer tubes are coated with silicone.

Buffer composition- We have used the HBSS without Calcium in the past as our wash buffer and subsequently added 25 uL of Lucigen in DI water to 200 uL of buffer. The superoxide values were about 400 RLU maximum and the cells died while on ice within 2 hours due to the lack of Ca. While it is necessary to deprive the cells of Ca during the lysis process to limit activation (transient), the absence of Ca, for extended period of time depletes the internal Ca stores and kills the cells.

The addition of more than 1/10 Ca media as a reconstitution buffer provides a calcium pulse that generally activates the leukocytes with values of 1000 RLU for
ND animals and 2000-3000 for diabetic animals. A stepwise approach increasing the calcium by about 1/10 every 5 minutes on ice can be used to increase Ca levels. I found that 1/10 seems to be a reasonable value but it is better to add it at the lysis step so that the Ca build up in the solution is more gradual (lysis buffer contains edta).

Allowing the cells to sit on ice for 15-20 minutes prior to use also helps to reduce activation but allow internal stores to maintain Ca.

The number of washes- I have also found that each wash reduces the number of RBC and WBC. If the cells are to be used right away with only 1 wash try to aspirate down to the pellet and then add the wash to decrease the residual concentration of the lysis buffer.

RBC lysis time- If less than 5 minutes is used for RBC lysis prior to centrifugation the RBC tend to clump and attach to the WBC which then shear and look like they have dots (chunks of cell) on the outside as observed under the microscope. Longer incubation times decrease both the number of WBC and RBC.

Centrifugation speed and time- Centrifugation stronger than (950 rpm) 200g tends to clump the cells which cause them to break apart upon resuspension. Long Centrifugation times tend to do the same thing, however an extended exposure to the lysis buffer is worse. Once the cells have been washed they can sit on ice for 15 minutes while you catch up on other tubes.

Multiple mice- We have found by trial and error that we can draw blood from 2-4 mice prior to the addition of the lysis buffer. The key is to let the tubes sit for at least 2 minutes on ice and then add the lysis buffer. Allowing the blood to sit on
the ice for an hour seems to not be a good thing yet we have not robustly tested this theory. Lysing the blood of 2 mice at a time allows the user to collect blood while the previous 2 tubes are in the centrifuge.

Triple cocktail seems to activate leukocytes, especially transient iNOS expression in vivo, therefore we have switched to anesthetizing the mice with CO₂ for about 45 seconds.
Profusion of mouse procedure (Alex Veenstra V2)

Location: C:\Documents and Settings\aav2.ADS\My Documents\kerns lab\procedures\basic lab procedures\Profusion of mouse procedure for retina.doc

**Perfusion procedure** (note this procedure was approved by the Institutional Animal Care and Use Committee IUCAC).

**Methods section for publication**

Animals were perfused (18ml/min) with 40 mL of saline, 1.5mg of Concavalin A in 10 mL of PBS, and then again with 40 mL of saline.

**Reagents**

Profusion apparatus

- Pressure infuser (Infusurge, 4010, Ethox Buffalo NY)
- IV Catheter set with regulating clamp (2C5417s, 70 inches, Baxter)
- Gavage Needle (7920 22g, 1.5”, 1.25mm bal straight cadence science)
- Saline Buffer (0.9% Veterinary grade, 1000 mL Baxter 04925-04-10)
  
  a. Option Heat saline bag to 37C
  
  b. Hang bag at 34 inches above counter top in pressure infuser
  
  c. Connect catheter set and gavage needle
  
  d. Option connect a 3 way valve at end of catheter set. Close port to the side, Connect two 3 foot extensions of IV line together and then to the valve.
Connect second three way valve at end. Connect 1 foot extension to bottom of valve. Connect gavage needle to end of short extension. Note can also put in 0.22 micron filter between third extension and the 3 way valve.

e. Set pressure cuff to 150, 200 or 250 mmHG

f. Set flow rate to 18 mL/min

g. Purge line of all air bubbles

h. Option Attach syringe filled with dye to 3 way valve closest to the gavage needle, turn the valve to shut off flow to the extension and gavage needle allowing flow to fill the syringe a little to push bubble from port into syringe. Turn off flow to syringe

Optional reagents

Concanavalin A Rhodamine Vector RL-1002 25mg in 5mL, use 300uL/ms in 10 mL of PBS

