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The Peroxisome Proliferator-Activated Receptor-β/δ antagonist GSK0660 mitigates Retinal Cell Inflammation and Leukostasis

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Abstract

Diabetic retinopathy (DR) is triggered by retinal cell damage stimulated by the diabetic milieu, including increased levels of intraocular free fatty acids. Free fatty acids may serve as an initiator of inflammatory cytokine release from Müller cells, and the resulting cytokines are potent stimulators of retinal endothelial pathology, such as leukostasis, vascular permeability, and basement membrane thickening. Our previous studies have elucidated a role for peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) in promoting several steps in the pathologic cascade in DR, including angiogenesis and expression of inflammatory mediators. Furthermore, PPARβ/δ is a known target of lipid signaling, suggesting a potential role for this transcription factor in fatty acid-induced retinal inflammation. Therefore, we hypothesized that PPARβ/δ stimulates both the induction of inflammatory mediators by Müller cells as well the paracrine induction of leukostasis in endothelial cells (EC) by Müller cell inflammatory products. To test this, we used the PPARβ/δ inhibitor, GSK0660, in primary human Müller cells (HMC), human retinal microvascular endothelial cells (HRMEC) and mouse retina. We found that palmitic acid (PA) activation of PPARβ/δ in HMC leads to the production of pro-angiogenic and/or inflammatory cytokines that may constitute DR-relevant upstream paracrine inflammatory signals to EC and other retinal cells. Downstream, EC transduce these signals and increase their synthesis and release of chemokines such as CCL8 and CXCL10 that regulate leukostasis and other cellular events related to vascular inflammation in DR. Our results indicate that PPARβ/δ inhibition mitigates these upstream (MC) as well as downstream (EC) inflammatory signaling events elicited by metabolic stimuli and inflammatory cytokines. Therefore, our data suggest that PPARβ/δ inhibition is a potential therapeutic strategy against early DR pathology.

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Keywords

Peroxisome proliferator-activated receptors; Diabetic Retinopathy; Inflammation

1.1 Introduction

Diabetic retinopathy (DR) is a leading cause of blindness in the United States (1). DR is a complication of diabetes consisting of two stages: an early stage called nonproliferative diabetic retinopathy (NPDR) characterized by microvascular changes, such as development of microaneurysms and hemorrhages; and a later stage called proliferative diabetic retinopathy (PDR) characterized by neovascularization. PDR is responsible for the irreversible vision loss observed in diabetics. Current treatments target PDR; however, providing therapies for early disease pathology could prevent the irreversible damage that accompanies progression to this later disease stage.

DR is a neurovascular inflammatory disease associated with metabolic dysfunction and chronic elevation of retinal inflammatory mediators. Systemic metabolic changes are associated with retinal cell activation, leading to increased synthesis and release of inflammatory cytokines. Notably, we have recently identified that metabolic stimuli associated with dyslipidemia specifically activate Müller cells (MC) to increase their production of inflammatory cytokines (2, 3). This supports a conceptual framework in which MC-derived inflammatory cytokines exert their actions through autocrine amplification of these cytokines and paracrine effects on retinal endothelial cells, among others. These amplification steps would then ultimately reach levels commensurate with severe inflammation-mediated tissue damage (4–6). Retinal endothelium respond to these inflammatory cytokines with increased expression of chemokines and leukocyte adhesion molecules that facilitate leukocyte homing, tethering and rolling, all leading to leukostasis (firm adhesion of leukocytes) (7–10). Leukostasis can cause vaso-occlusion, or the arrested leukocytes can transmigrate into perivascular tissues further escalating chronic vascular inflammation. Leukostasis has been linked to EC tight junction dysfunction, blood-retina barrier breakdown, and capillary dropout (11–13). Since leukostasis occurs before any other clinical sign of DR and is linked to DR progression, it constitutes an attractive target for early DR therapy (14).

Mounting evidence supports the investigation of fatty acids as relevant stimuli to model diabetes-linked dyslipidemia in DR (2, 3, 15–17). Serum and tissue profiles from diabetic patients and experimental models of diabetes demonstrate that one saturated fatty acid, palmitic acid (PA), is elevated above others (17–19). Increased retinal levels of PA, as well as the unsaturated fatty acids oleic acid (OA) and linoleic acid (LA), have been observed early in rodent models of diabetes (17, 20). Notably, there is evidence that these fatty acids elicit inflammatory responses in Müller cells and retinal microvascular endothelial cells (RMEC) (3, 15, 16). These data suggest that elevated fatty acids may be causally linked to retinal inflammation occurring early in DR pathogenesis, and provides a novel target for DR therapeutic intervention.

Our data and those from other labs, have shown that MC amplify tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) interleukin-8 (IL-8) and Angptl4 through autocrine and paracrine signaling pathways and each has been linked to angiogenic and/or inflammatory component of DR (4, 21–24). Tumor necrosis factor alpha (TNFα) induces vascular inflammation, and increased serum and vitreous levels of TNFα correlate with the onset and progression of DR (25–29). TNFα stimulates retinal endothelial cells to express inflammatory chemokines and the leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin (30, 31). IL-1β, IL-6 and IL-8 are inflammatory cytokines and increased retinal/vitreous levels have been observed in DR animal models and patients (25, 32–36). Thus, these cytokines constitute rational targets for therapeutic intervention in order to limit DR progression. Furthermore, drug targets that manipulate the production of multiple cytokines may be more efficacious than targeting any one cytokine alone.

PPARβ/δ is a transcription factor that is primarily known for its role in lipid metabolism, however our group and others have shown that it can regulate both inflammation and angiogenesis (37–40). Angiopoietin-like 4 (Angptl4) is one of its known target genes (41). Notably, Angptl4 has been firmly established as a potent angiogenic/permeability factor in DR (24, 42–44). GSK0660 is a selective antagonist of PPARβ/δ, which also has inverse agonist activity when used alone (45). Our lab generated evidence that GSK0660 inhibits the TNFα-induced expression of multiple chemokines human retinal microvascular endothelial cells (HRMEC) (40), however the functional significance of these transcriptional changes has not been evaluated. Additionally, using an unbiased approach, we recently observed that several target of PPARβ/δ activity were altered in PA-treated Müller cells (2). As such, we hypothesized that PPARβ/δ constitutes a rational therapeutic target for DR because it may exert its effects at multiple steps in the pathologic cascade. Specifically, we used GSK0660 to interrogate the role of PPARβ/δ in: 1) the induction of pro-inflammatory cytokines in human MC (HMC), 2) the paracrine effect of one of these cytokines, TNFα, on leukocyte adhesion, and 3) TNFα-stimulated retinal leukostasis. These findings represent novel extensions of our previous work (2, 40). Importantly, we identify a role of GSK0660 in the upstream production of inflammatory mediators by Müller cells. Moreover, we demonstrate that transcriptional changes associated with GSK0660 treatment in retinal endothelial cells elicits significant changes in inflammatory behaviors that occur early in DR pathogenesis. These findings further support the clinical potential of PPARβ/δ manipulation in the treatment of DR.

