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Comparison of Different Modulators that Affect Macrophage Activation In Vitro

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Comparison of Different Modulators that Affect Macrophage Activation *In Vitro*

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science in Cell and Molecular Biology

By

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ABSTRACT

Inflammation is known as a mechanism to regulate and control infections as well as promote tissue repair. Macrophages (M ϕ) are known to be a major cell type in the initiation, sustainability and resolution of inflammation. Moreover, M ϕ are essential for the remodeling process that is also known as the wound healing response. The objective of this research was to compare five modulators (acetylsalicylic acid (ASA), dexamethasone (DEX), prostaglandin E₂ (PGE₂), iloprost, and resolvin D₁ (RvD₁) for their anti-inflammatory effects on macrophages *in vitro*. Then, M ϕ phenotype in terms of gene expression and secreted cytokine response was determined. Our study compared NR8383 cells induced with LPS versus a modulator. Using ELISA measurements of chemokine (C-C motif) ligand 2 (CCL2), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF- α) performed. Gene expression analysis for the following transcripts: arginase -2 (ARG-2), nitric oxide synthesis type II (iNOS-2), macrophage - associated antigen (CD163) is known to be expressed by M2c phenotype and mannose receptor C type 1 (CD206) is known to be expressed by the cells of the M2 phenotype. In conclusion, each modulator has shown to present an anti-inflammatory response and acetylsalicylic acid (ASA), dexamethasone (DEX) and prostaglandin E₂ (PGE₂) did express CD163. Future work, further analysis will be necessary any functional of these in *in vivo*.

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LIST OF SYMBOL/ABBREVIATIONS

Acetylsalicylic acid (ASA)

Arginase-2 (ARG2)

Chemokine (C-C motif) ligand 2 (CCL2)

Cluster of Differentiation (CD-163 or CD-206)

Dexamethasone (DEX)

Electrospray ionization (ESI)

Interleukin (IL-6 and IL-10)

Liquid Chromatography (LC)

Lipopolysaccharide (LPS).

Macrophages (M ϕ)

Mass Spectrometry (MS)

Nitric oxide synthesis type II (iNOS-2)

Nuclear factor kappa light chain enhancer of activation B cells (NF- κ B)

Prostaglandin E₂ (PGE₂)

Prostaglandin I₂ (PGI₂)

Resolvin D₁ (RvD₁)

Toll-like receptor (TLRs)

Ultra high pressure liquid chromatography (UPLC)

Tumor necrosis factor alpha (TNF- α)

SIGNIFICANCE

Macrophages are one of the major cells that affect inflammation. One role of macrophages is to promote phagocytosis, but also macrophages are essential for the remodeling process which requires the balancing of inflammation. The objective of this research was to induce macrophage to a more anti-inflammatory state with different well-known modulators to compare protein secretion and macrophage activation. With these *in vitro* comparisons, it is possible to obtain a better understanding of the cytokine secretion and macrophage activation induced by each modulator, and also obtains better understanding of these modulators as a counterbalance to pro-inflammatory responses. Acetylsalicylic acid (ASA), dexamethasone (DEX), prostaglandin E₂ (PGE₂), prostaglandin I₂ and resolvin D₁ (RvD₁) are modulators which are known to produce an anti-inflammatory response (El et al., 2002; Kopp and Ghosh, 1994; MacKenzie et al., 2013; Titos et al., 2011; Zhou et al., 2007). The anti-inflammatory properties of each of these modulators can be used to alter macrophage phenotype. By altering the macrophage phenotype, it may be possible to improve biomaterial longevity and decrease formation of scar tissue.

INTRODUCTION

Inflammation is a complex mechanism of the innate immune system that involves recruitment of leukocytes and plasma proteins into sites of infection or tissue injury. Moreover, inflammation can also be triggered by molecular signals that are common features of microbes or dead cells. These patterns are known as pattern recognition receptors; for example, toll-like receptors (TLRs) which recognize bacterial DNA, virus, and other pathogens. In addition, inflammation is regulated by the

body to control infections and promote tissue repair, but also can cause tissue damage and disease. To maintain and promote the recruitment of leukocytes, the cells must be able to be recruited and transported to the site of which can be explained in two steps (Abbas, 2012):

1. Recruitment and accumulation of leukocytes through the endothelium to the local site of the injury: this process of recruitment is receptor mediated. For example, TNF- α and LPS promotes the expression of P-selectin in murine of the endothelial cell surface, and also, cytokines are regulated from the site of the injury which promote the induction of the expression of the E-selectin on endothelial cells. The secretion of cytokines is one of the first responses of the body to an infection, tissue damage or cell death (apoptosis).
2. Migration across into the endothelium: chemokine gradients, which are presented in the surrounding tissue, display on the endothelial surface. Chemokines, which are produced around the area, are going to be displaced on the endothelium surface and then bind to the receptors of the rolling leukocytes. Then, the leukocytes crawl between the endothelium cells and migrate through the venular. Monocytes, neutrophils and T lymphocytes use this mechanism to emigrate out of the blood stream (Abbas, 2012).

One type of cell that rises up into the endothelium by the inflammation mechanism is the monocyte/macrophage. Monocytes can be described as white blood cells which are being produced from the bone marrow by the precursor monoblasts, and are being stored in the spleen. Monocytes are cells that can circulate through the bloodstream, and also can reach into the tissue. Also, monocytes move through the bloodstream and into the tissue where they differentiate into macrophages. Macrophages (M ϕ) are primary cells that can be differentiated from monocytes in tissue. M ϕ are known to be a major player in the initiation and resolution of inflammation. Also, the role of the M ϕ cell is to phagocytose cells by attacking foreign substances, and also stimulate lymphocytes and others immune cells to defeat pathogens (Abbas, 2012). Macrophages can be activated by microbial molecules. For instance, an endotoxin, such as lipopolysaccharide (LPS) can activate macrophages to secrete numerous pro-inflammatory cytokines such as IL-6, IL-12, and TNF- α . Moreover, M ϕ can also switch from a pro-inflammatory to an anti-inflammatory microenvironment by secreting anti-inflammatory cytokines such as IL-10. M ϕ can auto-regulate because macrophages are a type of cell which can switch the phenotype (activation) depending on the chemicals residing in the microenvironment (Martinez et al., 2008).

In addition, macrophages are essential for the remodeling process that is also known as the wound healing response. In others studies, the M ϕ has shown a shifting of polarization which regulates the remodeling and inflammatory response. These wide-spectra of the macrophage polarity are handled by the body as either a mechanism of defense which can be expressed as a wound healing or an immune response. Moreover, M ϕ cells are known to play a central role in the regulation of immune responses and also in the acute and chronic inflammation (Garg et al., 2013). Depending of the microenvironment that M ϕ are exposed to, these plastic cells can differentiate into M1s

(which is the classical activation) and M2s (which is the alternative activation). The M1 phenotype (classical activation) is known to secrete pro-inflammatory cytokines, and also M1s can be activated by inducing with LPS. To clarify, M1s are induced by interferon-gamma (INF- γ) or tumor necrosis factor alpha (TNF- α), and secrete pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-12 and IL-23 (Mantovani et al., 2013). On the other hand, M2 phenotype (alternative activation) is known to promote tissue remodeling. Moreover, M2 phenotype activation can be further classified by subtype M2a, M2b and M2c. M2a phenotype tends to initiate type II inflammation. This macrophage type is presented against parasites and can be induced by IL-4, IL-10 and IL-13. M2b phenotype is considered as an immune-regulate/immune-suppressed and can be induced by IL-10 and TGF- β . Lastly, M2c phenotype is known to participate in matrix remodeling and tissue repair and can be induced by IL-10 (Martinez et al., 2008). In addition, M1 phenotype should be expressed first and then a transition to the M2 phenotype (Murray et al., 2014) promote wound remodeling (Garg et al., 2013). Thus, macrophages are known to behave according to the chemical in the microenvironment; for these reasons M ϕ can shift polarization depending on the presence of stimulating molecules.

On the other hand, new guidelines are being proposed to classify macrophages by their activation. To illustrate, when the cells are induced with LPS, the new guidelines denotation is M (LPS) which represent as macrophages (M) and LPS as the inducer compound. Another example, when M ϕ cells are induced with dexamethasone, the new guidelines denote as an M (DEX).