Concanavalin A FITC Vector 25mg in 5mL, use 200uL/ms in 10 mL of PBS

Concanavalin unlabeled Vector 25mg in 5mL of ConA buffer

ConA buffer 10mM Hepes,0.15MNaCl,0.1mMCa,0.08%NaN3 0.01mMMn

3 way IV line valve ()

2x1 inch IV extension set (McGraw V5406, 3ml 21” Male Leur 060232396)

2 x36 inch IV extension set ()

10 mL syringe ()
Procedure

1. Set flow rate and then decrease pressure in cuff (not flow regulator) until only a trickle comes out.
3. Starting at groin pinch skin and lift up using scissors to cut through skin into abdominal cavity.
4. Extend cut to sides
5. Pull up skin on one side to separate from organs and fat and extend cut up the body towards the diaphragm. (note be careful not to cut liver, organs or fat)
6. Repeat on other side.
7. Cut diaphragm slightly at edge to allow air into cavity, then cut diaphragm.
8. Cut rib cage and extend past heart stopping a half inch from aorta
9. Use scissors to clear fibrous material from heart
10. Option- Draw Blood if leukocyte studies are desired. Open chest cavity of mouse and draw blood (Blood draw and wbc isolation.doc).
11. Cut left ventricle with scissors about 1cm
12. Insert gavage needle through heart just into aorta
13. Clamp heart with hemostats across lower half.
14. The right atria should begin to swell, cut it immediately to avoid pressure damage

If no flow coming out
i) check to see you are not inflating lungs (or water coming from nose) which means needle is not in aorta but in the pulmonary vein
ii) check if there is flow coming out of needle, by observing dripping in catheter set.

iii) release hemostats and reposition so as not to cut off flow to the atria

15. Increase pressure to 150, or 200 mm Hg (whatever pressure was used to set the flow rate)

16. Allow mouse to profuse for 2 minutes during which

a) Move gavage needle around slightly

b) Roll mouse onto right and then left side

c) Between heart and kidney on your right, mouse’s left, push down on aorta next to spine for 3-4 seconds

Option to inject dye into animal

17. Stop flow with quick shut off

18. Turn 3 way valve near needle to closed into mouse open to port and incoming line

19. Turn three way valve at other end to stop output from saline bag, open to port and open to line between valves

20. Starting at the port near the needle, use syringe to inject dye through the port and into the extension line between valves. Allow displaced fluid to exit port closest to saline bag. Close the 3 way valve near the saline bag. Turn on flow so a small amount of fluid moves back into syringe clearing any bubbles. Turn off port to syringe opening flow into the mouse.

21. Profuse for 2 minutes to stain with dye, and another 2 min to washout dye
22. Repeat dye loading procedure for formalin if desired, except when mouse hands clap immediately stop the flow of fluid into the mouse.

23. Remove hemostats

24. Turn off flow

25. Remove gavage needle and decrease pressure.

Average cardiac output of mouse = 500mL/min/kg (Janssen et al. 2002)
Retina isolation procedure

Methods section for publication
The anterior section of eye was removed just below the limbus with a Teflon coated razorblade (electron microscopy sciences) from enucleated eyes of saline perfused mice (18 mL/min) anesthetized with CO₂. The retina was carefully detached from the sclera at the ciliary body junction with a micro spatula prior to cutting the sclera with micro scissors from the limbus to the optic nerve, and cutting the optic nerve.

Reagents
RS 5983 micro dissecting scissors curved blunt 22mm (ROBOZ)
Kimwipes (fisher)
71970 Razor blades single edge stainless steel GEM Teflon coated 62-0165 (electron microscopy sciences EMS)
72670 Pink Dental wax (EMS)
72700-D Tweezers Dumont #5 Biological grade tip (EMS)
0103-2-PO Tweezers Dumont electronic grade tip (EMS)
RS-5620 VANNAS Micro dissecting spring scissors 3mm straight ROBOZ
Micro spatula FST 10091-12 (fine science tools)
Jenco dissecting scope
Light source MI-150 fiber lite