2.1 Materials and Methods

2.2 Reagents

The PPARβ/δ inhibitor GSK0660 and the PPARβ/δ agonist GW0742 were purchased from Tocris (Minneapolis, MN, USA). Palmitic acid and fatty acid-free BSA was purchased from Sigma (St. Louis, MO, USA). Human retinal microvascular endothelial cells (HRMEC) and attachment factor were purchased from Cell Systems (Kirkland, WA, USA). Peripheral blood mononuclear cells were purchased from Sanguine Biosciences (Valencia, CA, USA). TNFα, CCL8, and CXCL10 were purchased from R&D Systems (Minneapolis, MN, USA).

2.3 Culture and treatment of human Müller cells (HMC)

The vehicle for PA was prepared by dissolving fatty acid free BSA in PBS at 100mg/ml, hereafter referred to as BSA. PA was dissolved in EtOH at 200mM and incubated with BSA for 90 minutes at 37°C to achieve 5mM. BSA-conjugated PA was then diluted in culture media to a final concentration of 250μM, hereafter referred to as PA. GSK0660 and GW0742 were dissolved in DMSO to achieve a 1mM solution. These solutions were added to cell culture media to achieve 1mM concentration and 0.1% DMSO. Preliminary dose response experiments were performed with PA and GSK0660, confirming no significant changes in cell viability (data not shown).

Retinal tissues for Müller cell isolation were harvested from a 42 year old, caucasion male under NDRI protocol #ND06065 and isolated as described previously (46). HMC were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1x antibiotic/antimycotic solution hereafter referred to as HMC growth medium. Passages 4 through 6 were used for all experiments. HMC were cultured in growth medium in 6-well dishes until approximately 70% confluent. Growth medium was aspirated replaced with 2% FBS or 2% + GSK0660 for 12 hours. These media were aspirated and HMC were treated with fresh 2% FBS + BSA (vehicle), PA, PA + GSK0660, or GW0742 for 24 hours.

2.4 Culture and treatment of HRMEC

HRMEC were cultured on attachment factor-coated plates in endothelial basal medium (EBM) supplemented with 10% FBS. Cultures were grown to 70% confluency in a humidified cell culture incubator at 37°C in 5% CO_2. For studies using GSK0660, medium was changed to 2% FBS with either vehicle (0.1% DMSO) or GSK0660 for 24hrs. HRMEC were then stimulated with 1ng/ml TNFα in combination with vehicle or GSK0660 for an additional 4hrs. Similarly, in experiments using CCL8 or CXCL10, recombinant proteins (50ng/ml) were added concomitantly with GSK0660 24 hours prior to TNFα treatment. Concentrations of TNFα were determined from previous, published studies (47, 48). For studies using GW0742, medium was changed to 2% FBS for 12hrs, and then treated in 2% FBS plus vehicle (0.1% DMSO) or GW0742. For blocking antibody studies, anti-CCL8 (Fisher Scientific Company LLC; Suwanee GA, USA) was used at 1μg/ml and anti-CXCL10 (Fisher Scientific Company LLC) was used at 4μg/ml. These were added coincident with TNFα, 4 hours before use in the parallel plate flow chamber.

2.5 Quantitative real-time PCR (qRT-PCR)

Cells were lysed and total RNA was isolated from cell lysates using an RNeasy kit (Qiagen; Valencia, CA, USA) according to the manufacturer's directions. RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Carlsbad, CA, USA). The target genes (*TNFA, IL1β, IL6, IL8 (CXCL8), CCL8, CXCL10, CCL17, ANGPTL4*) vs *ACTB* or *18S* were amplified by qRT-PCR using gene-specific TaqMan Gene Expression Assays (Applied Biosystems). Primer ID and target exons are included in Table 1. Data were analyzed using the comparative Ct method and Ct values were normalized to *ACTB or 18S* levels.

2.6 Parallel plate flow chamber

Peripheral blood mononuclear cells were resuspended in Hank's Buffered Salt Solution (HBSS) at a concentration of 5×10^5 cells/ml and loaded into a syringe. HRMEC were grown in monolayers on glass slides coated with attachment factor and treated with drugs as outlined in the above sections. After treatment, slides were mounted in a rectangular parallel plate flow chamber with a silicon rubber gasket (GlycoTech; Gaithersburg, MD, USA). The chamber had a flow width of 1.00cm and a height of 0.0127cm. The chamber was connected to inlet and outlet syringes with Silastic™ tubing (GlycoTech) with an inner diameter of 1/16 inch. A syringe pump (World Precision Instruments; Sarasota, FL, USA) was used to pull PBMC across HRMEC monolayers at a rate of 150μl/min for 7min. Non-adherent cells were washed off the plate with HBSS at a flow rate of 300μl/min for 2min. Eight fields of view were captured using an IMT-2 inverted microscope (Olympus; Tokyo, Japan) and Q-Color3 digital camera (Olympus) at 20x magnification. Adherent cell counts were performed by masked observers using ImageJ (NIH; Bethesda, MD, USA) and averaged for each slide.

2.7 Intravitreal Injections

All experiments using mice were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week old, male C57BL/6N mice (Charles River Laboratories; Wilmington, MA, USA) were anesthetized by isoflurane (Butler Animal Health Supply; Minneapolis, MN, USA) inhalation. Before intravitreal injection, 0.5% proparacaine (Allergan, Troy Hills, NJ, USA) was topically applied to the cornea. The globe was penetrated approximately 0.5mm posterior to the ora serrata, using a 30-gauge needle with a 19° bevel and a 10μL syringe (Hamilton Co., Reno, NV, USA). The needle was advanced to the posterior vitreous at a steep angle to avoid contact with the lens. The injection bolus was delivered near the trunk of the hyaloid artery proximal to the posterior pole of the retina. After injection, a topical antibiotic suspension (Vigamox; Alcon, Fort Worth, TX, USA) was applied. Mice were given a 1μl injection of vehicle (0.1% DMSO in PBS), GW0742 (1μM), 50ng/ml TNFα plus vehicle, 50ng/ml TNFα plus 1μM GSK0660, or 50ng/ml recombinant CCL8 plus 50ng/ml recombinant CXCL10.

