

RNA-seq identifies a role for the PPAR β/δ inverse agonist GSK0660 in the regulation of TNF α -induced cytokine signaling in retinal endothelial cells

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Purpose: The peroxisome proliferator-activated receptor beta/delta (PPAR β/δ) is a transcription factor with roles in metabolism, angiogenesis, and inflammation. It has yet undefined roles in retinal inflammation and diabetic retinopathy (DR). We used RNA-seq to better understand the role of the antagonist and inverse agonist of PPAR β/δ , GSK0660, in TNF α -induced inflammation. Understanding the underlying mechanisms of vascular inflammation could lead to new treatments for DR.

Methods: RNA was isolated from human retinal microvascular endothelial cells treated with a vehicle, TNF α , or TNF α plus GSK0660. RNA-seq was performed with a 50 bp single read protocol. The differential expression was determined using edgeR and gene ontology, and a pathway analysis was performed using DAVID. RNA-seq validation was performed using qRT-PCR using the primers for *ANGPTL4*, *CCL8*, *NOV*, *CXCL10*, and *PDPK1*.

Results: TNF α differentially regulated 1,830 transcripts, many of which are involved in the cytokine–cytokine receptor interaction, chemokine signaling, and inflammatory response. Additionally, TNF α highly upregulated genes involved in leukocyte recruitment, including *CCL5*, *CX3CLI*, and *CXCL10*. GSK0660 differentially regulated 273 transcripts in TNF α -treated cells compared to TNF α alone. A pathway analysis revealed the enrichment of cytokine–cytokine receptor signaling. In particular, GSK0660 blocks the TNF α -induced upregulation of *CCL8*, a chemokine involved in leukocyte recruitment.

Conclusions: TNF α regulates several genes related to retinal leukostasis in retinal endothelial cells. GSK0660 blocks the effect of TNF α on the expressions of cytokines involved in leukocyte recruitment, including *CCL8*, *CCL17*, and *CXCL10* and it may therefore block TNF α -induced retinal leukostasis.

Diabetic retinopathy (DR) is currently one of the leading causes of irreversible blindness in working-age adults in the United States, and cases are expected to increase threefold within the next 35 years [1]. The pathology of DR consists of two main stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR, the early stage, is characterized by vascular dysfunction, including microaneurysms, lipid exudation, and vascular hyperpermeability. PDR, the later and more severe stage, is characterized by abnormal neovascularization. Retinal inflammation plays a critical role in the pathogenesis of both NPDR and PDR.

Retinal inflammation is a result of several inflammatory mediators, including TNF α , IL-8, IL-6, IL-1 β , and NF- κ B, which are upregulated in the retina or vitreous of patients with DR [2,3]. TNF α , in particular, is important to disease onset and progression. Increased TNF α levels in the

sera of children are predictive of NPDR and are observed in the vitreous of patients with PDR [4,5]. Furthermore, TNF α drives leukostasis, endothelial cell apoptosis, and blood-retinal barrier breakdown in DR [6,7]. Leukostasis is a critical pathologic feature of DR because adhered leukocytes can occlude capillaries, leading to focal areas of ischemia and eventual progression to PDR. Additionally, leukocytes may extravasate and promote local cytokine upregulation, increasing retinal inflammation [8]. TNF α contributes to leukostasis through the upregulation of adhesion proteins, including VCAM1, ICAM1, and E-selectin on the luminal wall of the vascular endothelium, as well as leukocyte recruiting chemokines, such as IL-6 and IL-8 [9,10]. While clearly important in DR, the mechanism of TNF α -induced inflammation in retinal endothelial cells has not been fully characterized.