Procedure

27. Profuse mouse if desired (Perfusion of mouse procedure.doc)
28. Proptose the eye. Turn mouse on side and place thumb on head just below eye but above jaw and index finger just above eye but not on top of head. Gently push down with fingers and spread them apart. The eye should pop out of the socket exposing the attachment of the muscle to the eye.
29. Enucleate one eye. While eye is proptosed use sharp curved scissors to scoop under the eye and cut the muscular attachment. Be careful not to pull the eye or to cut off the back of the sclera. Enucleated each eye and isolate each retina before enucleating the opposite eye.
30. Remove anterior segment of eye. (make an eye cup) The Retina in the mouse is attached to the sclera at the optic nerve and at the junction of the cornea, sclera, iris, and cilia body. The vitreous is loosely bound to the retina and often resembles a membrane bound sack in diabetic or old mice.
   a) Orient eye- Place eye on small piece of wet Kimwipe on top of dental wax under dissection scope with external lighting. Orient eye so that optic nerve faces left and pupil faces right with side pointing up. Use blunt end
tweezers to hold muscle attachment between the (clear) cornea and (blue) sclera.

b) Cut at limbus- Use fresh Teflon coated razor blade to make an incision 2-3 mm below the limbus (cornea sclera junction). Specifically while holding muscle with tweezers, draw blade across eye while pushing gently down. Rotate eye and repeat until over half the cornea is detached. Avoid sawing (back and forth motion). Pushing down to finish cut often results in cutting the retina close to the optic nerve as the blade is deflected off the lens.

c) Remove kimwipe. Eye should be only on dental wax to avoid retina from sticking to kimwipe.

d) Remove lens- It is usually helpful at this point to carefully push the lens out of the eye noting that the vitreous may come with it.

e) Finish Cut. Use the razor blade or a pair of micro scissors to cut the retina-sclera just below the limbus. Note pushing the razor into the wax will dull the blade preventing a good initial cut with the same blade.

6) Detach retina from limbus junction.- Orient the eye so that optic nerve is facing down. Locate at least one area where retina is puckered or not attached to the sclera. Grab muscle on outside of sclera with tweezers or if possible the sclera but not the retina at the edge. Place spatula between sclera and retina working all the way around the eye cup. Where the retina is attached gently use spatula to break the connection by lifting up from below. Note that this will often leave a very sticky ribbon of vitreous and iris at the end of the retina. Alternatively the micro scissors can be used to cut the retina below the limbus so that the attachment is
avoided. Note avoid pulling on retina as it will cause activation of the tissue that lasts for hours, while cutting it does not.

7) Cut optic nerve- Either use the micro scissors to cut the sclera down to the optic nerve, and then cut the optic nerve, or scoop out the retina with the micro spatula.

8) Immediately transfer retina to a 1.5 mL tube with buffer or flash freeze on dry ice.
Methods section for publication

Retinae were quickly isolated from enucleated eyes of saline perfused mice (15 mL/min) previously anesthetized with CO₂, flash frozen on dry ice and stored at -80°C. Retina was homogenized in 300uL of Trizol (Invitrogen) with 50uL of 0.5mm RNase free stainless steel beads (Next Advance) in a Bullet blender (Next Advance) for 2x5 minutes at 4°C. mRNA was extracted from Trizol according to manufacturer’s instructions, resuspended in nuclease free water (Ambion), and stored at -80°C until use. cDNA was transcribed from mRNA using iScript
Reverse transcription super mix (BioRad) followed by RTqPCR (BioRad) using fast Start Universal SYBR Green master mix (Roche) with the primers listed below (Invitrogen). Products of the PCR reaction were checked for primer dimers on TAE 2.5% agarose gel.

iNOS F1 TCT-TTG-ACG-CTC-GGA-ACT-GTA-GCA,
iNOS R1 TAG-GTC-GAT-GCA-CAA-CTG-GGT-GAA

**Reagents (section A and B)**

1.5 mL locking Eppendorf tubes (Nuclease free sterile) 022363204

Trizol (Invitrogen 15596-026)

SSB05-RNA 0.5 mm RNase free stainless steel beads (next advance.com)

Chloroform (fisher C298-500)

Nuclease free water (AM9938 Ambion)

Glycogen (Glycogen blue Invitrogen am9515)

Isopropyl alcohol (Acros nuclease free 327272500)

Bullet blender homogenizer (Next Advance BBX24)

95% Ethanol

Nanodrop 2000 (Thermo Fisher)

RNase free pipette tips with filters (Biochemistry stock room)

Note: Clean exterior of all pipettes with soap and warm water to remove protein and DNA debris on them. The interior of the pipette is usually full of cellular debris. Wipe the pipettes with ethanol. Then place pipettes in
the culture hood with the UV light on for several hours to destroy residual DNA. Finally clean the work area in the culture hood and the pipettes with RNase away or a similar product. Store pipettes were co-workers will not use and contaminate them.