2.8 In vivo leukostasis

Retinal leukostasis was performed as described previously (47). Briefly, 6hrs after injection, mice were anesthetized with ketamine and xylazine and perfused through the left ventricle with 0.9% saline at physiological pressures followed by 2.5ml of 40μg/ml FITC-conjugated concanavalin-A (Vector Laboratories; Burlingame, CA, USA). Non-adherent leukocytes were washed out with 0.9% saline. Eyes were enucleated and retinas dissected in 4% paraformaldehyde. Retinas were flat-mounted and fluorescent images captured with an AX70 upright microscope (Olympus) with a DP71 digital camera (Olympus). Lumenal leukocytes were counted by masked observers using ImageJ and normalized to the retinal vascular area.

2.9 Statistics

The data collected were relative mRNA expression levels, leukocytes adhered per mm^2, and leukocytes adhered per retina. For all experiments, statistical significance was determined using either a one-way or two-way ANOVA with a student's t post hoc analysis using the software JMP (SAS Institute; Cary, NC, USA). Data are considered significant with $p < 0.05$.

3.1 Results

3.2 The PPARβ/δ inverse agonist GSK0660 attenuates palmitic acid-induced cytokine expression in HMC

Based on our previous findings that palmitic acid stimulates transcription of several inflammatory mediators (2), we tested the hypothesis that PA-induced cytokine expression was PPARβ/δ-dependent. Indeed, in HMC, PA increased the following cytokine transcriptional targets relative to vehicle value +/− SD: *TNFA*, 27.6 ± 12.3; *IL1B*, 5.9 ± 0.7; *IL6*, 33.2 ± 6.8; *IL8*, 835.3 ± 203; and *ANGPTL4*, 32.2 ± 2.1. Co-treatment of HMC with GSK0660 reduced PA-stimulated expression of *TNFA* by 55.8% (Fig. 1A); *IL1B* by 67.2% (Fig. 1B); *IL6* by 73.3% (Fig. 1C); *IL8 by* 69% (Fig. 1D); and *ANGPTL4* by 93.1% (Fig 1E). To test PPARβ/δ-dependency of these effects, HMC were treated with PPARβ/δ agonist GW0742. We observed a 45.3-fold increase in *ANGPTL4* mRNA (Fig. 1E). However, no increases in *TNFA*, *IL1B*, *IL6*, and *IL8* were observed (Fig. 1A–D), suggesting that GSK0660's effect on inflammatory cytokine transcription were, in part, PPARβ/δ-independent. Our observations reported herein are not likely to be related to any toxicity imposed by these compounds. In a previous study, we tested GSK0660 and GW0742 in HRMEC proliferation and tube formation assays with no evidence of toxicity (39). In the same study, we also tested these drugs on the normal retinal vascular development of rat pups and neither showed any effect.

3.3 PPARβ/δ inhibition by GSK0660 attenuates TNFα-induced chemokine expression

We next investigated the effect of one of the PA-stimulated cytokines, TNFα, on HRMEC to query the role of PPARβ/δ on inflammation from the cross-talk of HMC to HRMEC. While our previous studies identified and confirmed changes in CCL8 and CXCL10 with a single GSK0660 treatment by RNA-seq (40), we tested a range of doses in the present study to optimize further treatments of HRMEC. TNFα increased expression of *CCL8* and *CXCL10*. HRMEC pre-treated with GSK0660 attenuated TNFα-induced expression of *CCL8* and *CXCL10* in a dose-dependent manner (Fig. 2A–B, Supplementary Fig. 1). At the highest concentration of GSK0660 (10μM), *CCL8* and *CXCL10* were inhibited by 86.1% ($p<0.0001$) and 59.7% ($p<0.0001$), respectively. TNFα-induced *CCL17* was not significantly decreased with any dose of GSK0660 (Fig. 2C).

3.4 PPARβ/δ activation by GW0742 increases CCL8 and CXCL10 expression

To further examine the PPARβ/δ-dependency of the effects of GSK0660, we complimented our studies with use of a PPARβ/δ agonist. PPARβ/δ activation in HRMEC was accomplished using the agonist GW0742, and verified by expression of the target gene,

ANGPTL4 (23.5-fold; p<0.0001; Fig. 3A) (39, 49, 50). GW0742 induced the expression of *CCL8* by 2.2-fold (p=0.0021; Fig. 3B) and *CXCL10* by 2.3-fold (p=0.430; Fig. 3C) following 12hrs of treatment. Taken together, these data support the idea that GSK0660 exerts its effect on HRMEC chemokine expression at least in part through a PPARβ/δ-dependent mechanism.

3.5 Inhibition of PPARβ/δ reduces TNFα-induced cell adhesion

To determine the behavioral effects of PPARβ/δ manipulation, we assessed PPARβ/δ inhibition in leukocyte behavior. As shown in Fig. 4A, treatment of HRMEC with both 1μM and 10μM GSK0660 significantly inhibited TNFα-induced cell adhesion by 49.7% (p=0.0291) and 36.8% (p=0.0083), respectively. The addition of recombinant CCL8 and CXCL10 with GSK0660 to HRMEC monolayers prevented the effect of GSK0660 on TNFα-induced cell adhesion.

3.6 CCL8 and CXCL10 are involved in TNFα-induced leukocyte adhesion

HRMEC monolayers were treated with maximal doses of antibodies against CCL8 and CXCL10 (as determined from preliminary experiments), alone or in combination. Addition of anti-CCL8 did not significantly affect TNFα-induced PBMC adhesion to HRMEC monolayers (Fig. 5). However, addition of anti-CXCL10 significantly reduced TNFα-induced cell adhesion by 41.2% (p=0.0113). Combination treatment with anti-CCL8 and anti-CXCL10 further reduced TNFα-induced PBMC adhesion, inhibiting induction by 55.1% (p=0.0057).

3.7 PPARβ/δ is involved in TNFα-induced retinal leukostasis in vivo

Intravitreal injections of TNFα increased the number of adherent leukocytes in retinal vessels by 2.3-fold (p=0.0072; Fig. 6A). PPARβ/δ agonism by GW0742 induced leukostasis by 2-fold (p=0.0303) while co-treatment of TNFα and GSK0660 reduced TNFα-induced leukostasis by 47.1% (p=0.0486). Similar to TNFα injection, recombinant CCL8 (50ng/ml) and CXCL10 (50ng/ml) significantly increased adherent leukocytes by 1.7-fold (p=0.045; Fig. 6B).