NF- κ B (nuclear factor kappa light chain enhancer of activated B cells), which is a protein complex that controls DNA transcription, can be triggered by the stimulation of a bacterial infection, cytokines irradiation and free radical (Shackelford et al., 1997). NF- κ B is a *rel* family transcription

factor which is present in the cytosol as a heterodimer of 50 kDa (p50) and 65 kDa (p65, Rel-A), and it is also bound to an inhibitor to protein subunit I κ B (Shackelford et al., 1997). The NF- κ B is known to be stimulated by lipopolysaccharides (LPS), an endotoxin, and TNF- α (tumor necrosis factor), IL-6 (interleukin) and other pro-inflammatory cytokines in response to induction by LPS. To clarify, LPS stimulation triggers the release of NF- κ B from I κ B which results in the translocation of NF- κ B from the cytoplasm to the nucleus whereas NF- κ B binds to the DNA of the cell and regulate the transcription of different genes. As a result of activation, the cells release pro-inflammatory signaling molecules such as IL-1, IL-6, IL-8, IFN- β , and TNF- α and others cytokine molecules (Kopp and Ghosh, 1994). On the other hand, modulators can be used to inhibit the NF- κ B protein complex. For example, dexamethasone is known to inhibit NF- κ B which results in lowered production of TNF- α , IL-12 and other pro-inflammatory cytokines. However, several modulators can be used to inhibit the activation of this protein complex.

Aspirin known as acetylsalicylic acid is the most widely used drug. It is often used as an analgesic to relieve pain, antipyretic to reduce fever, and as an anti-inflammatory drug. Aspirin is the first known nonsteroidal anti-inflammatory drug (NSAIDs) of which there are now a host of similar drugs; on the other hand, aspirin differs from other NSAIDs in its mechanism. A treatment at 3 mM of ASA has showed to be a potent anti-inflammatory drug in alveolar macrophages (Duan et al., 2014). For this reason, concentrations of ASA that was used in our study were in the range between 0.1 to 10 mM of ASA. In others studies, aspirin has been reported to inhibit NF- κ B through the stabilization of I κ B molecule. In cells such as macrophages, NF- κ B is known to encode inflammatory mediator such as TNF- α (Shackelford et al., 1997).

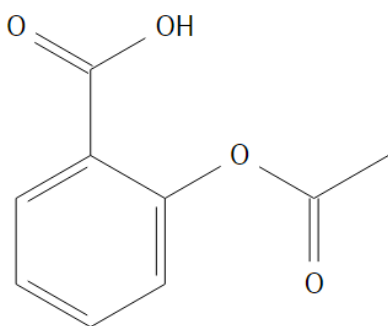


Figure 1: Molecular structure of acetylsalicylic acid (ASA)

Dexamethasone (DEX) is known to be a potent glucocorticoid, which is a steroid drug with anti-inflammatory properties and immunosuppressant effects. Glucocorticoids are known to inhibit the expression of inflammation mediators in cells such as macrophages. The anti-inflammatory response of glucocorticoid has shown to be an effective inhibitor of NF- κ B and activator protein 1 (AP-1), which are known to induce an inflammatory effect (Abraham et al., 2006). The activation of NF- κ B and activator protein 1 (AP-1) is induced by LPS which releases TNF- α into the microenvironment. To illustrate, dexamethasone is known to reduce the pro-inflammatory molecules by abolishing the pro-inflammatory cytokines: TNF- α , IL-8 and IL-6, but also exaggerating the anti-inflammatory cytokine: IL-10 (El Azab et al., 2002). In addition, the concentration of DEX and LPS were obtained from a previous study (Bajpai, 2015).

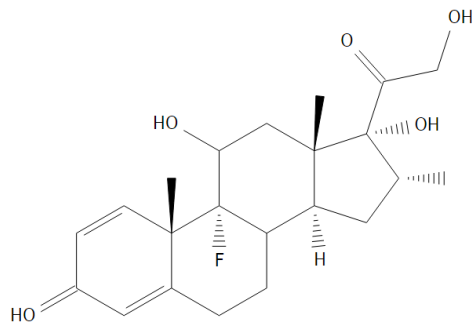


Figure 2: Molecular structure of dexamethasone (DEX)

Eicosanoids are lipid-signaling molecules which are known to be present mainly in inflammation and also these molecules are known to be a messenger in the central nervous systems (Balazy, 2004). Eicosanoids are derived from either omega-3 (ω -3) or omega-6 (ω -6) fatty acids. Prostaglandins (PGs) are a group from the ω -6 fatty acid family. Prostaglandins are known compounds which consist of 20 carbons with a 5 carbon ring (Funk, 2001). Moreover, PGs are known to be secreted in different places in the body, and also PGs can be secreted by mast cell or basophil and M ϕ (Abbas, 2012). The prostaglandins are in a subclass of a group called eicosanoids. The biosynthesis of the prostaglandin starts with the stimulation of toll-like receptor 4 (TLR4) by induction of LPS which leads to the activation of mitogen-activation protein kinase (MAP kinase) and IKK pathways. Then, the membrane phospholipid is hydrolyzed, and catalyzed by the enzyme phospholipid A₂ (PLA₂) (Sakata et al., 2010). As a result, macrophages or mast cells synthesize arachidonic acid (AA) which can hydrolyze to obtain prostaglandin G₂ (PGG₂), and subsequently prostaglandin H₂ (PGH₂) by the synthesis of prostaglandin H, which is also known as cyclooxygenase (COX). COX can be further derived into COX-1 (PTGS-1) and COX-2 (PTGS-2). Prostaglandin H₂ is an unstable lipid mediator which is soon converted into prostaglandin D₂ (PGD₂), E₂ (PGE₂), F (PGF_{2 α}), I₂ (PGI₂), and thromboxane (TXA₂) (Fukata and Abreu, 2008).

In other studies, the receptors that recognize the presence of PGE₂, can be further divided into four types EP₁, EP₂, EP₃, and EP₄. The PGE₂-EP₄ is known to be essential to the transcription factor. The effect of PGE₂ can direct the process of the suppression of inflammation and the enhancement wound healing (Takayama et al., 2002). Moreover, PGE₂ has been shown to inhibit chronic and acute inflammation *in vivo* and also can inhibit pro-inflammatory cytokine response in cells such as macrophages (Vassiliou et al., 2003). In addition, PGE₂ (at ~ 10⁻⁹ M) can inhibit TNF- α , and IL-6 and increase IL-10 in peritoneal macrophages (PM) which were stimulated with LPS 0.5 μ g/ mL for 24 hours (Strassmann et al., 1994). Moreover, LPS and TNF- α can be used to induce the production of PGE₂, on the other hand, the induction of PGE₂ with LPS is known to stimulate cells to inhibit TNF- α mRNA expression and protein secretion (Strassmann et al., 1994).

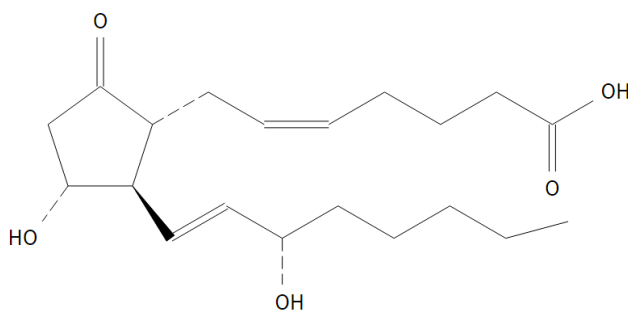


Figure 3: Molecular structure of prostaglandin E₂ (PGE₂)

Prostaglandin I₂ (PGI₂) is a lipid mediator member of the prostaglandin family known as an eicosanoid. PGI₂ is known as a prostacyclin which is a metabolite from arachidonic acid (AA), and also recent studies have shown it to have anti-inflammatory properties (Zhou et al., 2007). Prostacyclin receptor (IP₁) is a member of the G-protein coupled receptor family. The function of

PGI₂ exerts through G-proteins couple IP1 and signaling which leads to the raising levels of intracellular cAMP. However, PGI₂ is an unstable molecule in aqueous solution (Cho and Allen, 1978); for this reason, an analog of PGI₂ is used iloprost or cicaprost. Iloprost and cicaprost have shown to inhibit the production of pro-inflammatory signaling molecules such as TNF- α (Eisenhut et al., 1993). However, iloprost and cicaprost show an increase IL-10 in human peripheral mononuclear cells *in vitro* as well (Zhou et al., 2007). Moreover, iloprost and cicaprost also have shown to inhibit the production of GM-CSF, IL-1, IL-6, and TNF- α and blocked NF-kB nuclear translocation in human alveolar macrophages (Zhou et al., 2007). Thus, PGE₂ and PGI₂ are known to reduce pro-inflammatory response such as TNF- α and IL-6. However, PGI₂ analogs (iloprost) can block the activity of NF-kB.