**Procedure (A mRNA extraction from retina)**

(modified from manufacturer’s instructions for Trizol from Invitrogen)

1) Anesthetize mouse with CO₂ (Anesthesia Protocols.doc).

2) Profuse mouse if desired (Perfusion of mouse procedure.doc).

3) Enucleate eye and remove retina (Retina Isolation.doc).

4) Isolate retina, transfer into 1.5 mL tube with 300uL of Trizol, flash freeze on dry ice, and store at -80°C until step 5.

5) Remove tube from storage at -80°C, add 50uL stainless steel beads to each tube, immediately transfer to wet ice and homogenize in bullet homogenizer power level 8.5 for 2x 5 min at 4°C.

6) Transfer lysate to new tube using magnet to retain beads, wash beads with 50uL nuclease free water and add water to Trizol/supernatant solution.

7) Allow supernatant to sit RT for 5 min.

8) Add 70uL chloroform (20/100uL Trizol).

9) Shake for 60 seconds and then let stand for 2-3 min.

10) Centrifuge at 12,000xg, 4C, for 15 min (do not spin at max speed).
11) Tilt tube at 45 degrees and transfer only the upper aqueous layer to a new tube on wet ice (about half of volume of Trizol) (note ok not to get it all, exclude middle and lower pink phase).

12) Add 1ug of glycogen to supernatant (0.75uL of 1.5ug/uL), and mix gently by inversion

13) Add 50uL Nuclease free water to lower phases, shake, and re centrifuge.

14) Pipette off supernatant (upper phase only) and add to that previously collected.

15) Transfer the majority of the lower phase (about 160uL of pink liquid) to new tube (pipette from bottom of tube).

16) Centrifuge remaining middle upper and lower phase at 12,000xg , 4C, for 15 min

17) Pipette off supernatant (upper phase only) and add to that previously collected. Pipette up and down to mix remaining lower and middle phases. Transfer to lower phase tube. Use 100uL of pink lower phase to wash out remaining middle phase (sticky) and combine the lower and middle phases in a tube.

18) Freeze lower phase at -80°C for later use in the extraction of protein from this sample.

19) To upper phase supernatant add 500uL of isopropanol, mix by inversion, and let stand at RT for 10 min.

20) Centrifuge at 12,000xg , 4C, for 15 min (do not centrifuge at maximum speed).

21) Help precipitate residual RNA from solution by incubating at 4°C for 10 min, then at -80°C for 4 min, then centrifuge at 12,000xg , 4°C, for 15 min (do not centrifuge at maximum speed).
22) Looking at the pellet on the side of the tube carefully remove supernatant from the tube without displacing pellet. It is a good idea to transfer supernatant to another tube just in case you suck up the pellet. Also check the pipette tip for the pellet.

23) Resuspend the pellet in 75% cold ethanol.

24) Centrifuge at 12,000xg, 4°C, for 15 min (do not centrifuge at maximum speed).

25) Remove supernatant and allow pellet to air dry for 10 minutes (over drying causes RNA not to resuspend).

26) Resuspend in 130μL of nuclease free water, pipette gently to resuspend.

27) Cook at 55-60°C for 15 min, flick by hand to resuspend.

28) Measure RNA concentration with Nanodrop spectrometer (pharmacology)
   
   Note if frozen Re heat 55-60 for 15 min and flick by hand to resuspend.
   
   Rinse Nanodrop aperture with clean water.
   
   Blank the Nanodrop with one drop of sterile water on the aperture.
   
   Add 1μL RNA solution to Nanodrop aperture to measure RNA.
   
   Sample the RNA solution at the bottom and the top of the solution. If the concentration is > 100ng/μL dilute to 70 ng/μL.
   
   Re-heat 55-60 for 15 min and flick by hand to resuspend.
   
   Re-measure concentration.