Discussion

The homeostatic functions of Müller cells decline in animal models of DR, perhaps leading to their activation (gliosis) and release of angiogenic growth factors and/or inflammatory cytokines. Gliosis, as defined by elevated glial fibrillary acidic protein (GFAP) occurs early in disease progression, preceding the vascular changes that characterize DR (51). *In vitro* studies support the theory that diabetes-relevant stimuli elicit these events. For example, Müller cells cultured in high glucose media increase expression of the activation marker glial fibrillary acidic protein (GFAP) and increase their release of inflammatory cytokines (52). However, the interpretation of these and related studies is sometimes obscured depending on the species, the use of normal or transformed Müller cells, and/or the incorporation of an osmotic control into the experimental design (2). Informed by studies suggesting a role for dyslipidemia in DR pathogenesis (2, 3), we used saturated fatty acids as a way to model an initiating step in the DR pathogenic cascade (Fig. 7).

PA is consistently elevated in the sera and tissues of diabetics, and we found it to be a potent inducer of *TNFA*, *IL1B*, *IL6*, *IL8*, and *ANGPTL4* in non-diabetic donor HMC. Each plays a significant role in DR pathogenesis. Increased concentrations of Angptl4 are observed in the aqueous of PDR patients and correlates with neovascular disease and retinal edema, independent of VEGF (42, 44). Experiments in hypoxic Müller cells and in a mouse model of oxygen-induced retinopathy revealed that increased Angptl4 expression was hypoxia–inducible factor (HIF)-dependent suggesting that hypoxia is the primary inducer of Angptl4 in PDR (44). However, free-fatty acids (FFAs) bind and activate PPARβ/δ, and Angptl4 is one of its target genes. Therefore, our findings suggest that PA induces a PPARβ/δ-dependent increase in Angptl4 that may elicit pathologic changes in response to dyslipidemia, independent of ischemia-induced hypoxia. Notably, we observed that GSK0660 mitigated *ANGPTL4* expression, suggesting a potential therapeutic strategy to intervene at the earliest stages of disease progression. As further support of PPARβ/δ as a drug target, *ANGPTL4* expression was increased in HMC by GW0742, suggesting that transcriptional regulation of this particular target is PPARβ/δ-dependent.

We also observed that GSK0660 decreased PA-induced *TNFA*, *IL1B*, *IL6* and *IL8* expression in HMC. However, unlike *ANGPTL4*, GW0742 did not increase expression of these inflammatory cytokines, suggesting that GSK0660 has off-target, but nonetheless beneficial effects. Further studies are warranted to elucidate these GSK0660 targets to facilitate the design, synthesis and implementation of novel inhibitors.

Since inflammatory cytokines released from Müller cells may activate inflammation pathways through paracrine signaling to other retinal cells, we investigated whether GSK0660 reduces TNFα-mediated inflammatory changes HRMEC. In previous experiments, we found that GSK0660 mitigates TNFα-induced *CCL8* and *CXCL10* gene expression in HRMEC. As part of this study, we confirmed these expression changes by qRT-PCR, and also determined similar effects at the protein level (Supplementary Fig. 1). There are data suggesting roles for CCL8 and CXCL10, also called MCP-2 and IP-10, respectively in DR. For example, in experimental DR, VEGFR1 blockade reduced retinal leukostasis and permeability in part through down-regulation of *ccl8* and *cxcl10* (53). Interestingly, CCL8 activates CCR5, and leukocytes expressing high levels of CCR5 were found to be the primary leukocyte subset involved in retinal capillary leukostasis (54, 55). CXCL10 may also contribute to DR pathogenesis, because its levels are significantly elevated in the vitreous of DR patients (56, 57). Guided by these studies, we performed additional experiments to investigate the functional significance of these transcriptional changes. We observed that GSK0660 reduces CCL8- and/or CXCL10-dependent leukocyte adhesion in *in vitro assays* (Fig. 5) and leukostasis in mice (Fig. 6B). Since CCL8 and CXCL10 have not been previously characterized as direct targets of PPARβ/δ, we performed additional tests to determine if the observed effects of GSK0660 were PPARβ/δ-dependent. Pharmacologic agonism with the well-characterized PPARβ/δ agonist, GW0742 increased *CCL8* and *CXCL10* expression (Fig. 3), suggesting PPARβ/δ-dependency. We also identified peroxisome proliferator response element (PPRE) motifs in the upstream 5’ promoter regions of the human *CCL8* and *CXCL10* genes by screening against a collection of 235 PPRE motifs (58). Six PPREs were found in *CXCL10* and 3 were found in *CCL8*.

This further suggests that reduced *CCL8* and *CXCL10* expression by GSK0660 is PPARβ/δ-dependent.

PPARβ/δ has been studied in the context of vascular inflammation, with a majority of work suggesting that PPARβ/δ agonism is anti-inflammatory. In human umbilical vein endothelial cells (HUVECs), PPARβ/δ agonists inhibited TNFα-induced expression of VCAM-1 and ICAM-1 (37, 59). Furthermore, PPARβ/δ agonists have been shown to inhibit the expression of some chemokines. In HUVECs, the PPARβ/δ agonist GW501516 reduced TNFα-induced release of CXCL10, and in cultured human monocytes it prevented VLDL-induced expression of MIP-1α (CCL3) (37). Activation of PPARβ/δ prevented TNFα-induced inflammation in adipocytes, HUVECs, and proximal tubular cells (37, 59–61). Additionally, PPARβ/δ agonists have been shown to be protective against inflammation associated with hyperoxia-induced lung injury and spinal cord injury in rodents (62, 63). Interestingly, in the present study we observed that PPARβ/δ agonism with GW0742 stimulates both chemokine expression (Fig. 3) and retinal leukostasis (Fig. 6A).

There has been little work testing the PPARβ/δ antagonist GSK0660 on molecular and cellular events that promote inflammation. Notwithstanding the clear effects of agonism on reducing inflammation, there also exist reports suggesting that GSK0660 might be anti-inflammatory. In monocytes, GSK0660 reverses the effect of carbaprostacyclin (a PPARβ/δ agonist) and TNFα on CXCL8 expression (64). GSK0660 also reduces an inflammatory psoriasis-like skin condition in mice, perhaps by reversing the upregulation of IL-1β (65). In our study in HRMEC, GSK0660 had no effect on TNFα-induced ICAM-1 or VCAM-1 expression (data not shown), but it reduced chemokine expression (Fig. 1), leukocyte adhesion to endothelial monolayers (Fig. 4), and leukostasis. Opposite effects were observed when using PPARβ/δ agonist GW0742, suggesting they were indeed PPARβ/δ-dependent. It is therefore possible that PPARβ/δ is differentially regulated depending on the cell type and tissue queried.

