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1. Introduction

Prostaglandin glyceryl esters (PG-Gs) are endogenous lipid mediators that are derived from the arachidonic acid derivative 2-arachidonoylgylcerol (2-AG) by the action of cyclooxygenase (COX) enzymes (**Figure 1**). PG-Gs act in the body by interacting with cellular receptors, triggering Ca^{2+} mobilization, inositol 1,4,5-triphosphate synthesis, and activation of protein kinase C (Kingsley *et al.*, 2019; Nirodi *et al.*, 2004). Some of the biological effects of these bioactive molecules are due to the action of their corresponding free (i.e., non-esterified) prostaglandins, which complicates their study (Kingsley *et al.*, 2019; Turcotte *et al.*, 2017). Prostaglandins, in both their glyceryl ester and free prostaglandin forms, are known to play roles in the polarization of macrophages. Our past work considered the effects of prostaglandin D_2 glyceryl ester (PGD_2-G) on macrophages that had been stimulated with lipopolysaccharide (LPS), a pathogen-associated molecular pattern (PAMP) component of Gram-negative bacteria that switches the phenotype of macrophages to a pro-inflammatory phenotype (Scheaffer *et al.*, 2020). PGD_2-G and its carboxylesterase 1 (CES1)-derived hydrolysis metabolite, prostaglandin D_2 (PGD_2), were shown to decrease the levels of the LPS-induced pro-inflammatory cytokine interleukin (IL)-6, thereby diminishing the pro-inflammatory activity of the macrophages. In contrast, PGE_2-G and PGE_2, regioisomers of PGD_2-G and PGD_2, were found to augment the production of LPS-induced IL-6, further enhancing the pro-inflammatory activation of the cells. Regioisomers have the same chemical composition, but different physical arrangements.

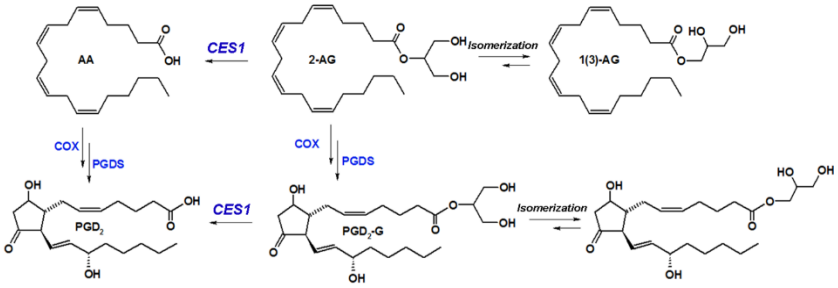

Figure 1: The formation of prostaglandins PGD_2-G and PGD_2 from 2-arachidonoylgylcerol (2-AG). CES1, carboxylesterase 1; COX, cyclooxygenase; PGDS, prostaglandin synthase; AG, arachidonoylglycerol; AA, arachidonic acid; PGD_2, prostaglandin D_2; PGD_2-G, prostaglandin D_2 glyceryl ester.

In the current study, we have further considered the role of the PGD_2-G metabolite PGD_2 in macrophages that were skewed to an anti-inflammatory phenotype by the Th2 cytokine IL-4. Macrophages are cells of the immune system that are known for their phagocytic properties (Ley, 2017). They ingest dead cells and cellular debris, induce the production of growth factors to aid tissue repair, and recognize and eliminate pathogens. IL-4 is a small soluble protein (~15 kDa) that has several different effects on immune cells, like macrophages. For example, it promotes the differentiation of naive helper T cells (Th0 cells) to Th2 cells, which is a crucial cellular transition in adaptive immunity. IL-4 can also influence macrophage phenotypes. When stimulated with IL-4, macrophages undergo 'alternative (M2) activation' and display an anti-inflammatory profile that is important in inflammation resolution and wound healing processes (Dubourdeau *et al.,* 2008; Bhattacharjee *et al.,* 2013). One of the most strongly upregulated genes in M2-activated human macrophages is arachidonate 15-lipoxygenase (*ALOX15*) (Bhattacharjee *et al.,* 2013); in fact, the expression of ALOX15 mRNA and protein typically occurs only after IL-4 stimulation (Han *et al.,* 2014), making it an effective M2 polarization indicator or marker. ALOX15 is an enzyme that is best known for oxygenating polyunsaturated fats and phospholipids in biological membranes. Its enzymatic action leads to the production of small signaling molecules, including 15-*S*-hydroxyeicosatetraenoic acid [15-(*S*)-HETE], that are involved in various inflammatory diseases and cancer (Han *et al.,* 2014). Therefore, understanding the mechanisms that regulate its production in different contexts is important for understanding the body's response to inflammatory stress.

In normal cell physiology, both ALOX15 mRNA and protein expression are tightly controlled and regulated (Han *et al.,* 2014). In the canonical IL-4 signaling cascade, IL-4 binds to its cell-surface receptor causing it to dimerize (or heterodimerize with the IL-13 receptor). This