The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear hormone receptors that have roles in metabolism, cell differentiation, cell proliferation, and inflammation [11]. Of the three isoforms, PPAR β/δ is the least well studied and understood, particularly in terms of its effect on proliferation and inflammation. To date, most

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reports in the literature on PPAR β/δ and inflammation have focused on the roles of its agonists. Activation of PPAR β/δ inhibits TNF α -induced expression of adhesion molecules and leukocyte adhesion to umbilical vein endothelial cells [12,13]. However, few studies have been done using the specific antagonist of PPAR β/δ , GSK0660, which also has inverse agonist effects when used alone [14]. We have previously shown GSK0660 to be antiangiogenic in the context of oxygen-induced retinopathy [15]. As inflammation, as well as angiogenesis, is an important component of DR, we chose to examine the effect of GSK0660 on TNF α -induced inflammation. In this study, we used RNA-sequencing (RNA-seq) to determine the effect of GSK0660 on TNF α -induced inflammation in human retinal microvascular endothelial cells (HRMECs). RNA-seq has advantages over microarrays in that it is more sensitive, has a broader dynamic range, and allows for the identification of novel transcripts [16].

METHODS

Culture of human retinal endothelial cells and RNA isolation:

Primary HRMECs were purchased from Cell Systems (Kirkland, WA) and grown in an endothelial basal medium (Lonza; Walkersville, MD) with 10% fetal bovine serum (FBS) and endothelial growth supplements (EGM SingleQuots; Lonza). Cultures were kept in a humidified cell culture incubator at 37 °C with 5% CO₂. Cells were plated in six-well dishes coated with attachment factor (Cell Signaling; Danvers, MA) and grown to 70% subconfluency. The medium was changed to 2% FBS with one of the following treatment schemes: a vehicle (0.1% DMSO) for 24 h then a vehicle for 4 h, a vehicle for 24 h then 1 ng/ml TNF α (Sigma-Aldrich; St. Louis, MO) + a vehicle for 4 h, or 10 μ M GSK0660 (Tocris; Minneapolis, MN) for 24 h followed by TNF α + GSK0660 for 4 h. The work area was cleaned using RNaseZap® (Life Technologies; Grand Island, NY) and then total RNA was isolated from cell lysates using an RNeasy kit (Qiagen; Valencia, CA) according to the manufacturer's directions.

Library preparation and RNA-sequencing: RNA samples were submitted to the Vanderbilt VANTAGE core for RNA-seq. RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA). The RNA integrity number (RIN) of each sample was 10. Libraries were prepared using the TruSeq RNA Sample Prep Kit (Illumina; San Diego, CA) to enrich for poly(A)-containing mRNA and generate cDNA. Library quality was also confirmed using the 2100 Bioanalyzer. The libraries were sequenced using a 50 bp single read protocol on the Illumina HiSeq 2500 (Illumina). Sequence data were deposited

at the NCBI Short Read Archive under the accession number SRP053124.

Sequence alignment and differential expression: Sequence alignment and differential expression were performed by the Vanderbilt VANGARD core. TopHat v2.0.9 was used to align sequences to the UCSC human reference genome hg19 using default parameters [17]. Raw counts of mapped reads were generated and then used by the MultiRankSeq program, which utilizes the edgeR algorithm to determine differential expression [18]. Comparisons were made between vehicle- and TNF α -treated HRMECs, as well as between TNF α -treated HRMECs and TNF α -treated HRMECs with GSK0660. Transcripts were considered significant with an adjusted p value of <0.05. The list was further reduced to transcripts with a fold change greater than or equal to 2. The Euler diagram was generated using the R package utility, VennDiagram [19].

Gene ontology and pathway analysis: The lists of differentially expressed genes were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 for gene ontology (GO) and pathway analysis [20,21]. GO was determined using the GOTERM_BP_FAT data set, which includes the lower levels of the biologic process ontology. GO terms were considered enriched with an EASE score of <0.05. Pathway enrichment was determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway annotation. Pathways were considered enriched with an EASE score of <0.05.

qRT-PCR validation: RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's directions. Quantitative real-time PCR (qRT-PCR) was performed by amplification of the gene of interest (*ANGPTL4*, *CCL8*, *NOV*, *CXCL10*, or *PDPK1*) versus β -actin using gene-specific TaqMan Gene Expression Assays (Applied Biosystems). Data were analyzed using the comparative Ct method and Ct values were normalized to β -actin levels. Statistical significance was determined using the statistical software JMP (SAS Institute; Cary, NC) and an ANOVA with a student's t post-hoc analysis. Data were considered significant at p<0.05.