The exact mechanism by which PPARβ/δ agonists and GSK0660 affect inflammation is still unknown. However, there are many reports suggesting these small molecules activate different pathways depending on concentration, duration, cell type, and context. For example, the PPARβ/δ agonist GW0742 reduced TNFα-induced VCAM-1, ICAM-1, and E-selectin expression in a BCL6-dependent manner in umbilical vein cells (59). In the same experiment, GW0742 had no effect on NF-κB translocation, nor did it affect phosphorylation of the three different MAPK pathways JNK, ERK, or p38 MAPK. However, in another endothelial cell line, a PPARβ/δ agonist inhibited TNFα-induced VCAM-1 expression by inhibiting NF-κB translocation (66). Lastly, GSK0660 and PPARβ/δ might act through a non-genomic mechanism to inhibit ERK signaling. PPARβ/δ has been shown to directly activate the Akt signaling pathway by binding to the regulatory subunit of the kinase PI3K (67). Further work should be done to determine the differential activation of signaling pathways by agonists and antagonists of PPARβ/δ in retinal endothelial cells.

In this study we used the C57BL/6 mice that are known to carry the rd8 mutation in Crb1. This gene causes retinal degeneration that could affect ocular inflammatory processes. Schnabolk et al. 2014, investigated potential differences between rd8 positive and negative

substrains to susceptibility to laser-induced choroidal neovascularization (LCNV) and inflammatory gene expression(68). They found that LCNV was not affected by the rd8 mutation and that the inflammatory gene expression was blunted. Therefore, it is reasonable to assume that this mutation did not skew our endpoints and if there were any minor effects, they were properly controlled in our experimental design.

In the present study, we demonstrated that GSK0660 is anti-inflammatory in both PPARβ/δ-dependent and -independent manners. Notably, one limitation to the present study is that Müller cells from a single donor were used. Despite this, these findings are an important extension of our previous work, because we confirm that the transcriptional pathways modulated by GSK0660 lead to significant attenuation of DR-related inflammatory processes. We propose a model in which GSK0660 intervenes at multiple steps in DR progression (Fig. 7). The notion of using GSK0660 as an intervention for DR is further bolstered by our previous work in which we demonstrated that PPARβ/δ inhibition with GSK0660 was angiostatic (39), which would be beneficial against the late, proliferative stage of DR as well. Taken together, our findings demonstrate that PPARβ/δ inhibition may be an ideal therapeutic strategy for DR because it is efficacious in multiple pathogenic steps of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Angptl4	Angiopoietin-like protein 4
CCL8	C-C motif chemokine ligand 8
CXCL10	C-X-C motif chemokine ligand 10
DR	Diabetic retinopathy
EC	Endothelial cell
HMC	Human Müller cell
HRMEC	Human retinal microvascular endothelial cell
IL-1β	Interleukin 1β
IL6	Interleukin 6
IL8	Interleukin 8
NPDR	Non-proliferative diabetic retinopathy

MC	Müller cell
PA	Palmitic acid
PBMC	Peripheral blood mononuclear cells
PDR	Proliferative diabetic retinopathy
PPARβ/δ	Peroxisome proliferator-activated receptor β/δ
TNFα	Tumor necrosis factor α

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Highlights

- The PPARβ/δ inhibitor GSK0660 inhibits palmitic acid-stimulated inflammatory mediator production by Müller cells.
- GSK0660 inhibits TNFα-induced leukocyte adhesion in both HRMEC and mouse retina.
- GSK0660 blocks TNFα-induced leukostasis by modulating the levels of CCL8 and CXCL10.
- GSK0660's effects on retinal cells are mediated by both PPARβ/δ-dependent and PPARβ/δ-independent pathways.

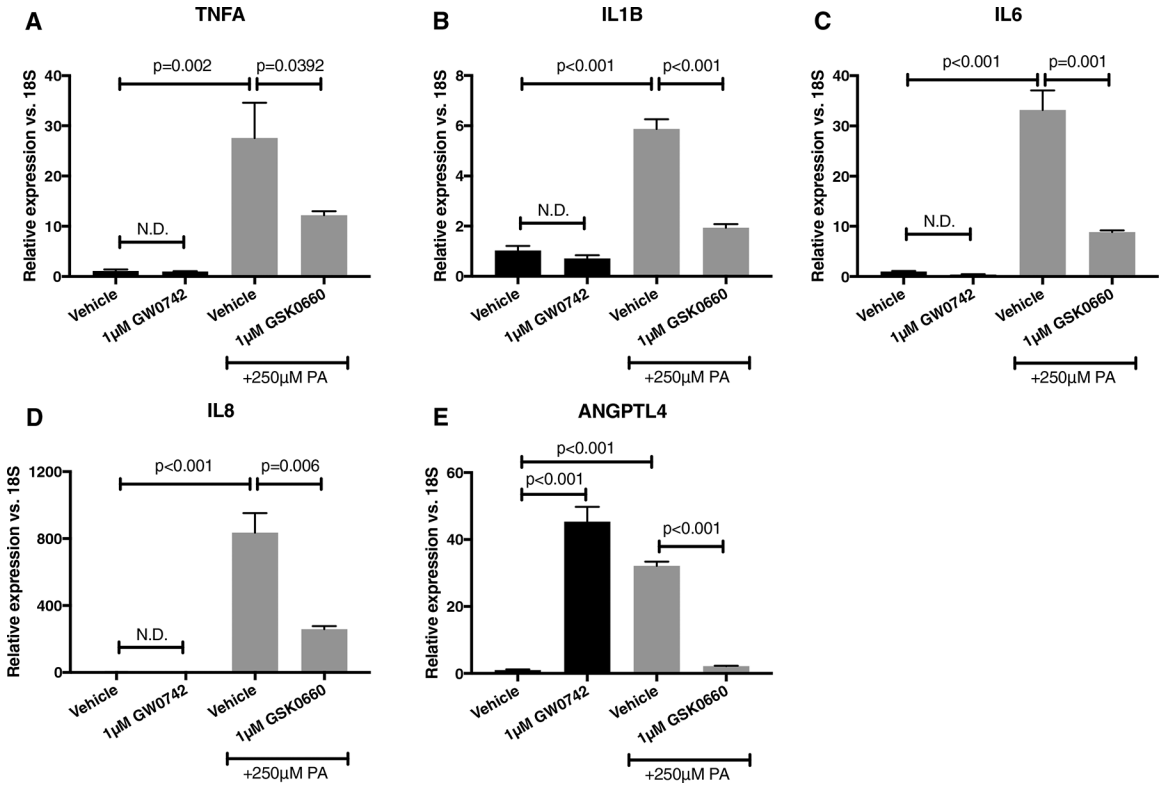

Figure 1. GSK0660 inhibits PA-stimulated inflammatory cytokines in HMC in a PPARβ/δ-independent manner.