dimerization leads to the auto-phosphorylation of the Janus kinase 1 (JAK1) enzyme that is associated with the IL-4 receptor and the subsequent JAK1-mediated phosphorylation of IL-4Rα subunit and signal transducer and activator of transcription 6 (STAT6) proteins. Phosphorylated STAT6 dimerizes and moves into the nucleus thereby activating the transcription of the *ALOX15* gene (Shankaranarayanan *et al.*, 2001). This process is described in more detail in **Figure 2**. In the experimental model described in this thesis, a human macrophage cell line was stimulated with IL-4 in the presence or absence of PGD_2. Because our previous results showed an attenuation of pro-inflammatory IL-6 levels when LPS-stimulated macrophages were treated with PGD_2, the expectation was that PGD_2 would augment the effects of anti-inflammatory IL-4. However, the addition of PGD_2 caused a profound decrease in IL-4-induced *ALOX15* mRNA and ALOX15 protein production. Therefore, the main goal of this research was to investigate the mechanisms that account for this unexpected finding. This study of *ALOX15* was also paired with a study of fatty acid binding protein 4 (*FABP4*), another gene induced by IL-4. *FABP4* is a target gene for PPARγ, a receptor that when activated has anti-inflammatory activity (Han *et al.* 2017). We also anticipated an increase in *FABP4* expression in the presence of PGD_2. This increase was noted, yet the method of regulation was unclear. By pairing the study of these two genes, *ALOX15* and *FABP4*, we sought to describe two different methods by which PGD_2 acts in the body.

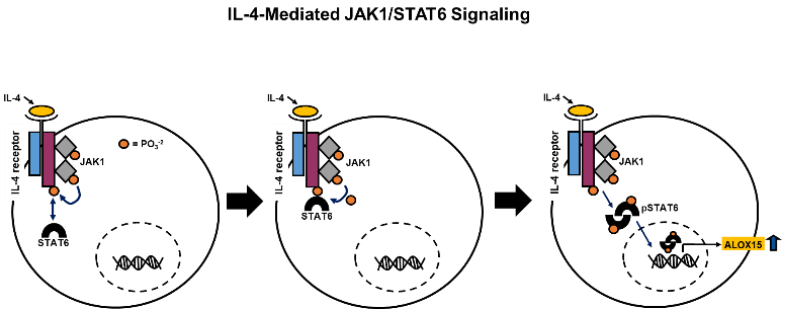

Figure 2: In normal macrophages, *ALOX15* mRNA is produced through a signaling cascade involving JAK1 and STAT6 phosphorylation. IL-4 binds to its receptor and JAK1 becomes phosphorylated. It, in turn, phosphorylates the IL-4 receptor and can phosphorylate STAT6. The activated STAT6 dimerizes and moves into the nucleus, acting as a transcription factor for *ALOX15*. IL-4, interleukin-4; JAK1, Janus kinase 1; STAT6, signal transducer and activator of transcription 6; pSTAT6, phosphorylated STAT6; ALOX15, arachidonate 15-lipoxygenase.

2. Hypothesis and Objectives

Our previous work, which used a cell culture model (human THP-1 monocytes/ macrophages), showed that the bioactive lipid PGD_2 and its glyceryl ester derivative (PGD_2-G) exerted anti-inflammatory properties in the setting of LPS-mediated inflammation (Scheaffer *et al.*, 2020). When LPS-stimulated macrophages were incubated with PGD_2 and PGD_2-G, the amounts of pro-inflammatory cytokine IL-6 were attenuated. In an anti-inflammatory environment, such as that promoted by the Th2 cytokine IL-4, we hypothesized that these lipid mediators would strengthen the effects of IL-4 by enhancing its anti-inflammatory responses. However, our initial studies showed an opposite effect in that PGD_2 attenuated IL-4 signaling in macrophages; that is, instead of enhancing IL-4's anti-inflammatory activity PGD_2 inhibited it, which was reflected by decreased *ALOX15* gene expression. We further hypothesized that PGD_2 must somehow prevent the production of anti-inflammatory molecule ALOX15 by interfering in the IL-4/STAT6 signaling axis. At the same time, it appeared that PGD_2 also augmented the IL-4-induced *FABP4* gene expression, a gene that is regulated by the anti-inflammatory PPARγ receptor. Using experimental approaches such as RT-qPCR to determine mRNA levels, immunoblotting to measure protein levels, and other techniques to measure gene activity, the purpose of the present study was to determine the mechanism by which PGD_2 interfered in the IL-4-induced responses in macrophages, thus altering the anti-inflammatory gene expression response IL-4 normally activates.

3. Materials and Methods

3.1 Cells, Chemicals, and Reagents

Human THP-1 monocytes and high-glucose RPMI medium were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Gentamicin sulfate solution (50 mg/mL) and low endotoxin-containing fetal bovine serum (FBS) were from Gibco, Life Technologies Corporation (Grand Island, NY), and phorbol 12-myristate 13-acetate (PMA) was from Fisher Scientific (Houston, TX). Lentiviral particles containing *PPARγ* shRNA and scrambled (nonspecific) shRNA constructs and puromycin hydrochloride were from Santa Cruz Biotechnology (Dallas, TX). PGD_2-G, PGE_2-G, PGD_2, and PGE_2 were from Cayman Chemical (Ann Arbor, MI). The PPARγ receptor antagonist GW9662 and the DP1 receptor (prostaglandin D_2 receptor) antagonist MK0524 were also from Cayman Chemical. The carboxylesterase inhibitor WWL113 was from Sigma. RNA isolation kits and SYBR green qRT-PCR master mix were from Qiagen (Valencia, CA), and the cDNA First-Strand Synthesis reagents from ThermoFisher Scientific (Waltham, MA). The pre-validated Quantitect primer assays for qRT-PCR experiments were from Qiagen (Valencia, CA) and the self-designed custom oligonucleotides were from Invitrogen (Carlsbad, CA). Additional primers were purchased from Eurofins Genomics (Louisville, KY). All primary antibodies used in Western Blot experiments were purchased from Abcam (Cambridge, MA). Secondary antibodies were from Santa Cruz Biotechnology.