29) Use mRNA to make cDNA as detailed below or freeze at -80°C and then repeat steps 27-28.
B) mRNA extraction from whole blood

1) Anesthetize mouse with CO₂ (Anesthesia Protocols.doc).
2) Open chest cavity and draw blood into syringe filled with EDTA solution (Blood draw and wbc isolation.doc).
3) Transfer contents to 7.2 mg EDTA vacutainer and mix by inversion a few times.
4) Add 200 uL of whole blood immediately to 800 uL of Trizol.
5) Shake mixture and pipette a few times to resuspend and break cells.
6) Flash freeze on dry ice and store at -80C.
7) Follow steps 5-29 above in section A to extract mRNA.

C) Protein isolation from lower phase of Trizol (note does not work well)

Note: There is protein in the upper phase of Trizol mixture as well.

1) Transfer frozen lower phase of Trizol to wet ice and add 1/10 volume of 10% SDS and 5uL of protease inhibitor
2) Dialyze phenol out of solution with 200 mL of 0.1%SDS, EDTA, EGTA (20 mL 10% SDS, 800uL 0.5M EDTA, 200uL of 0.5M EGTA) at 70-80 C using 2000MWCO regenerated cellulose membrane, not cellulose ester membrane which will be degraded by the phenol.
3) Transfer to new dialysis buffer and dialyze overnight.

Notes: Solubility of the protein drops quickly as phenol is slowly removed, red color (not phenol) remains with the protein and sticks to membrane.
Remaining reagents interfere with western blot causing the protein to smear in the gel.

D) cDNA synthesis

Reagents

Nuclease free water (AM9938 Ambion)
iScript Reverse transcription super mix for RT-qPCR (Biorad 170-8840)
Total mRNA (from procedures A and B above)
PCR strips (LPS L232450 0.2mL 8 snap flat cap strip)
RTqPCR machine (BioRad)

Procedure

Note: if primers correspond to DNA sequence then the sample must be treated with DNase prior to making cDNA. Then the DNAse needs to be neutralized by heating.

1) Combine 4uL master mix, 1 ug total RNA, and nuclease free water to a final volume of 20 uL.
2) Transfer to PCR vial strip and close cap. Mark top of cap with colored marker on end for future reference.
3) Mix contents of strip by gentle flicking.
4) Centrifuge briefly to force contents of the vial to the bottom (note use plastic insert from 200uL tip refills to hold strips in 96 well plate adaptor for the centrifuge.)
5) PCR at 5 min 25°C, 42 min 60°C, 5 min 85°C
   a) Turn on machine using switch by the electrical plug in the back of the
      machine.
   b) Open the machine using the bar and insert strips in the wells
   c) When prompted to log on, press ENTER to pull up the name menu.
   d) Use the Arrow key to scroll down/up to QL and press ENTER to select QL.
   e) Press NEXT key to move to password and press ENTER key.
   f) Press the ENTER key again top open up files.
   g) Use the Arrow key to scroll down to rt254885 and press the ENTER key to
      select program.
   h) Press the start key to start program.
   i) When finished press exit from screen, Press enter to close file, select log out,
      then select turn off and turn off the power switch when prompted by screen

6) Store cDNA at -80°C

E) qPCR procedure

Reagents

Primers Stock 25nM desalted (Invitrogen).

Reconstitute vial with 212uL of nuclease free water and store –at -80°C.

Nuclease free water (AM9938 Ambion)

Primers working dilution
Make 1/10 dilution of stock primer (10 uL primer to 90 uL water) and store at -20C.

master mix 2x fast Start Universal SYBR Green master (Roche 04 913 850 001)(-20 storage)
cDNA from procedure D above
96 well PCR plate (Biorad MLL9601)
Adherent 96 well plate sealing sheet Biorad (micro seal B film MSB1001)

Procedure

1) For each well, combine 10 uL master mix with 1uL of cDNA, 1 uL of each primer and 8 uL of water. Minimize error by making a batch and then pipette to each well.

2) Load samples into plate in triplicate.

3) The qPCR parameters for the run are 50C for 2 min, 95C for 10 min, then repeat 40 times heat to 95C for10s, cool to 55C for 30 s, and then take a picture, after the end of 40 cycles, heat to 95 for 10 s, 65 for 10s, then calculate 95C melt curve.