HMC were treated for 24 hours with BSA alone, BSA with 1μM GW0742, 250μM PA, or 250uM with 1μM GSK0660. (A) *TNFA*, (B) *IL1B*, (C) *IL6*, (D) *IL8*, or (E) *ANGPTL4* were assessed by qRT-PCR. All data are relative to BSA alone. Bars represent mean ± SEM (n=3).

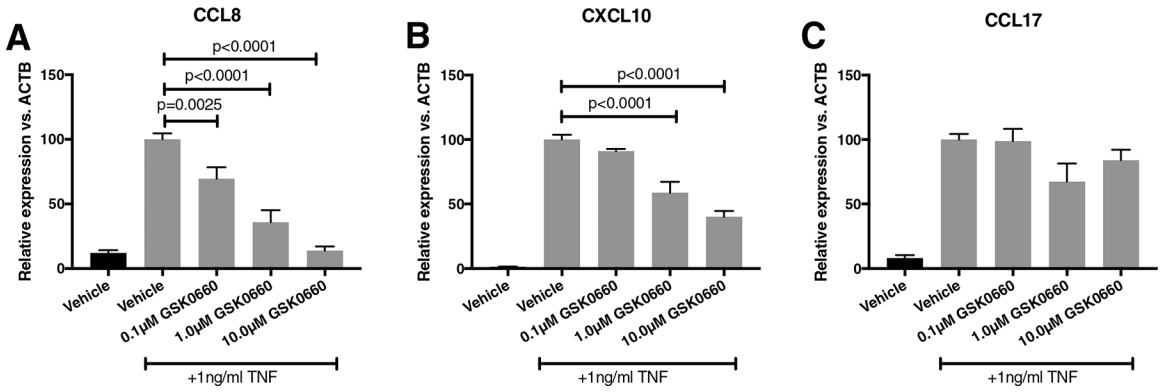

Figure 2. GSK0660 inhibits TNFα-stimulated CCL8 and CXCL10 in HRMEC.

HRMEC were pre-treated with increasing concentrations of GSK0660 for 24hrs before stimulation with 1ng/ml TNFα for 4hrs. (A) *CCL8*, (B) *CXCL10*, and (C) *CCL17* were assessed in treated HRMEC by qRT-PCR. All data are relative to the TNFα plus vehicle-treated samples. Bars represent mean ± SEM (n= 6).

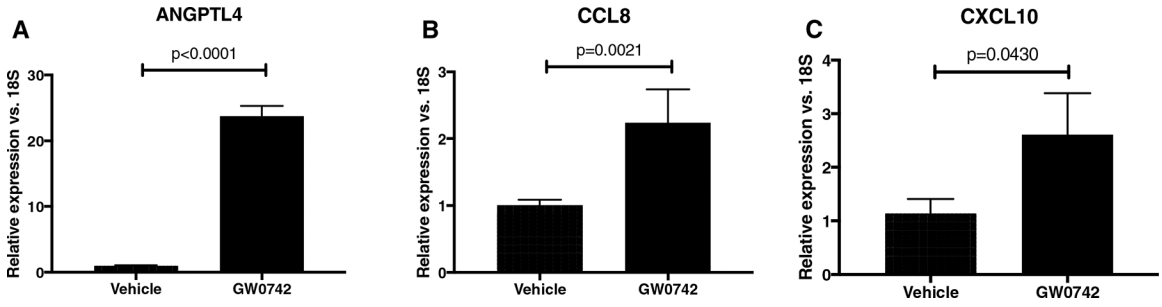

Figure 3. GW0742 stimulates CCL8 and CXCL10 expression in HRMEC.

HRMEC were treated with vehicle or 1μM GW072 for 12hrs. PPARβ/δ activation was confirmed by (A) *ANGPTL4* expression. (B) *CCL8* and (C) *CXCL10* were assessed by qRT-PCR. Bars represent mean ± SEM (n=6).