3.2 Cell Culture Conditions

THP-1 monocytes were cultured in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium

bicarbonate, and 50 μg/mL gentamicin (complete growth medium) and maintained at 37°C at a density of 1×10^6 cells/mL with medium changes every 48-72 h. THP-1 monocytes were differentiated into macrophages by the addition of PMA (final concentration, 20 or 100 nM) to the complete growth medium for 24 or 72 h (Mangum *et al.*, 2018). For simplicity, THP-1 monocytes/macrophages are henceforth termed as monocytes when not treated with PMA or macrophages when treated with PMA.

Peroxisome proliferator-activated receptor-gamma (PPARγ) expression was knocked down in THP-1 monocytes by transduction with lentiviral particles containing *PPARγ* shRNA constructs and subjected to puromycin selection, as described previously (Mangum *et al.*, 2018); these are termed "PPARγKD" cells. Monocytes were also transduced using lentiviral particles containing scrambled shRNA constructs and are termed "control" cells. Control and PPARγKD monocytes were cultured in a complete growth medium with 5 μg/mL puromycin hydrochloride. Only puromycin-resistant cells were used for subsequent experiments. In this report, wildtype (WT) cells, control cells, and PPARγKD cells refer to cells that were non-transduced, transduced with scrambled shRNA, or transduced with PPARγ-specific shRNA, respectively.

3.3 RT-qPCR Analysis of THP-1 Macrophages Following Treatment with PGs/PG-Gs in the Presence and Absence of the CES1 Inhibitor WWL113

THP-1 monocytes (1×10^6 per well of a 12-well plate) were differentiated in complete RPMI by the addition of 100 nM PMA (final concentration) for 72 h at 37°C. After differentiation, the media was removed and replaced with 1 mL of serum-free RPMI. The 12-well plate was divided into three treatment groups (n=4). Cells were treated with prostaglandins (PGD_2, PGE_2, or PGD_2-G; final concentration of 10 μM) in the presence and absence of

WWL113 (final concentration of 3 μM WWL113, cells were preincubated with inhibitor for 30 min before adding the prostaglandin). Separate experiments indicated that neither the prostaglandins nor WWL113 are cytotoxic to the macrophages. Cells underwent a 1-h incubation with inhibitor and prostaglandins before human IL-4 (final concentration of 100 ng/mL) was added to all wells, followed by a 24-h incubation at 37°C. After the treatment period was over, total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen) according to the provided protocol. Total purified RNA amounts were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and cDNA was synthesized using protocol and reagents provided in the PreverAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR of the cDNA products was performed on a Stratagene Mx3005P thermal cycler with QuantiFast SYBR Green PCR master mix (Qiagen) or self-designed custom oligonucleotides (Invitrogen). The thermocycler program used for all target genes consisted of a 5 min hot start at 95°C prior to 40 cycles of 10 s at 95°C, followed by 30 s at 60°C, as recommended by the manufacturer. PCR product quality was assessed *via* dissociation curve analysis immediately following amplification. Differential expression of target genes was assessed by the $\Delta\Delta C_T$ method using *GAPDH* as the reference gene. Results are presented relative to the vehicle or control samples run within each experiment.

3.4 PGD_2 and PGE_2 Concentration Response Study in THP-1 Macrophages

In a 24-well plate, THP-1 monocytes (minimum of 5×10^5 per well in 18 wells) were differentiated in complete RPMI by a final concentration of 100 nM PMA for 72 h at 37°C. Following differentiation, the existing media was removed and replaced with 1 mL of serum-free RPMI. Each group (n=3) was treated with a different concentration of prostaglandin to give the final concentrations: 3.0 μM, 1.0 μM, 0.3 μM, 0.1 μM, 0.01 μM, and 0.0 μM. The

prostaglandins used were either PGD_2, PGE_2, or PGD_2-G. After a 1 h incubation, IL-4 (final concentration 100 ng/mL) was added to all groups, followed by a 24-h incubation at 37°C. Total RNA was extracted and cDNA created as described above, and RT-qPCR was conducted as detailed above. The genes examined were *ALOX15*, *FABP4*, and *JAK1*.

3.5 PPARγ Luciferase Reporter Gene Assay

COS-7 cells were transfected with a PPRE-luciferase reporter plasmid (PPRE x 3 firefly luciferase), a PPARγ expression plasmid, and a *Renilla*-luciferase plasmid (transfection efficiency control); all plasmids were obtained from Addgene. Cells were treated with the following lipid mediators: PGD_2, PGE_2, PGD_2-G, and PGE_2-G. After 24 h, the extent of luminescence was assessed in cell lysates by standard procedures. Firefly luciferase readings were normalized on *Renilla* luciferase readings to correct for plasmid transfection efficiency.

3.6 Inhibitor Studies – GW9662 and MK0524

To study the effect of small-molecule antagonists GW9662 and MK0524 on gene expression in THP-1 macrophages, THP-1 monocytes were differentiated for 48 h at 37°C in complete RPMI and 20 nM PMA. For the first experiment, cells were treated with: a) IL-4 alone, b) IL-4 and GW9662, c) IL-4 and PGD_2, d) IL-4, GW9662, and PGD_2. Final concentrations are as follows: IL-4, 100ng/μL; GW9662, 25μM; and PGD_2, 10μM. The IL-4 and PGD_2 concentrations remained consistent throughout this set of experiments.

A second set of differentiated THP-1 monocytes were treated to test the effects of DP-1 receptor antagonist MK0524. Cells were treated as follows: a) IL-4 alone, b) IL-4 and MK0524, c) IL-4 and PGD_2, d) IL-4, MK0524, and PGD_2 with the final concentration of MK0524 set to 10 μM.