RESULTS

RNA-seq quality and alignment: To determine the effect of GSK0660 on the TNF α -dependent gene expression in HRMECs, we treated HRMECs with a vehicle, TNF α plus a vehicle, or TNF α plus GSK0660 and performed a transcriptomic analysis using RNA-seq with three samples per treatment. The total number of reads ranged between 28,275,640 and 33,252,277, which covered 32,009 different transcripts

(Appendix 1). There was no difference in the total number of reads across the nine samples (ANOVA, $p = 0.07$). On average, 96.5% of the reads mapped to the UCSC human genome hg19 and between 311 and 1,084 reads were removed due to low quality.

Differential expression: The differential expression of transcripts was determined using the MultiRankSeq program, which determines the differential expression from raw read counts using the edgeR algorithm. Comparisons were made between the TNF α - and vehicle-treated cells, as well as between the TNF α - and TNF α plus GSK0660-treated cells. Transcripts were considered significant with an adjusted p value of <0.05 and a fold change of at least 2.0. Using these parameters, 1,830 transcripts were differentially expressed in the TNF α -treated cells compared to vehicle-treated cells. TNF α plus GSK0660 treatment altered the expression of 273 transcripts compared to TNF α alone (Table 1).

Effect of TNF α on HRMECs: Stimulation of HRMECs with TNF α resulted in large changes to the gene expression

TABLE 1. SUMMARY OF RNA-SEQ DIFFERENTIAL EXPRESSION ANALYSIS.

Treatment comparison	Transcripts with adj $p < 0.05$	Upregulated Transcripts (≥ 2)	Down-regulated Transcripts (≤ -2)
TNF α versus Vehicle	1830	746	1084
TNF α + GSK0660 versus TNF α	273	169	104

Differential expression was determined using edgeR and transcripts were considered significantly changed with adjusted $p < 0.05$ and fold change of at least 2.0.

(Appendix 2), with the top 10 upregulated and downregulated transcripts of protein-coding genes summarized in Table 2. Notably, TNF α increased the expressions of *CCL5*, *CX3CL1*, and *CXCL10*, all of which play roles in leukocyte recruitment. Additionally, TNF α increased its own transcription.

TABLE 2. TOP 10 UPREGULATED AND DOWNREGULATED PROTEIN-CODING GENES BY TNF α IN HRMEC.

Gene Symbol	Log ₂ Fold Change	Adjusted P Value	Ensembl ID
Upregulated Genes			
LAD1	6.421452313	1.10E-07	ENSG00000159166
CSF2	6.39995987	<0.000001	ENSG00000164400
TNFAIP6	6.358926206	2.18E-154	ENSG00000123610
CX3CL1	6.121874962	<0.000001	ENSG00000006210
CXCL10	5.706389982	6.14E-222	ENSG00000169245
HLA-DOB	5.667011352	5.01E-13	ENSG00000241106
ETV3L	5.616009893	1.07E-05	ENSG00000253831
CCL5	5.487915198	5.76E-257	ENSG00000161570
TNF	5.486418289	4.71E-52	ENSG00000232810
GBP7	5.455106479	1.78E-05	ENSG00000213512
Downregulated Genes			
RBM20	-4.29030613	0.009588413	ENSG00000203867
PAK6	-4.29073104	0.026177447	ENSG00000137843
OR1F1	-4.291371288	0.00855682	ENSG00000168124
MYO18B	-4.474679753	0.004370114	ENSG00000133454
CSRNP3	-4.474760229	0.004370114	ENSG00000178662
CR1	-4.634084683	0.003662894	ENSG00000203710
ARL14	-4.783629776	0.001145234	ENSG00000179674
STOX2	-4.915666474	0.000890805	ENSG00000173320
FAM151A	-4.916414121	0.000585614	ENSG00000162391
MUC20	-5.040180048	0.000485122	ENSG00000176945

Transcript fold change and adjusted p value of transcripts affected by TNF α -treated HRMEC compared to vehicle-treated were determined by the edgeR algorithm.