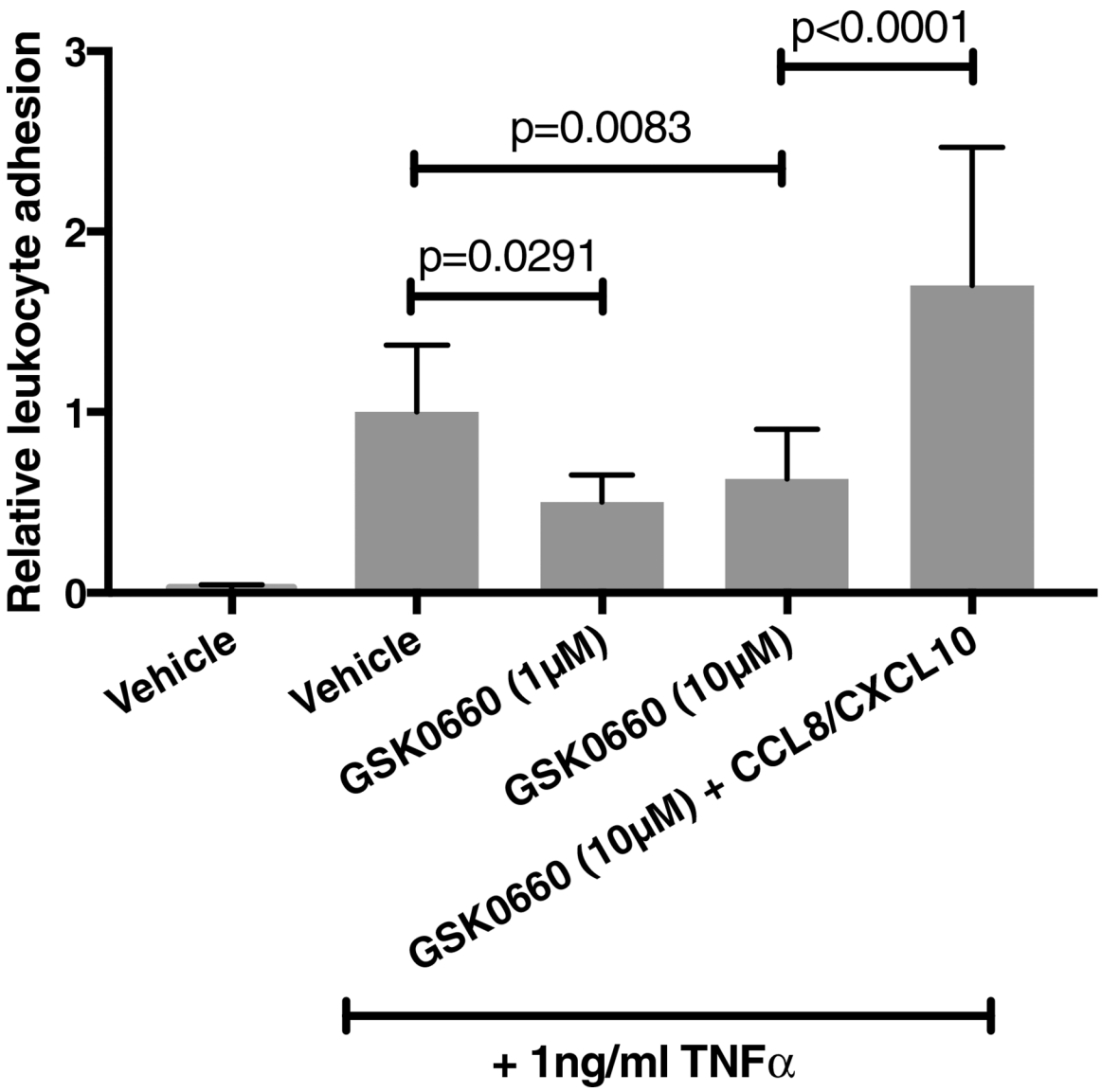

Figure 4. GSK0660 inhibits TNFα-induced PBMC adhesion to HRMEC via its effect on CCL8 and CXCL10.

HRMEC monolayers were treated with vehicle, 1μM, or 10μM GSK0660 for 24hrs before stimulation with 1ng/ml TNFα. An additional group was treated with 50ng/ml of both CCL8 and CXCL10 in addition to 10μM GSK0660. PBMC were flowed over treated monolayers in a parallel plate flow chamber and adherent leukocytes were quantified. Data are shown as relative to vehicle control. Bars represent mean ± SEM (n=6).

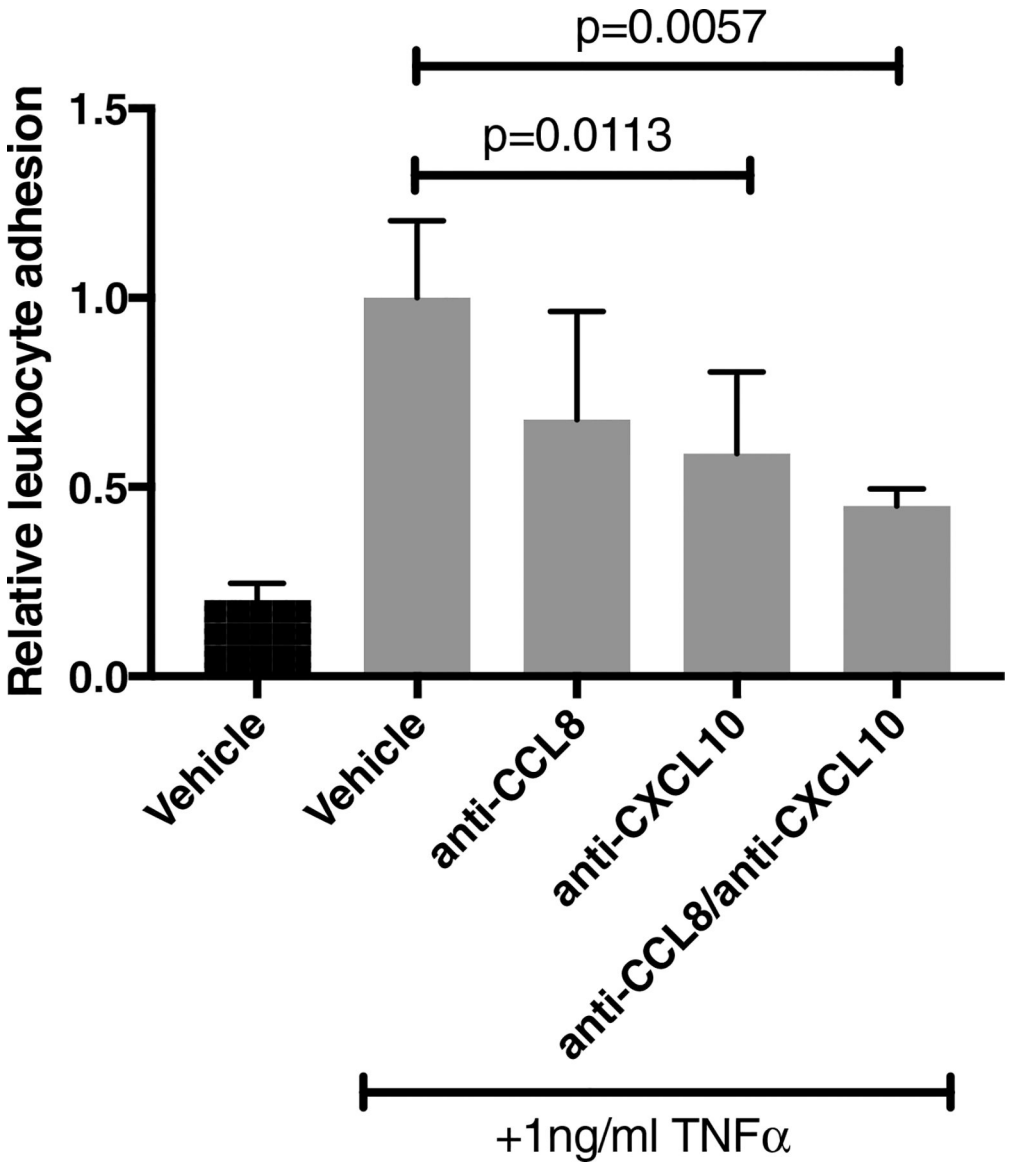

Figure 5. CCL8 and CXCL10 mediate TNFα-induced PBMC adhesion to HRMEC.

HRMEC monolayers were treated with 1μg/ml anti-CCL8, 4μg/ml anti-CXCL10, or a combination of both CCL8 and CXCL10, concomitant with 1ng/ml TNFα stimulation. PBMC were flowed over treated monolayers in a parallel plate flow chamber and adherent leukocytes were quantified. Data are shown as relative to vehicle control. Bars represent mean ± SEM (n=6).