Finally, the last set of THP-1 monocytes were differentiated to macrophages and were treated with a combination of GW9662 and MK0524. The treatment groups were a) IL-4 alone, b) IL-4 and PGD_2, c) IL-4, GW9662, and MK0524 d) IL-4, GW9662, MK0524, and PGD_2 all with the final concentrations mentioned above.

For all three experiments, cells were incubated for 16 h at 37°C. Following the incubation, RNA was extracted, cDNA prepared, and RT-qPCR analysis completed as described above.

3.7 Western Blot Analyses

All Western blot experiments described below were conducted according to the same general protocol.

Wild-type monocytes were differentiated over three days into macrophages by the addition of 20 nM PMA. They were then divided into 4 treatment groups (n=3): vehicle control, IL-4 (100 ng/μL), IL-4 (100 ng/μL) + 10 μM PGD_2, and 10 μM PGD_2 alone. These macrophages were incubated for 16 h at 37°C. Following incubation, cell lysates (proteomes) were prepared in ice-cold lysate buffer (RIPA buffer with added protease and phosphatase inhibitors) by sonication (3×10 s, on ice), followed by protein quantification (BCA reagent, Thermo-Pierce). 6× SDS-PAGE loading buffer (reducing) was added to proteomes (1 mg protein/mL of lysis buffer), and all samples heated at 90°C for 5 min to denature all proteins. The proteomes were separated by electrophoresis in a 10% SDS-PAGE gel and transferred to an Immuno-Blot PVDF Membrane (Bio-Rad, Hercules, CA). The membrane was blocked in a 5% non-fat milk solution in Tween buffer for 2 h at room temperature. Next, the membrane was incubated with an anti-phosphorylated STAT6 antibody (1:1,000 v/v in 5% non-fat milk solution

in Tween buffer) overnight at 4°C, then washed at least 3 times with Tween buffer. This was followed by incubation with 1:6,000 v/v goat anti-rabbit secondary antibody for 1.5 h, followed by membrane washes. Finally, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate solution (Thermo Scientific) for 5 min and chemiluminescent signals captured using a BioRad gel documentation system.

This same membrane was used to visualize the presence of STAT6, ALOX15, and GAPDH (loading control) proteins. After reading the membrane for pSTAT6, the membrane was incubated in a BioRad stripping buffer (~10 min) and blocked again in a 5% non-fat milk solution for 1.5 h. It was next placed into a solution of 1:1,000 anti-STAT6 antibody for 1.5 h and washed 3 times with Tween. After incubation with the same secondary antibody as mentioned above, and more washes, the membrane was incubated again with the chemiluminescent substrate solution for 5 minutes and visualized in the gel documentation system. For analyses of ALOX15 and GAPDH, the membrane was not stripped after reading because these proteins have different molecular weights than pSTAT6/STAT6 but rinsed for 1-2 min in Tween buffer before incubation with the next primary antibody. Anti-ALOX15 primary antibody was diluted 1:1,000 and the membrane was probed overnight at 4°C, while anti-GAPDH primary antibody was diluted 1:25,000 and the membrane probed for 1.5 h at room temperature. For each antibody, the procedure of membrane washes, incubation with the secondary antibody, and membrane visualization was the same.

In a separate experiment, Western blot was used to determine the presence of PPARγ protein. The same procedure was followed. The anti-PPARγ primary antibody was diluted to 1:1,000 v/v in 5% non-fat milk and the membrane was soaked in this solution overnight at 4°C.

The secondary antibody was a 1:6,000 v/v goat anti-mouse solution in 5% non-fat milk in Tween buffer and the membrane incubation lasted 1 h and 15 min.

3.8 RT-qPCR Analysis following THP-1 Macrophage Treatment ± IL-4 and PGD_2

THP-1 WT macrophages were divided into 4 treatment groups and treated as described for the Western blot analyses. Following treatment, RNA was extracted, cDNA produced, and qPCR run as mentioned previously for the following genes: *JAK1, SOCS1, SHP1, STAT6, PPARγ, FABP4, ALOX15, CYP27A1*. Primers were from either Qiagen, Invitrogen (self-designed), or Eurofins.

3.9 Statistical Analysis

SigmaPlot version 11.0 was used to perform all statistical analyses and results are presented as means ± SD. For quantitative RT-PCR gene expression results, data were converted to linearized $\Delta\Delta C_t$ values, as described (Schmittgen and Livak, 2008). Statistical comparison between cell types and treatment groups was performed through the Student's t-test and one- or two-way ANOVA with Student-Newman-Keuls *post hoc* testing. In most cases, a p value of less than 0.05 ($P < 0.05$) between groups was used to indicate significant statistical difference.

4. Results

4.1 WWL113 treatment attenuates both ALOX15 and FABP4 expression

In our previous work, it was shown that PGD_2-G and its CES1-mediated hydrolysis metabolite PGD_2 exhibited anti-inflammatory effects when present in an inflammatory cellular environment stimulated by LPS (Scheaffer *et al.*, 2020). The presence of the CES1 inhibitor WWL113 protected PGD_2-G from hydrolytic degradation thereby augmenting the anti-inflammatory activity of this prostaglandin derivative (Scheaffer *et al.*, 2020). The same trend was expected to occur in the setting of IL-4-mediated stimulation of macrophages; however, the opposite effect was observed. That is, when PGD_2-G was added to cells that had been stimulated by IL-4, an anti-inflammatory cytokine, the level of *ALOX15* mRNA, a gene commonly used to study alternative human macrophage polarization, was found to decrease, **Figure 3**. The addition of WWL113, instead of repairing the decreased *ALOX15* mRNA level, further attenuated its expression. In addition, the levels of *FABP4* mRNA, a PPARγ target gene that provides an excellent readout of the activity of this nuclear receptor, were assessed. *FABP4* encodes a protein that transports water-insoluble fatty acids around the cell. *FABP4* increased following treatment with PGD_2-G, whereas it decreased in the presence of WWL113. Similar trends are seen when the cells were treated with