F) qPCR product verification

Reagents

Agrose (HS molecular biology grade sdt/high melt denville CA3510-8 500g)
TAE 1x (10x 48.4g Tris base, 11.4mL glacial acetic acid 17.4 M 3.7g EDTA, 800mL H2O up to1L)
Ethidium bromide 10 mg/mL

1Kb DNA ladder

Loading dye

**Procedure**

1) Cast new Gel
   
   a) Combine 2.5 g agarose in 100 mL 1xTAE.
   
   b) Microwave 3x2 min.
   
   c) Allow to cool for 2 min.
   
   d) Add 2uL of Ethidium bromide solution and mix gently (note EtBr causes cancer and migrates through both skin and latex gloves in seconds).
   
   e) Pour gel and allow 20 minutes for it to solidify.
   
   f) Immerse gel into TAE in running apparatus.

2) Load 3 uL 1kB ladder in lane 1

3) Remove samples from 96 well plate and load into agarose gel.

4) Run at 100V for 20 to 40 minutes (dimers are near dye front).

5) Check band size using UV light box.

6) Discard results that have primer dimers and change conditions during PCR run (warmer) or redesign primers.
Tail vein injection protocol (Alex Veenstra V1)

Location C:\Documents and Settings\aav2.ADS\My Documents\kerns lab\procedures\basic lab procedures\ Tail Vein Injection Protocol.doc

**Reagents**

- Syringe- Thermo 1cc with 27gx1/2” non removable needle #SS*01A2713 note can use 28g for smaller animals
- Heat lamp 11.25 inches from counter top, GE infrared heat reflector 250W (250R40-10)
- Mouse tail vein isolator (Rotating tail injector (RTI) Braintree scientific inc)
- line sides of slope with tape to prevent light from blinding you. Line tube with ½ inch wide paper towel for small mice or buy smaller tube
- Replacement bulb GE 120V 4W 38 lumens candelabra screw (E12), bulb shape C7
- Night light 57YL with adaptor and extension cord
- Sterile liquid for injection ie saline or DMEM 1x 10-013-cv cellgro

**Procedure**

a. Place heat lamp at 11.25 inches above benchtop.

b. Warm mice under heat lamp for 7 minutes to see tail vein. Mice jumping (like popcorn popping) indicates that they are too warm and may die from shock.

c. Place mouse in isolator and compress to avoid mouse movement
d. Locate tail vein by rotating mouse 45 degrees. Tail vein lines up with ears.

e. Clear syringe of all bubbles and load up to 200uL. Bubbles will kill mouse.

f. Starting near the end of the tail slide the needle just under the surface and then push back towards the surface and into the mouse about 3mm.

g. Plunger should move easily if needle is in tail vein.

h. Inject up to 200 uL into the tail vein of each recipient mouse.
Methods section for publication

Animals were anesthetized with CO₂

Reagents

1.5 mL sterile tubes (Eppendorf)

0.7 mL sterile tube (Eppendorf) with hole in bottom created by 18g needle

RBC lysis buffer (00-4333-57 ebioscience)

DMEM (1x 10-013-cv cellgro)

Syringe

PBS

Procedure

1) Irradiate recipient mice with two 600 rad doses spaced 3 hours apart in Cesium irradiator.

2) Anesthetize donor mice, cut off hind legs, remove foot and skin and place leg in ice cold sterile pbs
3) For each leg remove majority of muscle and cut tendons between joints to isolate femur and tibia. Don’t let bones dry out during process. (humerous and hip can be used but has a low marrow yield)

4) Remove muscle from bone using scissors and sterile chemwipes. Soaking in PBS will swell muscle tissue and help to identify residual tissue. 

5) Remove ends of bone and transfer to fresh PBS. 

6) Place bones in 0.7 mL tube with hole and place that tube in a sterile 1.5 mL tube

7) Spin at 2-3000 rpm for 45 seconds in benchtop micro-centrifuge. 

8) Suspend cell pellet in 0.6 mL RBC lysis buffer and incubate on ice for 2 min. Pipet up and down to break up clumps. 

9) Combine all cells in 10 mL of DMEM and add DMEM to achieve 14 mL. 

10) Mix 15 mL tube by inversion and look for clumps of muscle tissue. 

11) Allow clumps to settle to bottom of tube for 1-2 min and transfer supernatant to fresh 15 mL tube. 

12) Centrifuge at 300g for 5 min at 4 degrees. 

13) Discard supernatant, resuspend cells in 1mL of DMEM and transfer to 1.5 mL Tube. 