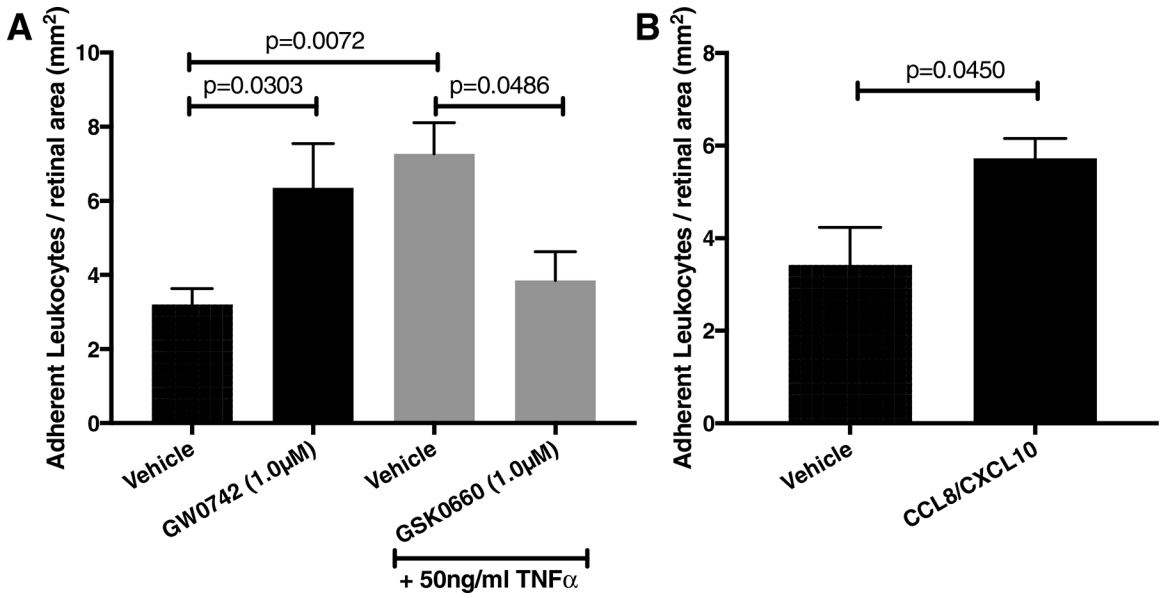

Figure 6. GSK0660 inhibits TNFα-induced retinal leukostasis in a PPARβ/δ-dependent manner.

(A) Mice were injected with vehicle, 1μM GW0742, 50ng/ml TNFα vehicle, or 50ng/ml TNFα with 1μM GSK0660. (B) Mice were injected with vehicle or 50ng/ml recombinant CCL8 with 50ng/ml recombinant CXCL10. Six hrs after intravitreal injection, mice were perfused with FITC-conjugated concanavalin-A to label adherent leukocytes. Bars represent mean ± SEM (A: vehicle n=13; GW0742 n=5; TNFα vehicle n=4; TNFα + GSK0660 n=8; B: vehicle n=5; CCL8/CXCL10 n=5).

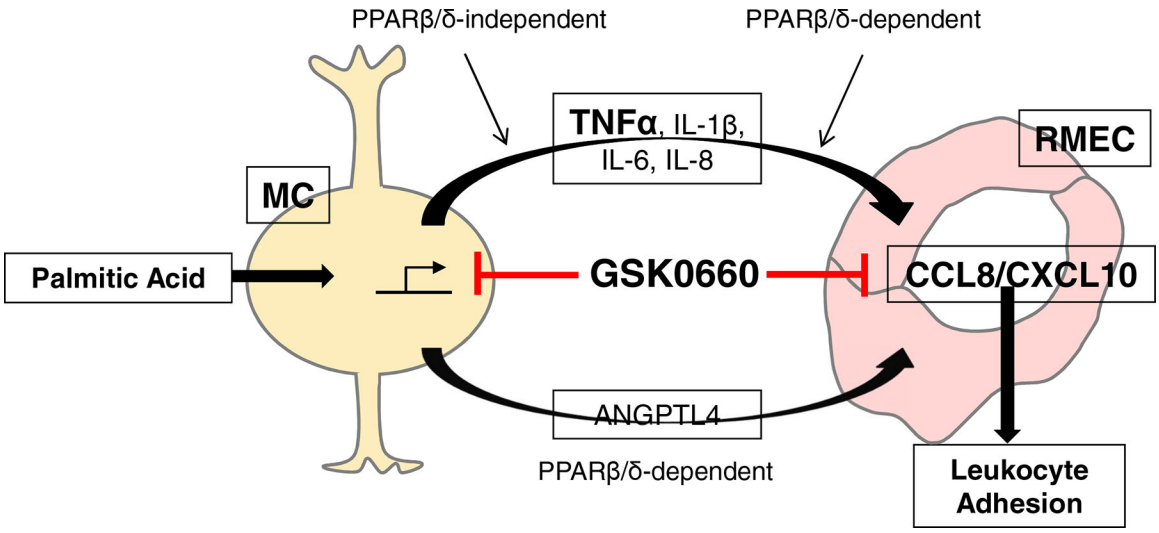

Figure 7. Model of GSK0660 on upstream (Müller cell) and downstream (endothelial cell) inflammation.

Palmitic acid stimulates the release of inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8) and a pro-angiogenic factor (Angptl4). GSK0660 reduces the stimulation of these factors in PPARβ/δ-independent and -dependent manners, respectively. TNFα can then act in a paracrine manner to stimulate CCL8 and CXCL10 production by endothelial cells. GSK0660 blocks the production of these chemokines in a PPARβ/δ-dependent manner, thereby limiting leukostasis.

Table 1.

Primer IDs, exons boundary, and amplicon length of Taqman primers used in this study.

Gene	Taqman Primer Assay ID	Exon boundary	Amplicon Length
TNFA	Hs00174128_m1	3–4	80
IL1B	Hs01555410_m1	3–4	91
IL6	Hs00985639_m1	2–3	66
IL8 (CXCL8)	Hs00174103_m1	1–2	101
ANGPTL4	Hs01101127_m1	6–7	92
CCL8	Hs04187715_m1	1–2	67
CXCL10	Hs01124251_g1	2–3	135
CCL17	Hs00171074_m1	2–3	51
ACTB	Hs99999903_m1	1–1	171
18S	Hs99999901_s1	1–1	187