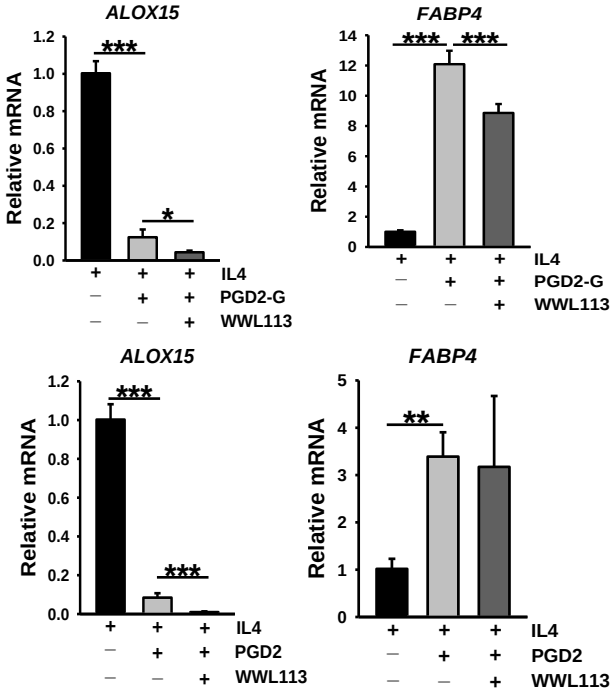

Figure 3: PGD_2-G and PGD_2 attenuate *ALOX15* expression, yet the addition of WWL113 does not repair these levels. Both prostaglandin molecules accentuate *FABP4* levels. IL-4, interleukin-4; PGD_2-G, prostraglandin D_2 glyceryl ester; PGD_2, prostaglandin D_2; WWL113, a CES1 inhibitor. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, relative to its corresponding non-PGD_2 treated group.

PGD_2, the PGD_2-G hydrolytic metabolite (see **Figure 3**). These results were unexpected and merited additional experimentation. Most of the further experiments were conducted with PGD_2, as the metabolite, however PGD_2-G was used to compare trends as was PGE_2-G, a documented pro-inflammatory molecule (Alhouayek *et al.*, 2013).

4.2 PGD_2 increases FABP4 mRNA levels and decreases ALOX15 mRNA levels in a concentration-dependent manner

In general, if there is an association between the presence of a certain compound and altered gene expression in the treated cells, increasing the amount of the compound in the cultured cells will cause a concentration-dependent change in gene expression. This was precisely what we observed for both *ALOX15* and *FABP4* expression when macrophages were treated with PGD_2 (**Figure 4**). *ALOX15* expression decreased steadily as PGD_2 concentration increased. In contrast, *FABP4* expression steadily increased as the concentration of PGD_2 increased. Not only does this confirm the results seen in the previous experiment, it also reaffirms that PGD_2 must be acting through two different mechanisms to affect the expression of these two genes. If PGD_2 is upregulating the activity of one receptor to increase *FABP4*, it must be downregulating transcriptional activity by other means leading to *ALOX15* reduction.

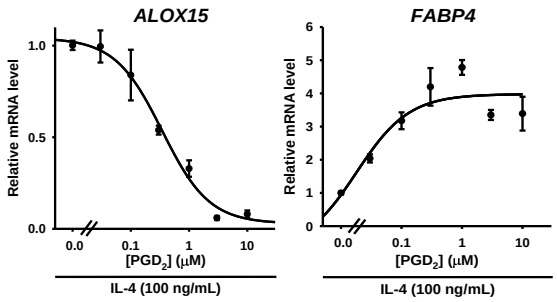

Figure 4: We observe a concentration-dependent affect in both *ALOX15* and *FABP4* mRNA levels. As PGD_2 concentration increases, *ALOX15* production decreases and *FABP4* is augmented. IL-4, interleukin-4; PGD_2, prostaglandin D_2.

4.3 FABP4 expression is controlled through a combination of PPARγ receptor and PGD_2 receptor (DP1) regulation

The results of the PPARγ luciferase reporter gene assay, an assay that ties light production to PPARγ activation so that its activation can be measured via luminescence, showed that PGD_2 is a significant PPARγ activator. In the presence of PGD_2, PPARγ activity increases to a greater extent than it does in the presence of GW1929 (**Figure 5)**, a well-known PPARγ agonist (Han *et al.*, 2017). This finding links PPARγ activity to PGD_2 presence, and therefore with *FABP4* expression, a canonical target gene for PPARγ.

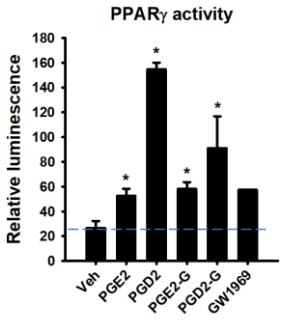

Figure 5: PPARγ transcriptional activity is enhanced by PGD_2. PPARγ, perioxisome proliferator-activated receptor-gamma; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2. $^{*}p < 0.05$ relative to vehicle (Veh).