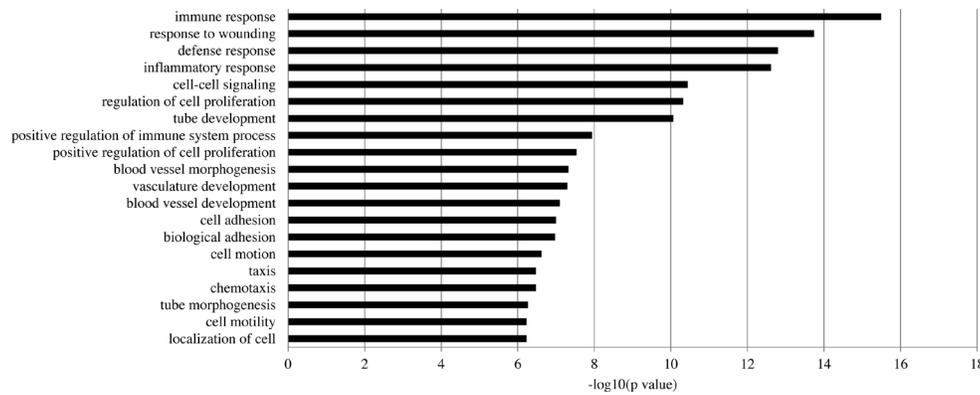


Figure 1. Top 20 biologic pathway GO terms enriched in TNF α -treated HRMECs. Biologic Pathway GO term enrichment in TNF α -treated HRMECs compared to vehicle-treated HRMECs was determined using DAVID. Terms were considered significant at p<0.05.

To determine the function of the transcripts differentially expressed by TNF α , we used the DAVID tool for functional annotation. In terms of gene ontology, 344 GO biologic pathway terms were significantly enriched, with the top 20 summarized in Figure 1. Significant terms included immune response, response to wounding, and inflammatory response. Another common theme included terms for regulating endothelial behavior, such as regulation of cell proliferation, vasculature development, cell adhesion, and chemotaxis.

To annotate further the transcripts, we used the KEGG database to determine enriched pathways. The pathways most highly enriched included the cytokine–cytokine receptor interaction, chemokine signaling pathway, and Jak-STAT signaling pathway (Figure 2). Additional pathways through which TNF α may affect HRMECs included calcium signaling, Toll-like receptor signaling, hedgehog signaling, and the complement cascade.

The effect of GSK0660 on TNF α -treated HRMEC: The effect of GSK0660 on the TNF α -regulated gene expression in HRMECs was determined; the top upregulated and downregulated protein-coding transcripts are summarized in Table 3, while the full data set can be found in Appendix 3. Among

those most highly downregulated by GSK0660 in TNF α -treated cells, *CCL8* is of interest due to its role in leukocyte recruitment. Also of note, GSK0660 prevented the TNF α -induced downregulation of *FAM151A*, *MUC20*, *STOX2*, and *ARL14*. These four transcripts were among those most highly downregulated by TNF α compared to the vehicle. Additionally, GSK0660 affected the transcription of one (*CXCL10*) of the top 10 genes upregulated by TNF α .

GO revealed 33 GO terms that were significantly enriched. Similar to TNF α treatment alone, TNF α treatment plus GSK0660 affected the regulation of cell proliferation and response to wounding (Figure 3). The terms also included reproduction, integrin-mediated signaling pathway, regulation of complement activation, and cell recognition. There was only one KEGG pathway enriched: the cytokine–cytokine receptor interaction (p = 0.01).

Of the 273 genes differentially expressed by TNF α co-treatment with GSK0660, 91 were also differentially expressed by treatment with TNF α compared to the vehicle (Figure 4). GSK0660 exacerbated the TNF α -induced upregulation of eight genes (*ATP2C2*, *GATA6*, *GCKR*, *LIF*, *SEMA3A*, *SERPINB2*, *TNFRSF18*, and *TSLP*) and TNF α -induced the