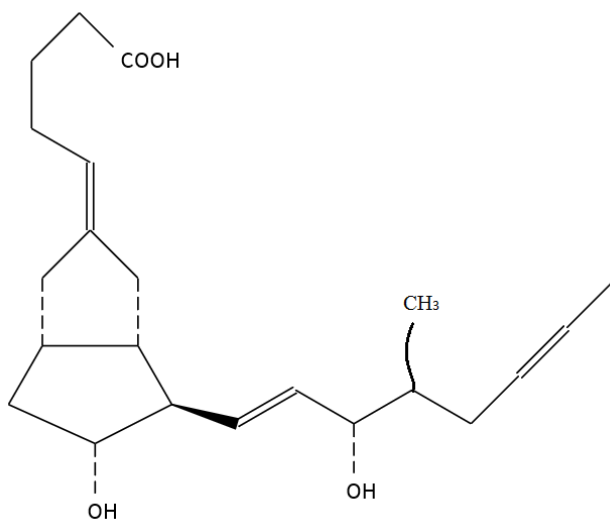


Figure 4: Molecular structure of iloprost

Resolvin is a potent lipid mediator which derived from docosahexaenoic (DHA) which is group from the ω -3 fatty acid family. In the inflammatory mechanism, phospholipase enzyme (cPLA₂) is known to release free polyunsaturated fatty acid (PUFA) including arachidonic acid (AA), docosahexaenoic acid (DHA), and omega-3 fatty acid eicosapentaenoic acid (EPA) which are

known to initiate a wide-variety of biosynthetic pathways which are known to be hydroxylated by two different enzymes: lipoxygenase (LOXs) and cyclooxygenase (COX-2). Recent studies have shown that the activation of the receptor by these lipid signaling mediators can affect levels of the expression of enzymes levels, chemokines, cytokines, and growth factors which play a predominate role in the inflammatory response and in the resolution of inflammation (Serhan and Petasis, 2011).

Resolvin D₁ (RvD₁) was recently shown to inhibit neutrophil activation, and regulate cytokine secretion. Recent studies, RvD₁ have shown to be a potent anti-inflammatory lipid mediator in different disease models such as colitis, periodontitis, acute kidney injury and peritonitis (Feng et al., 2012; Serhan et al., 2008; Serhan and Petasis, 2011). Moreover, RvD₁ has been shown to improve insulin sensitivity in obese diabetic mice (Hellmann et al., 2011). The protective effect is partially mediated by the suppression of tumor necrosis factor (TNF- α) and also can mediate the reduction of inflammatory activity of leukocytes. In addition, RvD₁ decreases the expression of the PGE₂ levels in *macaca mulatta* CREC cells line when the molecule is induced by IL-1 β (Tian et al., 2009). Moreover, RvD₁ and their precursor DHA have shown to promote the macrophage polarization to the M2 phenotype (Titos et al., 2011). According to Tito, RvD₁ have shown to promote similar result as IL-4 in the expression of TNF- α and IL-6.

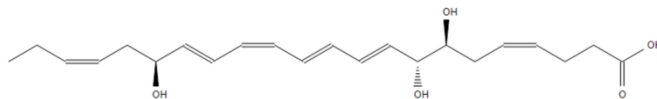


Figure 5: Molecular structure of resolvin D₁ (RvD₁)

Thus, the concentrations of PGE₂, iloprost and RvD₁ that was used in our experiment were chosen using previous studies (Panzer and Ugucioni, 2004; Sun et al., 2007; Yao et al., 2009; Zhou et al., 2007).

In this present study the aim was to compare five modulators (acetylsalicylic acid (ASA), dexamethasone (DEX), prostaglandin E₂ (PGE₂), iloprost and resolvin D₁ (RvD₁)) for their anti-inflammatory effects on macrophages in vitro, and also to understand how they affected the macrophage phenotypes in terms of gene expression and secreted cytokines. Four well-known anti-inflammatory and pro-inflammatory cytokines were analyzed:

➤ Pro-Inflammatory signaling molecules:

- Monocyte chemo-attractant protein-1 (MCP-1/CCL2) is a chemokine that is known to regulate migration and infiltration of monocytes.
- Interleukin-6 (IL-6) is known as an anti and pro-inflammatory cytokine responses. The principal cells sources of IL-6 are macrophages, endothelial cells and T-cells.

- Tumor necrosis factor alpha (TNF- α) is known to be involved in the systemic inflammation, and also TNF- α is known to stimulate acute inflammatory response. This cytokine is; also, known to increase collagen, and TNF- α can be secreted by T-cells, macrophages and other types of cells.

➤ Anti-Inflammatory signaling molecules:

- Interleukin-10 (IL-10) is known anti-inflammatory cytokine. In addition, IL-10 is a known cytokine which is known to drive macrophage to the M2 phenotype.

In addition to the protein secretion, macrophage polarization was analyzed by the gene expression. GAPDH is known to play a role in glycolysis and nuclear function inside of the cell. Others genes that were used for the experiment are listed and described as follows:

- Arginase -2 (ARG-2) is known to be a protein coding gene. Moreover, ARG-2 plays of catalyze hydrolysis of arginine to ornithine, and also in the down regulation of nitric oxide synthesis.
- Nitric oxide synthesis type II (iNOS-2) is the enzyme a radical which is known to be a biologic mediator as a neurotransmission and antimicrobial and antitumor activities. iNOS is expressed by the combination of lipopolysaccharide (LPS). iNOS is known to mediate tumoricidal and bactericidal actions.
- Macrophage - associated antigen (CD163) may play an anti-inflammatory role and may be used as a parameter for monitoring macrophage activation in inflammatory conditions.

Moreover, CD163 expression showed to suppress pro-inflammatory mediators like LPS and TNF- α and up-regulate mRNA in IL-6 and IL-10 in monocytes and macrophages (Buechler et al., 2000). CD163 is known to be expressed by cells of the M2c phenotype.

- Mannose receptor, C type 1 (CD206) is a protein coding gene, mediates the endocytosis of glycoprotein and acts as a phagocytosis receptor for bacteria and other pathogens. The protein is known to attach to high mannose structures on the surface of potential pathogens such as bacteria, fungi, and virus, and then neutralize by phagocytosis. Moreover, CD206 antigen is known to be expressed by the cells of the M2 phenotype.

METHODS

Materials

Experiments were conducted using NR8383 cells that were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Fetal bovine serum was obtained from Sigma-Aldrich (St. Louis, MO). F-12k media which contained 100 g/mL streptomycin, 0.25 g/mL amphotericin were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and 2 mM glutamine and antibiotic (penicillin) were obtained from Sigma (St. Louis, MO). The lipopolysaccharide *Salmonella typhimurium* (LPS) was obtained from Calbiochem (Massachusetts, MA). For the RT-PCR, the Trizol reagent was from Life Technologies (Grand Island, NY), RNeasy Kit from Qiagen (Germantown, MD) as a column clean up, high capacity RNA-to-cDNA kit from Life Technologies (Grand Island, NY) and finally primers such as GADPH (NM_017008.3) ARG-2 (NM_019168.1), iNOS (NM_012611.3), CD-163 (NM_001107887.1), CD-206 (NM_001106123.1), TNF- α (NM_012675.3), CCL2 (NM_031530.1) and IL-10 (NM_012854.2) from Life Technologies (Grand Island, NY). ELISA kits (CCL2, IL-6, IL-10, and TNF- α) were purchased from BD Biosciences (San Diego, CA).