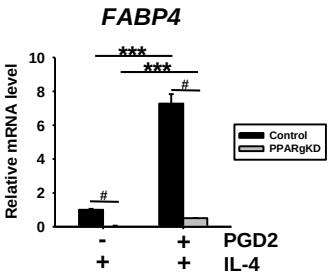

Figure 6: When PPARγ gene expression is knocked down, *FABP4* levels are abrogated. IL-4, interleukin-4; PGD_2, prostaglandin D_2. $^{***}p < 0.001$, $^{\#}p < 0.05$, relative to its corresponding non-PGD_2 treated group.

Additionally, our Western blot data shows that PGD_2 does not change the amount of PPARγ protein present in the cells (data not shown). Therefore, the observed activity change is not due to a change in the amount of PPARγ, but is because what is already there becomes more active. Whenever PGD_2 is added to the cells the levels of *FABP4* expression increases. If PPARγ gene expression is knocked down, as in PPARγKD cells, *FABP4* levels are markedly reduced (**Figure 6**). In the presence of PPARγ antagonist, GW9662, the induction of *FABP4* expression caused by PGD_2 is also significantly reversed (**Figure 7**). This leads us to conclude that PGD_2 must be influencing the PPARγ receptor to cause changes in *FAPB4* mRNA levels.

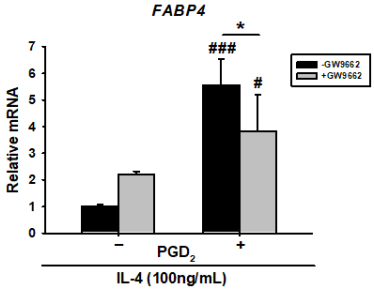

Figure 7: PGD_2 induces *FABP4* mRNA expression. The addition of antagonist GW9662 attenuates this expression. IL-4, interleukin-4; PGD_2, prostaglandin D_2. $^{*}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#}p < 0.05$, relative to its corresponding non-PGD_2 treated group.

The G-protein coupled receptor that recognizes PGD2 (termed DP1) was also considered as a possible means of PGD_2 control over *FABP4* production. When PGD_2 is used to treat cells, the DP1 receptor antagonist MK0524 acts to reduce *FABP4* expression in a manner similar to the PPARγ antagonist GW9662. A significant reduction in expression levels is seen (**Figure 8**). This same reduction is observed when both the PPARγ and DP1 antagonists were incubated with PGD_2-treated cells. Based on this, we believe that the DP1 receptor also contributes to *FABP4* gene expression control, because a similar decrease in activity was seen in *FABP4* expression when the DP1 and PPARγ receptors were blocked.

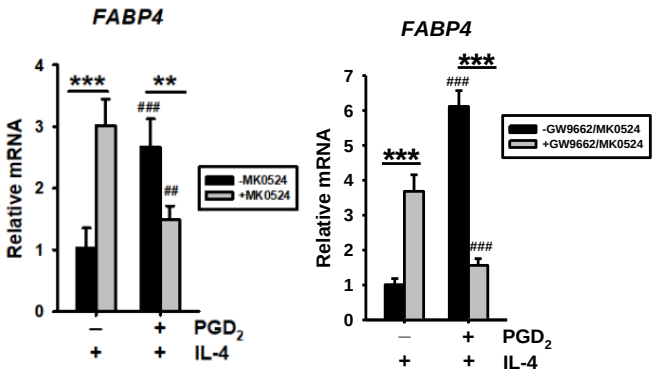

Figure 8: DP1 receptor inhibition causes a decrease in *FABP4* mRNA. When both antagonists are present, MK0524 and GW9662, the attenuation seems to be more similar to the results of DP1 antagonism alone. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, relative to its corresponding non-PGD_2 treated group.

4.4 Inhibiting the PPARγ and DP1 receptors does not affect the attenuation seen in ALOX15 when PGD_2 is present

Contrary to our studies with *FABP4*, the PGD2-mediated decreases in *ALOX15* expression were not altered by inclusion of either PPARγ or DP1 receptor antagonists. This holds true for *ALOX15* mRNA levels measured in PPARγKD cells, cells treated with the PPARγ receptor antagonist GW9662, and cells treated with the DP1 receptor antagonist MK0524. While some variation was seen in *ALOX15* expression in cells incubated with either GW9662 or MK0524 without PGD_2 treatment, there is almost no variation when PGD_2 is present. Its presence always results

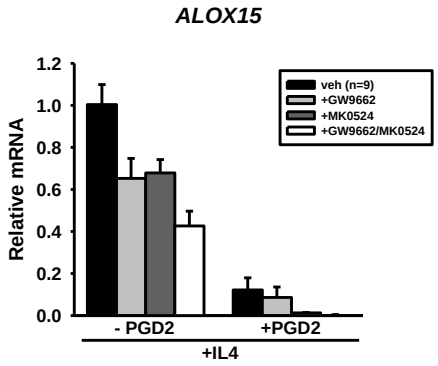

Figure 9: No matter the treatment, when PGD_2 is present, *ALOX15* mRNA expression decreases. IL-4, interleukin-4; PGD_2, prostaglandin D_2.

in decreased *ALOX15* expression when compared to the vehicle control (**Figure 9**). Because of this, we hypothesized that PGD_2 might be interfering with *ALOX15* production not through its binding to a receptor but by interfering directly in the IL-4 signaling pathway itself that leads to *ALOX15* production.