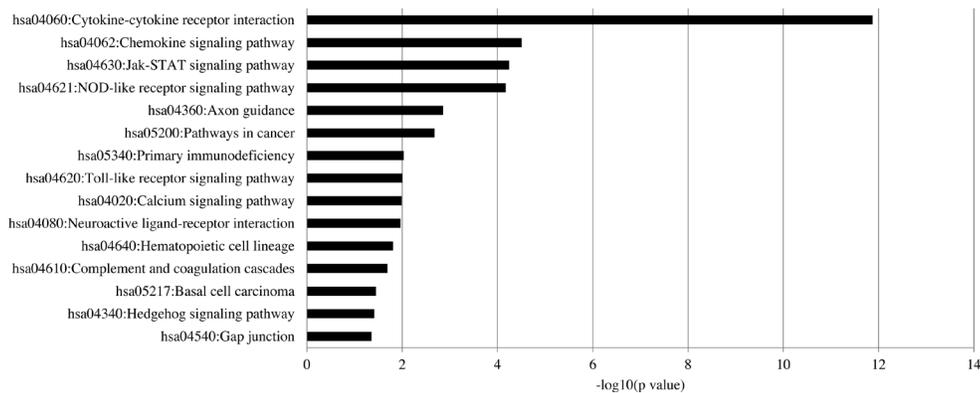


Figure 2. KEGG pathways enriched in TNF α -treated HRMECs. Pathway enrichment was determined for differentially expressed transcripts by TNF α compared to the vehicle using DAVID. Pathways were considered enriched at p<0.05.

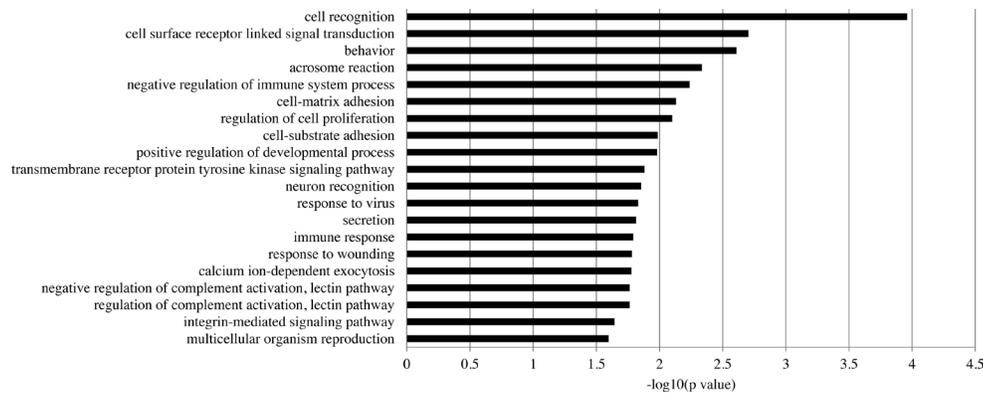


Figure 3. Top 20 GO terms enriched in GSK0660-treated samples. The list of differentially expressed transcripts in TNF α plus GSK0660-treated HRMECs compared to TNF α alone was submitted to DAVID. Terms were considered significant at $p < 0.05$.

downregulation of 11 transcripts. GSK0660 counteracted the effects of TNF α on the remaining 72 transcripts. A KEGG pathway analysis of these 91 transcripts included the enrichment of the cytokine–cytokine receptor interaction (*CCL8*, *CCL17*, *CXCL10*, *LIF*, *TSLP*, and *TNFRSF18*) and chemokine signaling (*CCL8*, *CCL17*, *CXCL10*, and *SHC3*).

qRT-PCR validation: For validation of the RNA-seq, we confirmed the expressions of *ANGPTL4*, *CCL8*, *NOV*, *CXCL10*, and *PDPK1* by qRT-PCR. *ANGPTL4* is a well-known PPAR β/δ target and is known to be downregulated by GSK0660. *CCL8* and *CXCL10* are chemokines and their TNF α -induced expressions were blocked by GSK0660 in

TABLE 3. TOP 10 PROTEIN-ENCODING GENES THAT WERE UPREGULATED OR DOWN-REGULATED BY GSK0660 IN TNF α -TREATED HRMEC.