Cell culture

NR8383 cells were cultured using 15 (v/v %) fetal bovine serum, 1 (v/v %) of 100U/mL penicillin, and 84 (v/v %) of F-12K which contained a 100 g/mL streptomycin, 0.25 g/mL amphotericin and 2 mM glutamine. Then, the cells were incubated at 37 °C and 5 % CO₂.

Treatment of cell culture for ELISA

Secreted CCL2, IL-6, IL-10, and TNF- α were measured using the procedure specified by the manufacturer. First, the cells were spun down by centrifugation for 7 min at 1000 rev/min, and washing using pH of 7.4 of 1x PBS which contained 1.7 M sodium chloride (NaCl), 33 mM potassium chloride (KCl), 67 mM disodium phosphate (Na₂HPO₄) and 7.07 mM monopotassium phosphate (KH₂PO₄) in 1 L. The cells were resuspended in 1 mL of media, cultured in a 24 well plates using 1.8×10^5 cells/ mL per well. For the cell count, we used the hemocytometer method which allows counting cells using 20 μ L of trypan blue and 20 μ L of cell suspension using a Countess automated cells counter.

The stock solution for acetylsalicylic acid was prepared by dissolving ASA in 1 mL of warm 50 °C ethanol 99% and then complete it with 1x PBS. The stock solution was prepared at 1 M of (ethanol and 1x PBS at pH 7.4). In this experiment were three different treatments:

- I. Treatment A was treated with the modulator first and 80 minutes later induced with endotoxin lipopolysaccharide (LPS) from *Salmonella typhimurium*.
- II. Treatment B was induced with the endotoxin LPS and 80 minutes later treated with the modulator later.
- III. Treatment C was induced with the endotoxin LPS and at the same time treated with the modulator.

Table 1: Induces and treats for acetylsalicylic acid and dexamethasone

Treatments	First Induction	Second Induction
A	Modulator (ASA or DEX), and 80 min later second induction	LPS
B	LPS, and 80 min later second induction	Modulator (ASA or DEX)
C	Modulator (ASA or DEX), and the second induction	LPS

Each modulator was induced using different concentration. Table 2 shows each modulator with the different concentrations. Finally, the plates were incubated for 24 hours.

Table 2: Modulators

Modulators	Concentration	Hypothesis Effects
Lipopolysaccharide (LPS)	50 ng/mL	Induce inflammation
Acetylsalicylic acid (ASA)	0.1, 1 and 10 mM	Anti-inflammatory modulator
Dexamethasone (DEX)	100 nM	Anti-inflammatory modulator
Prostaglandin E ₂ (PGE ₂)	10 nM, 100 nM, and 1 μM	Anti-inflammatory modulator
Iloprost	10 nM, 100 nM, and 1 μM	Anti-inflammatory modulator
Resolvin D ₁ (RvD ₁)	10 nM, 100 nM, and 1 μM	Anti-inflammatory modulator

ELISA (Enzyme Linked Immunosorbent Assay)

1.8 x 10⁵ cells/ mL of NR8383 cell were harvested. Then, the levels of CCL2, IL-6, IL-10, and TNF-α in the supernatant only were quantified using ELISA kit following the manufacture`s procedure.

Treatment of cell culture for the qRT-PCR measurement

Cells were spun down by centrifuge them for 7 min at 1000 rev/min, and washed using 1x of PBS. After centrifuged the cells and reconstituted using 1 mL of media, the plate were cultured in a 6 well plates using 2.8 x 10⁶ / mL of cells per well. For the PCR analysis were used the same treatment that was presented in table 1, and the concentration followed for treatment PGE₂, dexamethasone, RvD₁ and iloprost. Real time qRT-PCR is a technique that is commonly used for the quantification of DNA sequences.

qRT-PCR (Real Time Polymerase Chain Reaction)

After treatment of the cells, the RNA was extracted from the cells (2.8×10^6 cells/ mL) that were already treated using the Trizol reagent following the manufacture procedure. Then, the total RNA was quantified using a NanoDrop Spectrophotometer 2000c at 260/280 and 260/230 nm absorption ratio. Using an agarose electrophoresis gel, the quality of the RNA was checked before synthesized cDNA by reverse transcription (RT) using a high capacity RNA-to-cDNA kit. Then, the samples were run in a thermo-cycle PCR TC-3000. Subsequently, the relative levels of target molecules which were GAPDH as a housekeeping gene, and targeted DNA GAPDH (NM_017008.3) ARG-2 (NM_019168.1), iNOS (NM_012611.3), CD-163 (NM_001107887.1), CD-206 (NM_001106123.1), TNF- α (NM_012675.3), CCL2 (NM_031530.1) and IL-10 (NM_012854.2) were determined using qRT-PCR (7500 Real Time PCR System from Applied Bio systems). The following thermal cycle condition for the qRT-PCR were initial denaturation at 50°C, 40 cycles of amplification at 95 °C, and then steady at 95°C and finally 60 °C.

Quality of Iloprost

LC/MS analysis was performing on LCMS-8040 triple quadrupole Mass Spectrometer (MS) with an ESI ion source connected to a Shimadzu Nexera X2 UPLC. The column, for the experiment, was a Phenomenex Kinetix C18 column (100x 3.0 mm, 2.6 μ m particle sizes). Moreover, as a solvent was used water and methanol and each one contained 0.1% of formic acid. To measure the sensitivity of the equipment was run an isocratic run with 65% methanol, 0.6 mL/min as a flow rate and 40°C as a column temperature. Using a selected ion monitoring (SIM) in the MS, 325.3 $[M^+ H^- 2H_2O]^+$, 343.3 $[M^+ H^- H_2O]^+$, 378.3 $[M^+ NH_4]^+$ and 383.3 $[M^+ Na]^+$ m/z was followed. These ions are present in iloprost, respectively. Sample of 1 μ L of iloprost were injected over a 10

min run. Then, it was injected a higher concentration of iloprost 10 pg/ μ L and then a low concentration of 1 pg/ μ L.

For the purity of iloprost, the chromatographic condition were 10% v/v (H₂O/MeOH), then an increase to 90% v/v (H₂O/MeOH) over 13 min, and then was held at 90% v/v (H₂O/MeOH) for 2 min. The flow rate for this experiment was used 0.6 mL/min with a temperature column of 40°C. MS was scanned from 140 to 800 m/z with a concentration of iloprost of 13.87 mM.

Statistical analysis

Data are expressed as mean \pm SE. Statistical significance was estimated using origin software OriginLab Corporation (Northampton, MA). The method of analyses data for the ELISA was ANOVA followed by Tukey post-hoc test, and the differences were significant if [p<0.05]. In addition, for the qRT-PCR were used the relative expression software tool (Multiple condition solver) REST-MCS version 2 using pair wise fixed reallocation randomization test, and the differences were significant if (p<0.05).

RESULTS

1.1 Secretion of anti and pro-inflammatory cytokines treated with acetylsalicylic acid (ASA) in NR8383 cells.

To examine the concentration of proteins secretion of pro and anti-inflammatory cytokines responses, (50 ng/mL) LPS was used to induce an inflammatory response in the NR8383 cell line. Then, from the supernatants ELISA was used to quantify the protein secretion levels of a variety of cytokines including:

- Monocyte chemo-attractant protein-1 (MCP-1/CCL2)
- Interleukin-10 (IL-10) an anti-inflammatory cytokine.
- Interleukin-6 (IL-6) an anti and pro-inflammatory cytokine.
- Tumor necrosis factor alpha (TNF- α) is known to be involved in the systemic inflammation, and also it is known to stimulate acute inflammatory response.