14) Add additional DMEM as per the number of mice to be injected. In general 1 mouse is good for 8-10 recipients. Calculate (# recipients + 1 extra)* 200 uL per injection and add DMEM to achieve this total volume 

15) Remove 10 uL for counting with trypan blue
16) Warm irradiated mice for 7 minutes to see tail vein. (Tail vein injection procedure.doc).

17) Inject 200 uL of cells into the tail vein of each recipient mouse. (3-5*10^6 cells / ms)
Reagents

Lucigenin- N,N'-Dimethyl-9,9'-biacridinium dinitrate - (M8010, Sigma-Aldrich) Lucigenin is used to measure superoxide anion production. It emits light on reduction and interaction with the superoxide anion.

a) 2.5 mg lucigenin in 1 mL DI water, keep on ice and out of light until use. Make enough for 1 week to be stored at 4°C in the dark. Aliquot 1mL for daily use and spin out debris at 10,000 rpm (max speed) for 5-10 min. Don’t use last the 50 μL containing the pellet.

kimble borosilicate glass tubes, round bottom (fisher, 73500-107510x75mm)

Monolight 2010 luminometer. Warm up time for instrument 15 min

HBSS 500mL with calcium and magnesium but no phenol red (Cellgrow, 21-023-CV)

HBSS 500mL without calcium, magnesium or phenol red (Cellgrow, 21-022-CV)

D-(+)-Glucose solution - 45% in H2O, tissue culture grade, autoclaved, cell culture tested (Sigma, G8769)

HEPES Buffer Solution (1 M) 238.3 g/L prepared in distilled water (gibco, 15630-106)
a) Krebs-L (Low Glucose Krebs with Ca\(^{+2}\)) Combine 500mL of HBSS with Ca, 10.2mL Hepes, 1.02mL of glucose solution.

b) Krebs-LC (Low Glucose Krebs without Ca\(^{+2}\)) Combine 500mL of HBSS without Ca, 10.2mL Hepes, 1.02mL of glucose solution.

c) Krebs-HC (High Glucose Krebs without Ca\(^{+2}\)) Combine 500mL of HBSS without Ca, 10.2mL Hepes, 5.1 mL of glucose solution

d) Krebs-L 1/10 calcium Combine 45 mL of Krebs-LC (without Ca\(^{+2}\)) with 5 mL of Krebs-L (with Ca\(^{+2}\))

Note both Hepes and Lucigenin are temperature and light sensitive.

Procedure

1) Isolate tissue of interest. See specific tissues below for additional details.

2) Add tissue and 200uL (volume changes with tissue) Krebs-Hepes buffer to borosilicate glass tube and incubate for 25 minutes (incubation time changes with tissue) at 37C and 5% CO\(_2\) (cell culture incubator) (Note keep initial buffer temperature consistent with tissue temperature to avoid thermal shock.)

3) Add 25 uL of lucigenin buffer (per 200uL of Krebs buffer) to tube and incubate for 5 minutes in the incubator

4) Gently shake but don’t vortex each tube just before use. (Gently swirl retina in tube vortexing will ruin retina, Too much shaking will increase readout, and too little will give a poor reading. For leukocytes and endothelial cells clumps will increase the readout as they break apart (clumps are bad).
5) Read luminescence in lumoinometer three times at 3 second read time then replace in incubator. If one of the three values is too high or too low repeat a fourth time.

6) When finished reading all tubes allow at least 30 seconds and repeat twice more. Values between data sets will decrease slightly with time.

7) Save tissue for protein measurement.

8) Express results as RLU/mg protein or RLU/units of cells. See individual tissues for sample results.

   (RLUs for blank = RLU empty glass tube = RLU for Krebs buffer with lucigenin = 40 RLU)

A) Retina

1) Anesthetize mouse (Anesthesia Protocols.doc) 3x cocktail can cause changes in vascular dilation with short incubation times.

2) No need to perfuse mouse however historically we perfused with saline at 18 mL/min, (Perfusion of mouse procedure.doc).

3) Isolate retina (Retina isolation.doc) (Do not immerse in PBS as it decreases superoxide) (Don’t damage retina or it will increase superoxide). Eyes need to be harvested without being cooled or placed in PBS. Stretching the retina by pulling activates it! Remove epi-retinal membrane if present and cut pattern of clover leaf into retina to prevent sticking and curling.
4) Incubate retina in 200uL of prewarmed buffer for 7 (or 25 min) minutes 37°C, and 5% CO₂ (cell culture incubator) add 25 uL of lucigenin and incubate for 2 minutes before reading. Save retina for protein counting.