4.5 STAT6 and JAK1 mRNA expression levels do not change when THP-1 macrophages are treated with PGD_2

Because we speculated that PGD_2 must be interfering somewhere in the IL-4 pathway, we first assessed the levels of the key protein players themselves for a PGD_2-dependent change. No change was seen in STAT6 expression, either at the mRNA level or protein levels, as shown in **Figure 10**. For example, in our Western blot experiments we found that total STAT6 protein levels did not change in the presence of PGD_2. We also investigated *JAK1* gene expression levels. Like *STAT6*, the presence of PGD_2 did nothing to change its expression. We were not able to examine the JAK1 protein levels via Western blot (because the JAK1 antibody that we used was not effective), but we did conduct a concentration study to corroborate our gene expression data (**Figure 11**). We found that even at the highest concentrations of PGD_2, *JAK1* mRNA levels remained unaffected. Because STAT6 protein or *STAT6* and *JAK1* mRNA levels were not affected by PGD_2, we next moved to investigating the activation of the STAT6 protein by phosphorylation when PGD_2 was present.

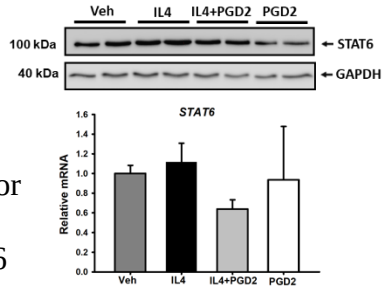

Figure 10: Western blot data depicts no change in STAT6 protein expression. This is also seen for *STAT6* mRNA levels. IL-4, interleukin-4; PGD_2, prostaglandin D_2.

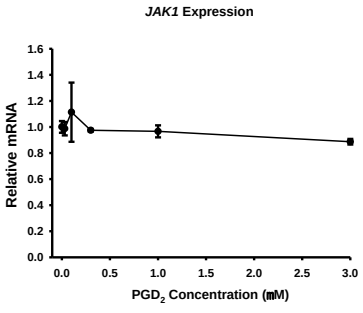

Figure 11: *JAK1* expression remains unchanged even at the highest PGD_2 concentrations. Therefore, there must not be a connection between PGD_2 and *JAK1* mRNA production. PGD_2, prostaglandin D_2.

4.6 PGD_2 decreases the levels of phosphorylated STAT6

The only significant change that we were able to detect when analyzing the possible modes of PGD_2 interference was in the phosphorylated STAT6 levels (**Figure 12)**. As previously mentioned, STAT6 levels were unchanged in the presence of PGD_2. However, the activity of this IL-4-induced phospho-protein is changed in the presence of PGD_2. It is worth noting again that in this instance phosphorylation of STAT6 activates its transcription factor activity, whereby it stimulates the synthesis of mRNA molecules of genes it positively regulates (by binding to the promoter and enhancer regions of those genes) (Heydeck *et al.*, 1998). Only the STAT6 proteins that contain a phosphoryl group will be officially active in this pathway and directly lead to *ALOX15* production. It should also be mentioned that IL-4 is necessary for STAT6 phosphorylation. The presence of IL-4 is what induces this pathway to begin working; STAT6 would not have a need to be activated through phosphorylation unless IL-4 was present, and the pathway turned on. As this change in STAT6 activation levels is the only detectable decrease that correlates with both the decrease in *ALOX15* gene production and ALOX15 protein presence (**Figure 12**), we conclude that PGD_2 interferes with STAT6 phosphorylation. It is by this interference that *ALOX15* production decreases, which explains our previous results. Interestingly, the effects of PGD_2 seem to be very specific, because the regioisomer PGE_2 did not alter IL-4-induced phosphorylated STAT6 amounts or *ALOX15* mRNA levels (data not shown).

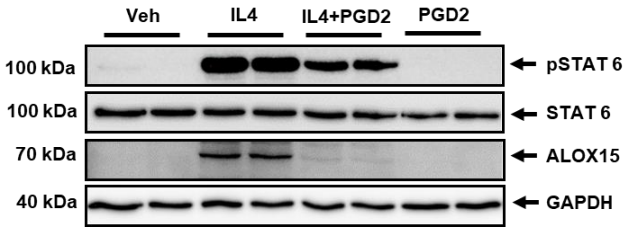

Figure 12: PGD2 affects the production of pSTAT6, thus leading to reduced levels of ALOX15. By eliminating the ability of pSTAT6 to be produced, there is less ALOX15 transcription factor available, and inevitably its production must also decrease. IL-4, interleukin-4; PGD_2, prostaglandin D_2; ALOX15, arachidonate 15-lipoxygenase .

5. Discussion

Our objective in conducting this study was to determine how the presence of PGD_2 modulates the anti-inflammatory affects that occur in macrophages stimulated with anti-inflammatory cytokine IL-4. Macrophages are key cells in the immune system and play a major role in controlling the inflammatory phenotype of the area of the body where they are found. By further understanding how PGD_2 plays an activating/deactivating role in helping to regulate the inflammatory phenotype, we gain knowledge of perhaps another approach by which it may be possible to control the inflammatory process.

The main findings for this study can be divided into two parts: (1) PGD_2's effect on *FABP4* gene expression, and (2) its ability to interfere in the IL-4 pathway. When macrophages were treated with PGD_2, we observed an increase in *FABP4* gene expression. By examining mRNA gene expression in both control and PPARγKD cells, it was noted that the knockdown of PPARγ expression in macrophages correlated with a decline in *FABP4* expression. A decline is also noted when THP-1 wild-type macrophages were treated with a PPARγ antagonist, GW9662, when exogenous PGD_2 was added. Because of this, we conclude that PPARγ, a nuclear receptor, is necessary for the expression of *FABP4*. However, there is also an additional route of *FABP4* activation through the DP1 receptor. In fact, the antagonism of this receptor, when compared to PPARγ antagonism, had a greater impact on *FABP4* expression when in the presence of PGD2. Therefore, because both receptors affect *FABP4*, each are involved in its regulation, although in the presence of PGD_2 the regulation by the DP1 receptor may be greater.