Gene Symbol	Log ₂ Fold Change	Adj P Value	Ensembl ID
Upregulated Genes			
CBFA2T3	4.970269827	0.001038075	ENSG00000129993
FAM151A	4.837332922	0.002008674	ENSG00000162391
MUC20	4.702950855	0.011947675	ENSG00000176945
STOX2	4.133641693	0.027737782	ENSG00000173320
ARL14	4.133638802	0.027737782	ENSG00000179674
BEAN1	3.364047225	0.004043429	ENSG00000166546
WISP2	3.130060871	0.012964106	ENSG00000064205
GPLD1	3.129996475	0.012964106	ENSG00000112293
UBASH3A	2.850826781	0.04000081	ENSG00000160185
GOLGA8R	2.850824538	0.04000081	ENSG00000186399
Downregulated Genes			
KIT	-1.992297751	3.64E-30	ENSG00000157404
PKD2L2	-2.016705824	0.026490386	ENSG00000078795
LCP2	-2.553894739	0.000489648	ENSG00000043462
TMPRSS9	-2.784724078	0.006155424	ENSG00000178297
FOXG1	-2.834209583	0.039176409	ENSG00000176165
CH25H	-3.087262332	0.014984916	ENSG00000138135
CCL8	-3.445451904	0.000244835	ENSG00000108700
HIST1H3J	-4.043009187	0.039661319	ENSG00000197153
CCDC73	-4.252616175	0.022003857	ENSG00000186714
SERPING1	-4.25281999	0.022002453	ENSG00000149131

Transcript fold change and adjusted p value were determined using the edgeR algorithm.

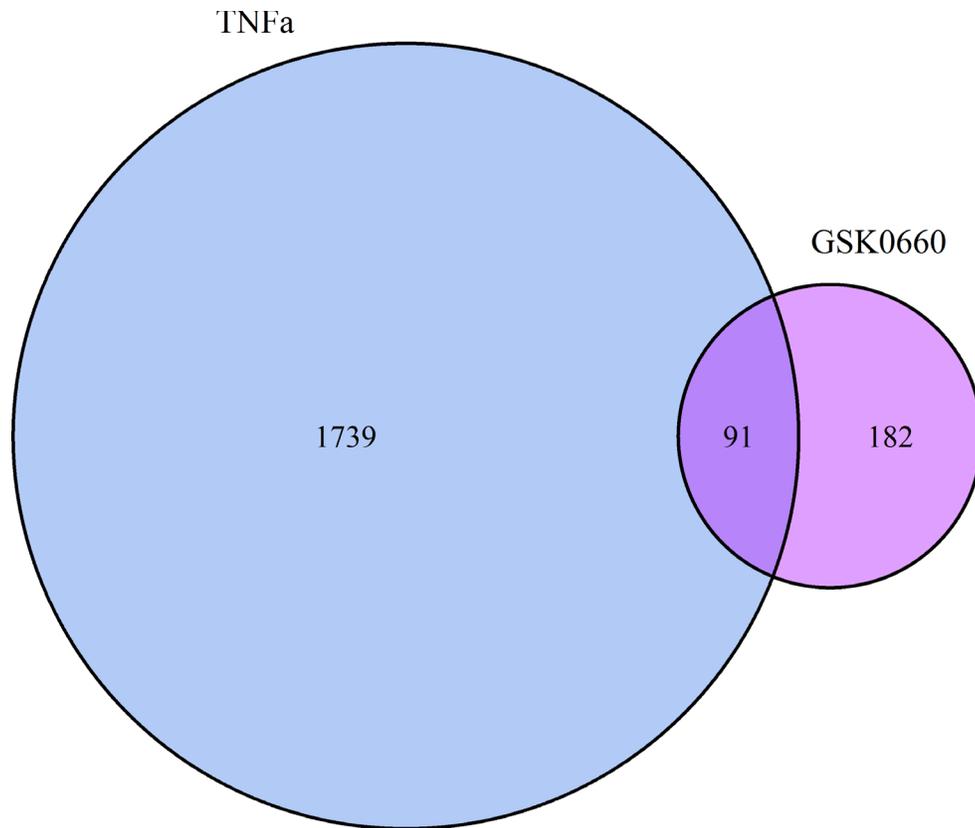


Figure 4. Euler diagram of transcripts differentially expressed by TNFα and GSK0660. TNFα treatment resulted in the differential expression of 1,830 transcripts compared to the vehicle. The addition of GSK0660 regulated 91 of these transcripts. In addition, co-treatment of GSK0660 and TNFα resulted in the differential expression of 182 transcripts that were not affected by TNFα alone.