5) Gently swirl retina in tube (vortexing will ruin retina)

6) Read luminescence in lumoinometer three times using a 3 second read time then replace in incubator

7) Gently shake each tube just before use. Read all tubes in lumonometer for 3 seconds three times and place tube back at 37 degrees. When finished reading all tubes allow at least 30 seconds and repeat twice more. Too much shaking will increase readout, and too little will give a poor reading. You should gently shake tubes. If there are clumps they will increase the readout as they break apart (clumps are bad).

8) Rinse retina in PBS and sonicate in 80 uL of PBS at setting 2.5. Freeze until measure mg protein. 1 retina is generally about 80 mg of protein.

9) Express as average RLU/mg protein

Average RLU values for blank are 40 RLU reading an empty glass tube or a glass tube with 200uL Krebs and 25uL Lucigenin.

Average RLU values for retina from non-diabetic mice is 130 RLU and 200 RLU or greater for retina from diabetic mice.

B) Bone marrow

1) Isolate bone marrow (Bone marrow transplant.doc). Resuspend bone marrow in 3mL of 1/10 Calcium Krebs buffer with appropriate glucose concentration,
incubate on wet ice and determine the number of cells per mL with a hemocytometer. Approximate concentration of cells is 400,000 cells per 100 uL using 1 femur and 1 tibia.

2) Resuspend 2 million bone marrow cells in 400uL 1/10 calcium Krebs buffer with appropriate glucose concentration.

3) Incubate at for 25 minutes 37°C, and 5% CO₂ (cell culture incubator) but flick tube every 10 minutes to break up clumping.

4) Add 50 uL of luciginin and incubate for 5 minutes.

5) Repeat steps 5-7 for the retina procedure above.

C) Spleen

1) Isolate spleenocytes- Remove spleen and place in petri dish with 600 uL of RBC lysis buffer. Cut off end of spleen and gently rub spleen towards cut end using a microspatula to push out cells from the encased spleen. Pipette lysis buffer and spleenocytes up and down to break up chunks. Add solution to pre chilled 15 mL tube with 10mL of Krebs buffer and store on ice for a few minutes. Centrifuge at 250x g for 7 minutes at 4°C. Aspirate wash solution to just above pellet and resuspend spleenocytes in 10mL of 1/10 Calcium Krebs buffer with appropriate glucose concentration, incubate on wet ice and determine the number of cells per mL with a hemocytometer. Approximate concentration of cells is 400,000 cells per 80-200 uL per normal spleen.

2) Resuspend 1 million spleenocytes in 400uL of 1/10 Calcium Krebs buffer with appropriate glucose concentration.
3) Repeat steps 3-5 for the bone marrow section above.

4) Average RLU for spleenocytes from non-diabetic mice are 140 RLU and 200 RLU for spleenocytes from diabetic mice.

D) Leukocytes

1) Isolate leukocytes (Blood draw and wbc isolation.doc), resuspend in 600uL of 1/10 Calcium Krebs buffer with appropriate glucose concentration, incubate on wet ice and determine the number of cells per mL with a hemocytometer.

2) Resuspend 400,000 leukocytes in 400 uL of 1/10 Calcium Krebs buffer with appropriate glucose concentration.

3) Repeat steps 3-5 for the bone marrow section above.

4) Average RLU for leukocytes from non-diabetic mice are 250 RLU and 500 RLU for leukocytes from diabetic mice.

E) Endothelial cells

1) Isolate endothelial cells- After 24 hours of co-culture with leukocytes (coculture assayprocedure.doc), gently rinse endothelial cells with PBS to remove leukocytes, incubate with trypsin for 2 minutes, and wash twice in PBS. Resuspend endothelial cells in 3mL of 1/10 Calcium Krebs buffer with appropriate glucose concentration, incubate on wet ice and determine the number of cells per mL with a hemocytometer.

2) Resuspend 80,000 endothelial cells in 400 uL of 1/10 Calcium Krebs buffer with appropriate glucose concentration.
3) Repeat steps 3-5 for the bone marrow section above.
Bibliography


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