Figure 6 shows the concentration of protein secreted for CCL2, IL-6, IL-10 and TNF- α . IL-10 did show significance differences at 0.1 mM of acetylsalicylic acid (treatment C), 1 mM of acetylsalicylic acid (treatment A and C), and 10 mM of acetylsalicylic acid (treatment A, B and C) compared to LPS treatment. Thus, IL-10 reduces significantly at higher concentration compared to LPS which is the concentration produce with cells induced with LPS. Moreover, TNF- α showed a significance reduction within the different treatments and concentration compared with LPS. Thus, TNF- α concentration significantly decrease at 10, 1 and 0.1 mM of acetylsalicylic acid.

CCL2 proteins secretion showed that using concentration at 10 mM of acetylsalicylic acid and 1 mM using treatment A and B.

The levels of CCL2 secretion decreased compare to LPS (Figure 6). Moreover, for IL-6 presented significance with all difference concentrations and treatments. Thus, IL-6 and CCL2 did revealed a reduced protein secreted by inducing cells with acetylsalicylic acid using at 10 and 1 mM, and also at 10 mM and treatment A at 1 mM the levels of IL-6 were bellowed of the detection limit of the ELISA kit for IL-6 (pg/mL).

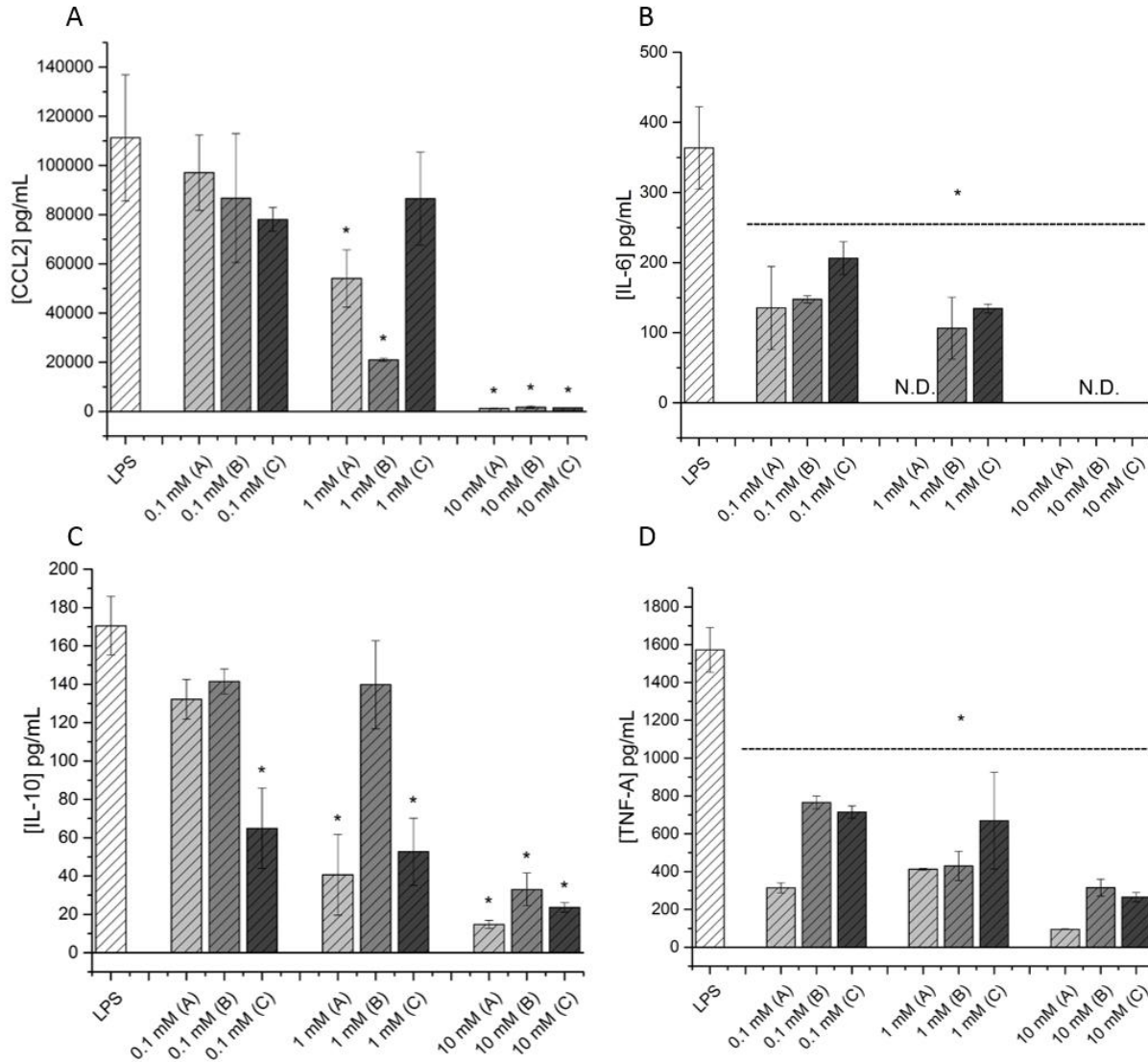


Figure 6: NR8383 treated induced with acetylsalicylic at three concentrations 0.1, 1 and 10 mM. (A) Concentration of CCL2 in cell induced with LPS and also treated it with ASA following treatment from Table 1. (B) Concentration of IL-6 in cell induced with LPS and also treated it with ASA following treatment from Table 1. (C) Concentration of IL-10 in cell induced with LPS and also treated it with ASA following treatment from Table 1. (D) Concentration of TNF- α in cell induced with LPS and also treated it with ASA following treatment from table 1. Data showed significances (*) compared to LPS as determined by using ANOVA and Tukey post-hoc ($p < 0.05$) $N=3$.

1.2 Gene expression from NR8383 cells treated with acetylsalicylic acid (ASA).

To examine gene expression in the induced cells, LPS was used to induce inflammatory response. To illustrate, Figure 7 shows each gene expression was compared resting cells (control) with LPS. Further experiments were measured using as a reference condition (LPS) and compared with each of the three treatments and each condition. GAPDH was used as a reference gene through all of the experiments. GAPDH is known to play a role in glycolysis and nuclear function inside of the cell. Other genes that were used for the experiment are listed and described as follows:

- Arginase -2 (ARG-2) is known to be down regulated when nitric oxide is up regulated because ARG-2 and iNOS-2 use a common substrate arginine. Inducing ARG-2 has showed to down-regulate NO production by depleting arginine (Mori, 2007).
- Nitric oxide synthesis type II (iNOS-2) is expressed by the treatment of lipopolysaccharide (LPS).
- Macrophage - associated antigen (CD163) may play an anti-inflammatory role and may be used as a parameter for monitoring macrophage activation in inflammatory conditions. Also, CD163 is known to be expressed by cells of the M2c phenotype.
- Mannose receptor, C type 1 (CD206) is known to be expressed by the cells of the M2 phenotype.