the RNA-seq. *NOV* was upregulated by GSK0660 in TNFα-treated HRMECs, but not by TNFα alone in the RNA-seq. In HRMECs, the TNFα increased the expressions of *ANGPTL4*,

CCL8, and *CXCL10*, but it had no effect on *NOV*. GSK0660 reduced the expressions of *ANGPTL4*, *CCL8*, and *CXCL10* in TNFα-treated cells and increased the expression of *NOV*. The

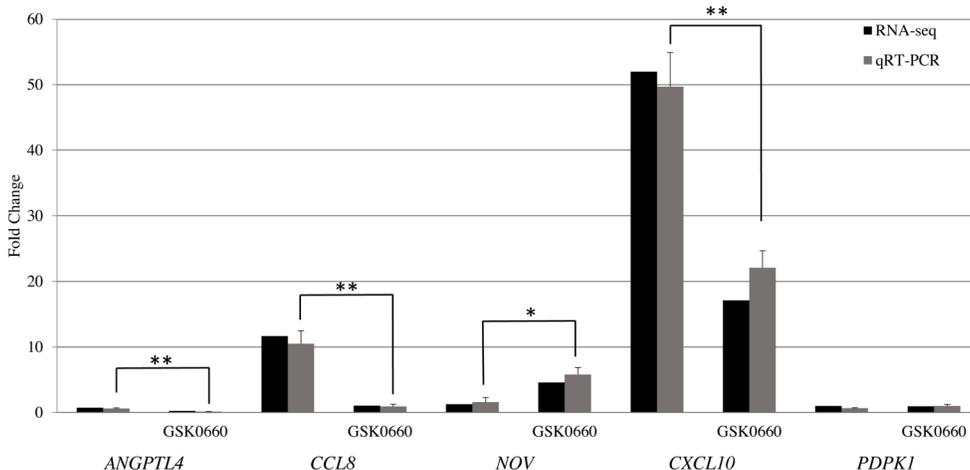


Figure 5. qRT-PCR validation of RNA-seq targets. HRMECs were pre-treated with the vehicle or GSK0660 for 24 h, followed by stimulation with 1 ng/ml TNFα for 4 h. The mRNA expression was evaluated by RNA-seq and qRT-PCR. The fold change for RNA-seq was determined by the edgeR algorithm, while the fold change for qRT-PCR was determined by the comparative Ct method and it is relative to the β -actin expression levels. All fold changes are relative to HRMECs treated with the vehicle alone. Error bars indicate standard deviation for three samples in each group. *p = 0.0003, **p < 0.0001.

changes are relative to HRMECs treated with the vehicle alone. Error bars indicate standard deviation for three samples in each group. *p = 0.0003, **p < 0.0001.

PDPK1 expression was not affected by either treatment using RNA-seq or RT-PCR (Figure 5). Taken together, these data confirm gene expression changes seen in RNA-seq.

DISCUSSION

Using RNA-seq, this study confirms further the effect TNF α has on HRMECs, as well as the role the PPAR β/δ inverse agonist GSK0660 plays in TNF α -induced inflammation. Using RNA-seq over microarray allows for greater sensitivity and a broader range of fold changes. Additionally, the data generated by the RNA-seq can be probed for the discovery of novel transcripts or can be queried in the future after novel transcripts have been characterized. In our study, TNF α had an effect on several signaling pathways including the Jak-STAT pathway, Toll-like receptor pathway, and the complement cascade, replicating findings in other cell types [22-24]. TNF α also differentially expressed several genes involved in cytokine–cytokine signaling and chemokine signaling, suggesting the role of TNF α in retinal inflammation. Further evidence suggests this role may be tied to retinal leukostasis, as TNF α induced the upregulation of several leukocyte adhesion genes, such as *VCAMI*, *ICAMI*, and *SELE*, the gene encoding E-selectin. Besides adhesion protein genes, TNF α also upregulated several leukocyte-recruiting genes, including *CCL8*, *CXCL10*, *CX3CLI*, and *CCL5*. Taken together, these data suggest a role for TNF α -induced inflammation in retinal endothelial cells that is likely to contribute to leukostasis.