FABP4 is a target gene for *PPARγ* gene expression (Janani and Kumari, 2015; Han *et al.*, 2017). PPARγ is significant because it plays a major role in the efflux of cholesterol from macrophages and in the storage, release, and transport of fatty acids. It also helps to regulate

lipid and glucose metabolism and is involved in transcriptional regulation of other genes involved in metabolic processes. Our study links PGD_2 to PPARγ. If further research continues to indicate a connection between these two, PGD_2 may be considered an important target for PPARγ regulation. This is a significant finding for those seeking to help regulate macrophage-mediated inflammatory processes.

The second half of our study explored possible modes by which PGD_2 interferes in the IL-4 pathway. It was initially noted that when wild-type macrophages were treated with PGD_2 *ALOX15* mRNA levels decreased. *ALOX15*, an M2 marker gene, encodes the ALOX15 enzyme, which is responsible for the oxidation of arachidonic acid into 15-(*S*)-HETE. 15-(*S*)-HETE then goes on to play a role in modulating further immune responses (Choe and Kwon, 2019). There are three possible ways that PGD_2 might affect *ALOX15* expression: 1) by acting as a ligand for PPARγ; 2) by activation of the PGD_2 receptor, DP1; or 3) by interfering directly in the IL-4 pathway (possible areas of interference are detailed in **Figure 13**). In addition to what has been mentioned above, PPARγ is also a transcription factor that is induced by IL-4 (Shankaranarayanan *et al.*, 2001; Dubourdeau *et al.*, 2008). We thought it logical that the activation of this receptor might initiate a signaling cascade that interfered with *ALOX15* expression. Similarly, PGD_2 can act through binding to the DP1 receptor. Again, we reasoned that this binding could have a downstream effect on *ALOX15*. Our initial results discouraged both these theories. We concluded that PGD_2 affects the activation, or

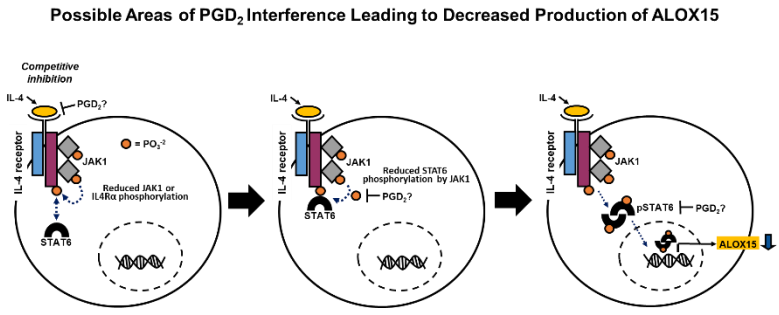

Figure 13: PGD_2 may interfere at multiple points along the IL-4 pathway: during (1) IL-4 binding, (2) JAK1, IL4Rα, and/or STAT6 phosphorylation, or (3) pSTAT6 dimerization and nuclear translocation. IL-4, interleukin-4; PGD_2, prostaglandin D_2, JAK1, Janus kinase 1; STAT6, signal transducer and activator of transcription 6; pSTAT6, phosphorylated STAT6; ALOX15, arachidonate 15-lipoxygenase.

phosphorylation, of the STAT6 protein. This protein has transcription factor activity and moves into the nucleus to directly control the synthesis of *ALOX15* mRNA. By inhibiting STAT6 phosphorylation, PGD_2 reduces *ALOX15* production, thereby decreasing its effects on further cellular functions.

As mentioned previously, *ALOX15* is a classic M2 marker gene in human macrophages, M2 indicating an alternative polarization to an anti-inflammatory phenotype, as opposed to the M1 inflammatory phenotype. Macrophages play a role in several diseases, such as atherosclerosis, auto-immune diseases, and cancer (Valledor *et al.*, 2010), and they have the ability to switch between these polarization states in order to best regulate cellular processes for the benefit of the body. In reality, as Mosser describes it, it is less of a firm switch between one phenotype and the other, and more like a shift along a linear gradient between the two extremes of full M1 or M2 polarization (Mosser and Edwards, 2008). We discovered that PGD_2 is a molecule that has a role in the process of macrophage polarization. By exploring the interference of PGD_2 in IL-4-induced ALOX15 biosynthesis, it may be that we have found one means by which cells become maladaptive in disease. Inducing PGD_2 production can keep macrophages from acting to their fullest anti-inflammatory potential, and thereby keep the immune interference of these cells to a minimum.

6. Conclusions

We have shown that the lipid mediator PGD_2 interferes in the IL-4 pathway in macrophages to decrease *ALOX15* mRNA expression. A decrease in *ALOX15* most likely translates to a decrease in production of the downstream immunoregulatory compounds, like 15-(*S*)-HETE. This is evident from gene expression and Western blot studies that show ALOX15 levels to be substantially decreased by PGD_2. On the other hand, *FABP4* expression levels increase when PGD_2 is present. Based on our results using receptor antagonists, we conclude that *FABP4* levels are controlled through a combination of the PPARγ and DP1 receptors. In the presence of PGD_2 it seems that *FABP4* expression depends on DP1 receptor activation. As to *ALOX15* mRNA and ALOX15 protein production, PGD_2 appears to interfere with phosphorylated STAT6 production. This interference causes less phosphorylated STAT6 to be available to move into the nucleus and induce *ALOX15* mRNA production. Understanding the way these signaling pathways work is important in the drug discovery process for inflammation treatment. By understanding the way that the body returns to homeostasis following inflammatory stress, the treatment strategies for targeting chronic unresolving inflammation may be improved, thereby increasing standards of care and ultimately standards of living.

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