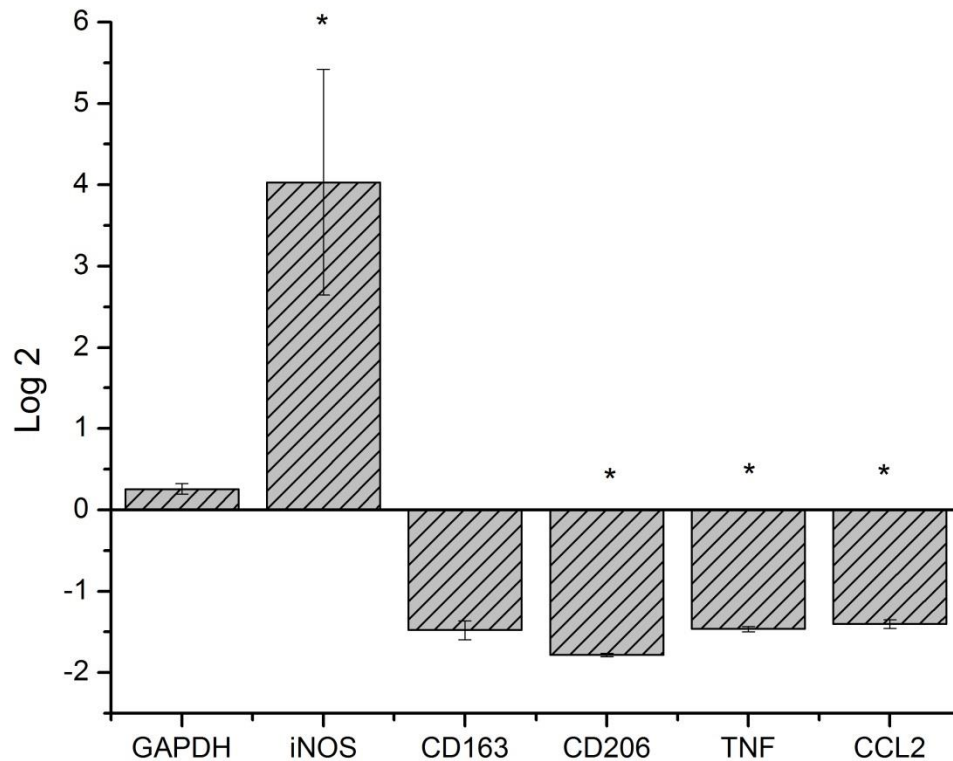


Figure 7: NR8383 resting cells (control) compare with LPS. Data showed significances difference (*). Data showed significances difference (*) compared to LPS using pair wise fixed reallocation randomization test (N=3). At 50 ng/mL of LPS, iNOS (p=0.05), CD206 (p=0.001), TNF- α (p=0.001) and CCL2 (p=0.001).

Figure 8 shows each gene expression was compared with the reference condition (LPS) and sample condition (modulator). At 10 and 1 mM of acetylsalicylic acid, data showed up-regulation CD206 and IL-6 in treatment C and IL-10 in treatment B. Thus, gene expressions did show an up-regulation for CD206 which is a known marker for the presence of M2. At 1 mM of ASA, data did not show a significance difference compare to LPS.

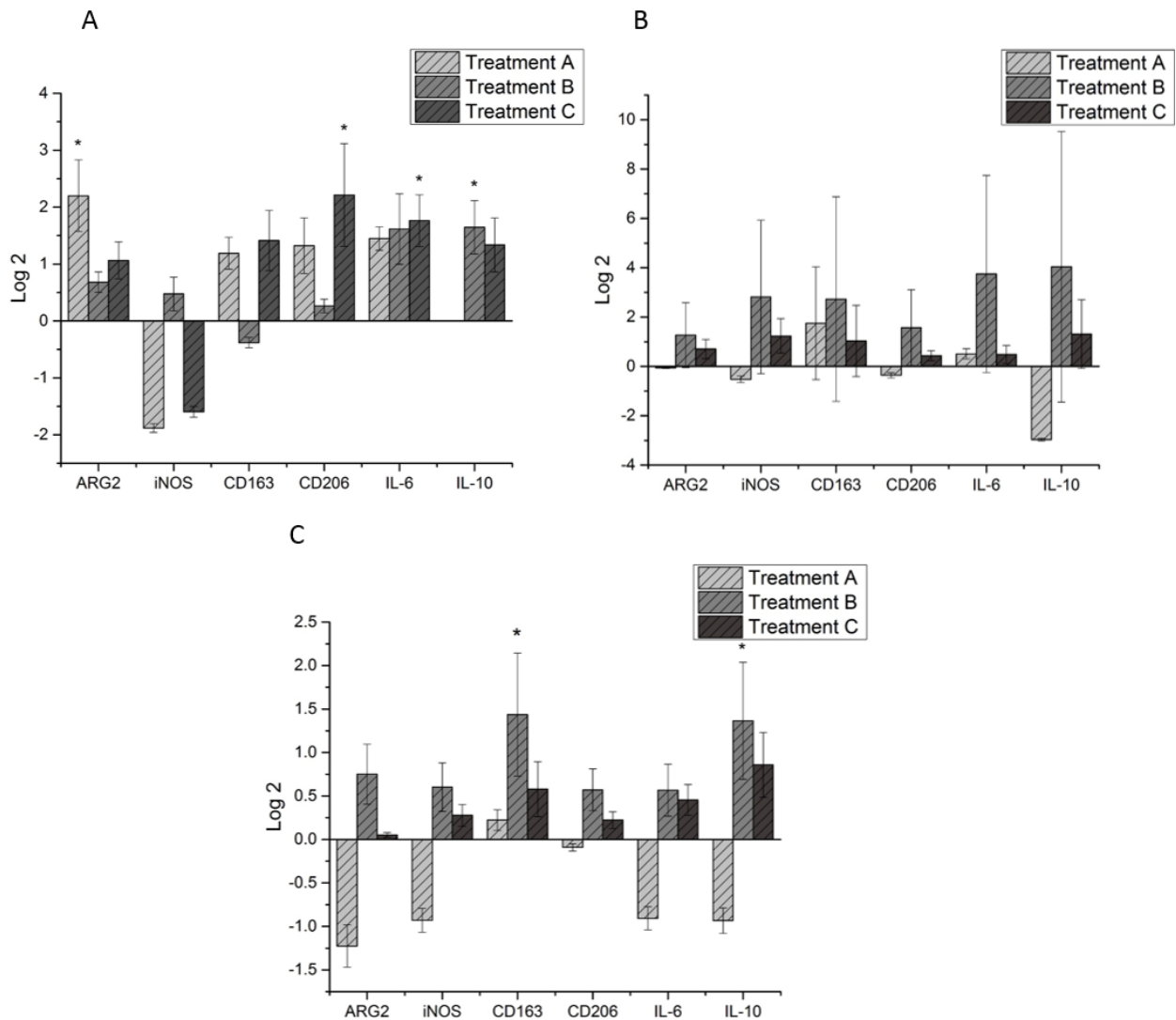


Figure 8: NR8383 treated with acetylsalicylic acid at 10, 1 and 0.1 mM. (A) Cell induced with LPS and treated with 10 mM of ASA following treatment of Table 1. (B) Cells induced with LPS and treated with 1 mM of ASA following treatments of Table 1. (C) Cells induced with LPS and treated with 0.1 mM of ASA following treatments of Table 1. Data showed significances difference (*) compared to LPS using pair wise fixed reallocation randomization test. At 10 mM of ASA, treatment B ($p=0.02$); treatment C CD206 ($p=0.02$) and IL-6 ($p=0.05$); at 0.1 mM of ASA treatment B CD163 ($p=0.001$) and IL-6 ($p=0.04$).

2.1 Secretion of anti and pro-inflammatory cytokines and gene expression in NR8383 cells treated with dexamethasone (DEX).

NR8383 rat alveolar cells lines were treated with dexamethasone. The concentration of DEX was use for this research was previous measure in our lab. At 100 nM, previous studies have showed to reflect differences in cells induced with LPS, and compare with cells induced with LPS and treated with DEX. The study used three different treatments at the concentrations displayed in Table 1. Each of the treatments was incubated for 24 hours.

In Figure 9 showed that with these three different treatments with dexamethasone, it significantly decreased cytokine protein levels of TNF- α and IL-6. Concentration of IL-6 were below of the limit of detection, and TNF- α were significantly lower compared to LPS. Moreover, CCL2 did not show significant change in treatment A and B. However, data showed that the proteins levels of CCL2 were significantly lower in treatment C. To rephrase treatment C, the cells were stimulated with LPS and dexamethasone at the same time. Finally, dexamethasone has shown to decrease the protein secretion of CCL2, IL-6, IL-10 and TNF- α in NR8383 cells lines.