PPAR β/δ has only recently been studied in inflammation, with most work suggesting that PPAR β/δ agonism is anti-inflammatory. According to the studies published, the activation of PPAR β/δ prevented TNF α -induced inflammation in adipocytes, human umbilical vein endothelial cells, and proximal tubular cells [12,13,25,26]. In addition, PPAR β/δ agonists have been shown to be protective against inflammation in both hyperoxia-induced lung injuries and spinal cord injuries in rodents [27,28]. There has been little work done, however, on the PPAR β/δ antagonist and inverse agonist GSK0660, and those studies typically co-administer it with the agonist. Studies that have used GSK0660 by itself have showed its role thus far in reversing ginseng-enhanced cardiac contractility, worsening spinal cord injury in diabetic rats, reducing retinal vascular permeability, and inhibiting psoriasis-like skin disease [29-32]. As well, our laboratory has shown that GSK0660 inhibits retinal endothelial cell proliferation and oxygen-induced retinopathy [15]. While the anti-angiogenic properties of GSK0660 make it a possible therapeutic for DR, it was not known whether GSK0660

would exacerbate or protect against inflammation, another component of this disease.

The RNA-seq analysis revealed GSK0660 differentially regulated several transcripts in TNF α -treated HRMECs. These transcripts have possible diverse roles in cell proliferation, wound response, cell recognition, and calcium ion-dependent exocytosis. Importantly, the only pathway significantly enriched was the cytokine–cytokine receptor interaction. This finding becomes even more significant when the list of transcripts is limited to those both differentially expressed by TNF α compared to the vehicle and by TNF α plus GSK0660 treatment compared to TNF α treatment alone. GSK0660 prevents the TNF α -induced upregulation of *CCL8*, *CCL17*, and *CXCL10*. *CCL8*, which is also known as *MCP-2*, *CCL17*, and *CXCL10* are chemokines that attract and activate leukocytes. Interestingly, these data suggest a possible role for GSK0660 in the prevention of TNF α -induced leukostasis, particularly related to chemokine recruitment. This result is unexpected, as the agonists of PPAR β/δ have been shown to inhibit TNF α -induced cell adhesion through the inhibition of the VCAM1 and ICAM1 expressions [12,13]. These results suggest that PPAR β/δ may have a contradictory effect on TNF α -induced leukostasis in that its activation inhibits cell adhesion through inhibition of the adhesion molecule expression, while the inhibition of PPAR β/δ prevents leukocyte recruitment.

TNF α is a potent inflammatory factor in HRMECs, with a role in leukocyte recruitment and adhesion to the vascular endothelium. Our RNA-seq revealed a novel role for GSK0660 in the regulation of the TNF α -dependent expression of cytokines known to be involved in leukostasis. GSK0660 may block some of the TNF α -dependent changes in expressions that facilitate leukocyte recruitment. Future studies will be aimed at investigating GSK0660 in this context.

APPENDIX 1. SUMMARY OF READS MAPPING TO THE HUMAN GENOME (UCSC HG19) USING TOPHAT V2.0.9.

To access these data, click or select the words “[Appendix 1.](#)” Total reads for 3 samples each of vehicle, TNF α , and TNF α + GSK0660 treated HRMEC were generated using RNA-seq and then mapped to the human genome UCSC hg19.

APPENDIX 2. TRANSCRIPTS DIFFERENTIALLY REGULATED BY TNFA TREATMENT IN HRMEC.

To access these data, click or select the words “[Appendix 2.](#)” Transcript fold change and adjusted p value were determined using the edgeR algorithm and were considered to be

significant with the adjusted p value <0.05 and a fold change ≥ 2 .

APPENDIX 3. TRANSCRIPTS DIFFERENTIALLY REGULATED BY CO-TREATMENT OF GSK0660 AND TNFA TREATMENT IN HRMEC COMPARED TO TNFA TREATMENT ALONE.

To access these data, click or select the words “Appendix 3.” Transcript fold change and adjusted p value were determined using the edgeR algorithm and were considered to be significant with the adjusted p value <0.05 and a fold change ≥ 2 .

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