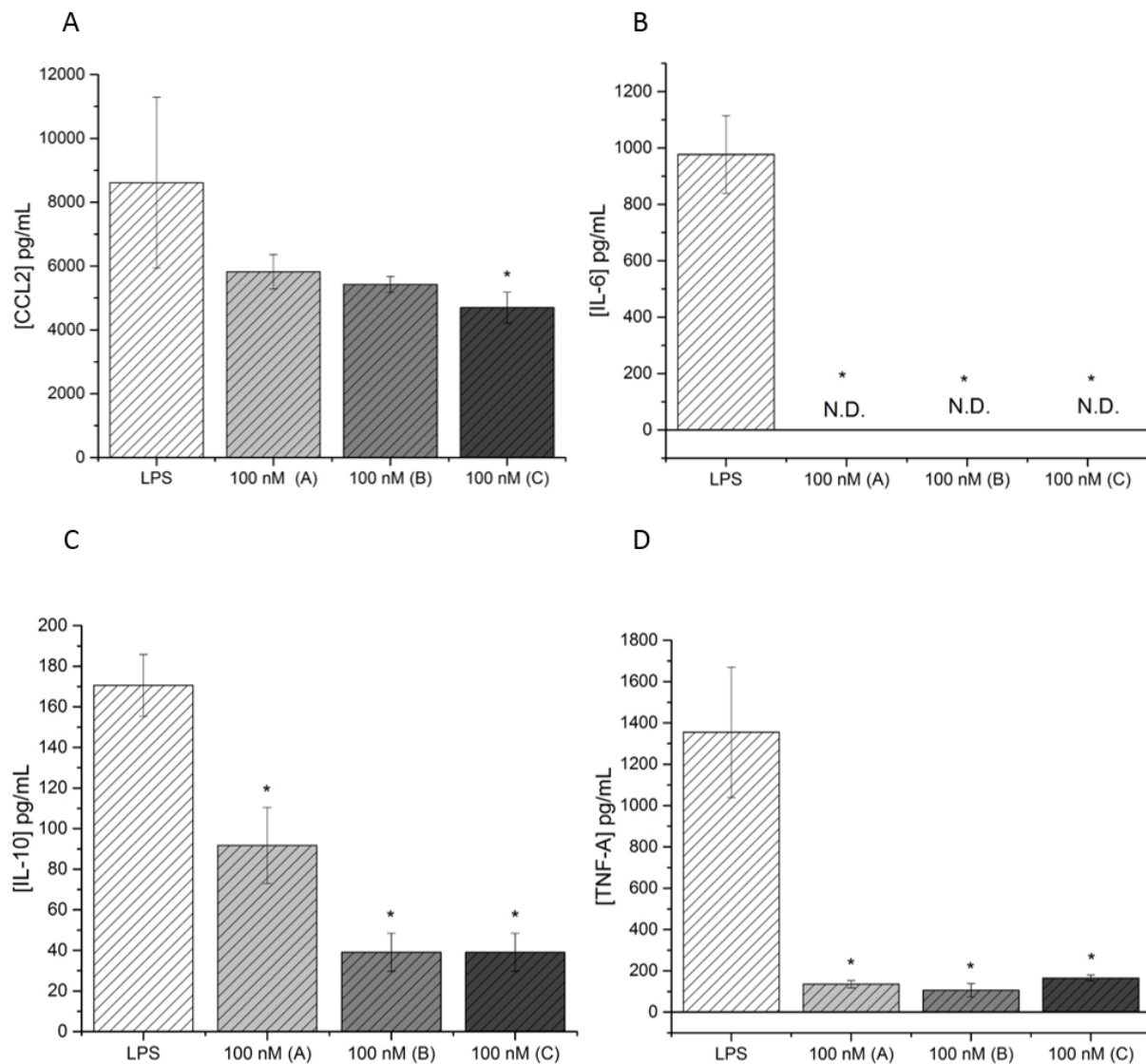


Figure 9: NR8383 induced with LPS and treated with dexamethasone at 100 nM. (A) Concentration of CCL2 in cell induced with LPS and also treated it with DEX following treatment from table 1. (B) Concentration of IL-6 in cell induced with LPS and also treated it with DEX following treatment from table 1. (C) Concentration of IL-10 in cell induced with LPS and also treated it with DEX following treatment from table 1. (D) Concentration of TNF- α in cell induced with LPS and also treated it with DEX following treatment from table 1. Data showed significances (*) compare with LPS using ANOVA and Tukey as a post-test ($p=0.05$) $N=3$.

2.2 Gene expression from NR8383 cells treated with dexamethasone (DEX).

NR8383 cells were treated with dexamethasone using 100 nM as shown in Table 1. To summarize, treatment A involved a first exposure with dexamethasone, and then 80 min later adding LPS. For treatment B, the cells were induced first with LPS, and then 80 min later treated with dexamethasone. Finally treatment C, dexamethasone and LPS delivered at the same time.

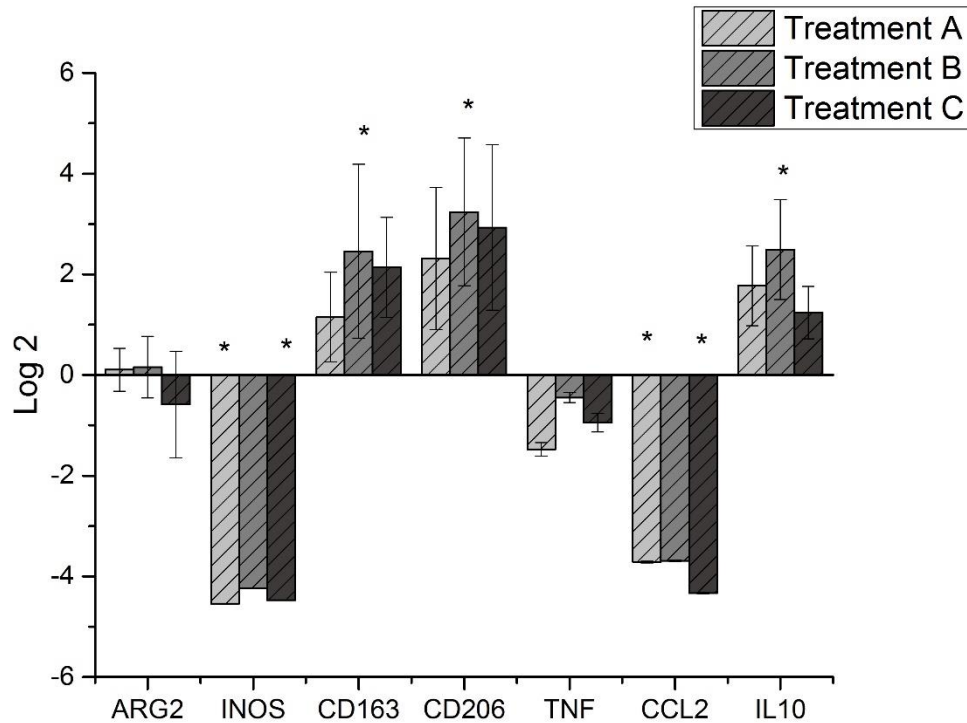


Figure 10: NR8383 induced with LPS and then treated with dexamethasone at 100 nM. treatment A was cells that were induced with dexamethasone and then 80 min later with LPS, treatment B was cells induced with LPS and then 80 min later with dexamethasone, and treatment C was cells induced with LPS and dexamethasone at the same time. Data showed significances compare to LPS using pair wise fixed reallocation randomization test N=4. At 100 nM, treatment A iNOS ($p=0.005$), CD163 ($p=0.05$), CD206 ($p\leq 0.05$), TNF- α ($p\leq 0.02$) and CCL2 ($p\leq 0.01$), treatment B iNOS ($p\leq 0.01$), CD163 ($p=0.001$), CD206 ($p=0.001$), CCL2 ($p=0.01$) and IL-10 ($p=0.05$) and treatment C iNOS ($p\leq 0.005$), CD163 ($p\leq 0.05$), CD206 ($p=0.05$) and CCL2 ($p\leq 0.01$).

In Figure 10 treatments A and C exhibited a down-regulation for iNOS and CCL2. iNOS is known to be up-regulate in cells that are induced with LPS, and CCL2 is known to be a chemokine that regulates migration and infiltration of cells. Moreover, treatment B showed up-regulation of CD163, CD206 and IL-10. Thus, dexamethasone up-regulates the anti-inflammatory response by

up-regulating CD163, CD206 and IL-10 and decreasing pro-inflammatory cytokine response such as TNF- α and IL-6.

3.1 Secretion of anti and pro-inflammatory cytokines and gene expression in NR8383 cells treated with prostaglandin E₂ (PGE₂).

For this experiment, NR8383 cells were treated with prostaglandin E₂ (PGE₂). The cells were treated using three different concentrations of PGE₂ [1 μ M, 100 nM and 10 nM]. PGE₂ was used to treat the cells that were induced of LPS. In Figure 11, data showed significantly difference (IL-6 and TNF- α) between cells induce only with LPS, and cells induced and then treated with 1 μ M of PGE₂.

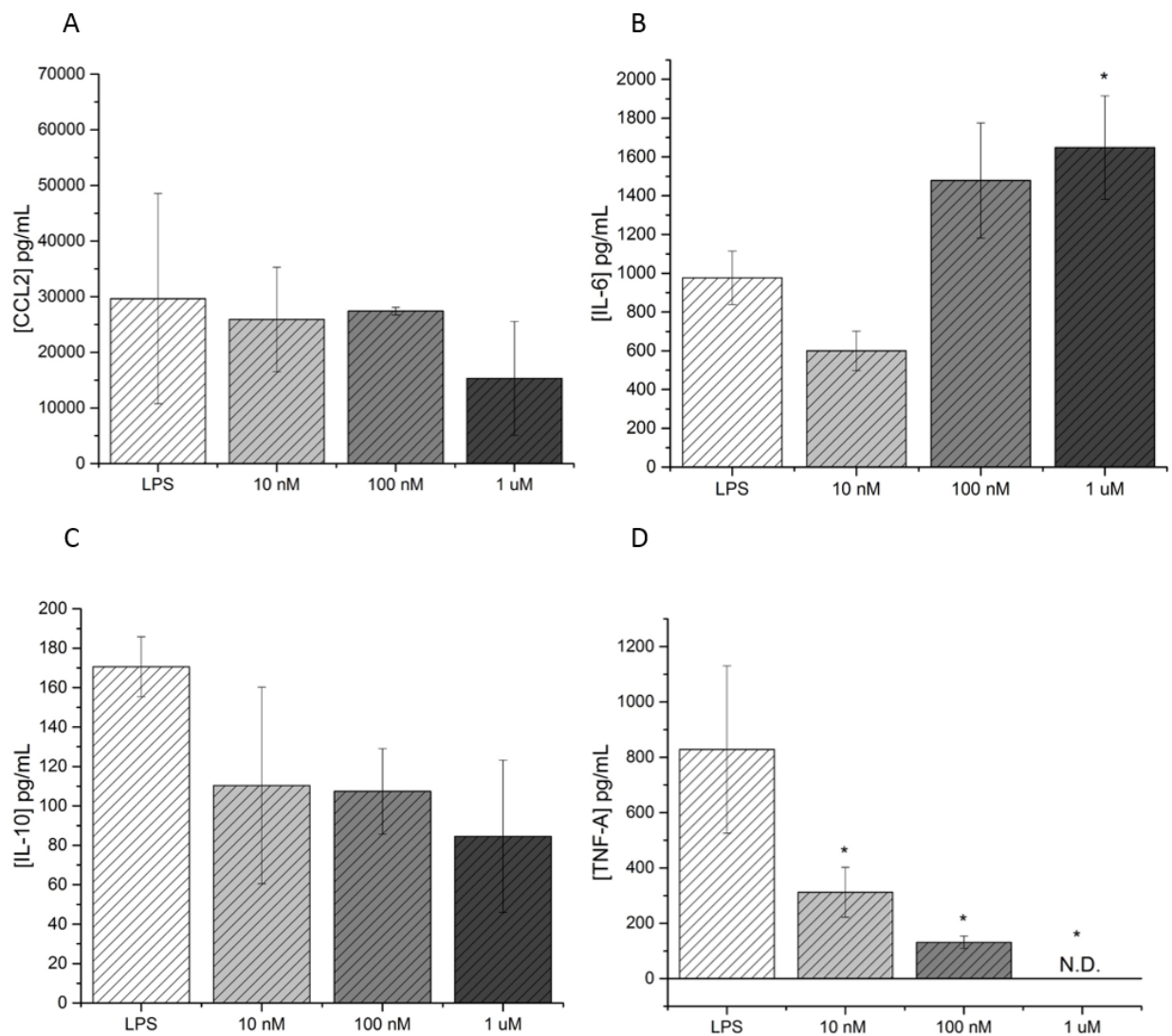


Figure 11: NR8383 induced with LPS and treated with PGE₂ following treatment from Table 1. (A) Concentration of CCL2 (B) Concentration of IL-6 (C) Concentration of IL-10 (D) Concentration of TNF- α . Data showed significance (*) compare to LPS as determined by using ANOVA and Tukey as a post-test ($p=0.05$) $N=3$.

In Figure 11 the graph shows, the three different concentrations of PGE₂ significantly decreased TNF- α . Also, the concentration of IL-6, known pro-inflammatory cytokine, was measured. The concentration of IL-6 resulting from treatment with 1 μ M of PGE₂ was significantly higher compare with LPS. In addition, Figure 11 revealed that level of CCL2 and IL-10 was not significantly different compared with LPS. Thus, PGE₂ shows a decrease of TNF- α ; however, IL-10 and CCL2 did not show significant differences.

3.2 Gene expression from NR8383 cells treated with prostaglandin E₂ (PGE₂).

In the gene expression patterns after PGE₂ treatments that are shown in Figure 12. At 1 μ M of PGE₂ showed significant gene expression levels of up-regulated ARG2, CD163, and CD206. Also, at 100 nM of PGE₂ showed significant up-regulated gene expression ARG2, CD163, CD206, and IL-10; however, at 10 nM of PGE₂ shown up-regulation ARG2, and CD206. Thus, these three different concentrations of PGE₂ revealed up-regulation for ARG2, and CD206. To recall, ARG2 is known to up-regulate when iNOS is down-regulated, and CD206 is known to be present in cells that are M2 macrophages. Importantly at 100 nM of PGE₂ shows up-regulation of CD163 and IL-10 which are known to be anti-inflammatory. Thus, PGE₂ is an anti-inflammatory modulator that decreases significantly the secretion of TNF- α and IL-6, and also up-regulates ARG2, CD206, CD163 and IL-10 in the gene expression assays.

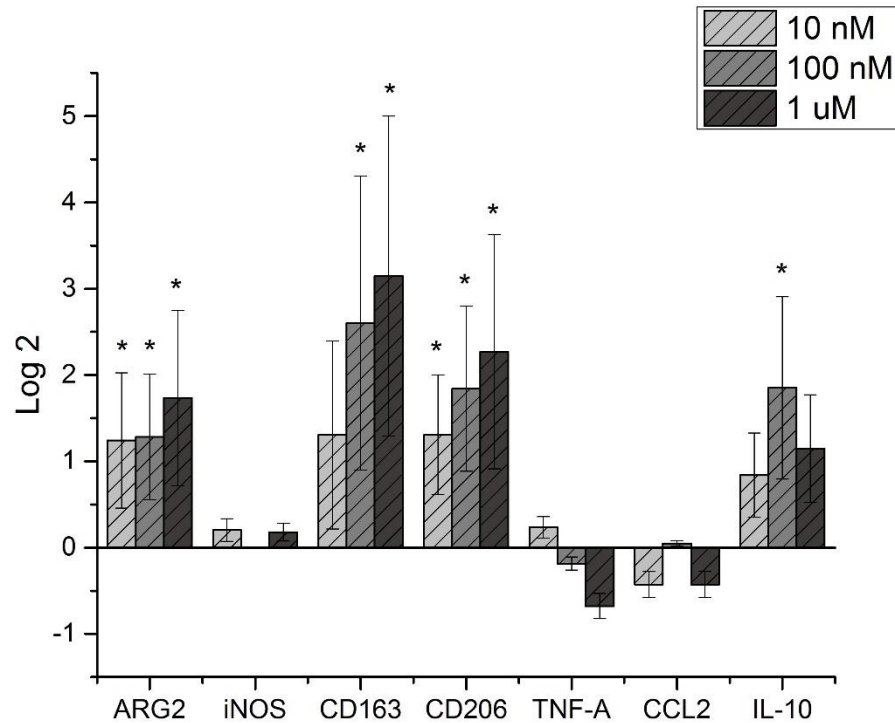


Figure 12: NR8383 induced with LPS and treated with PGE₂ at three concentration 10 nM, 100 nM and 1 μ M. Cells were induced with LPS and treated with prostaglandin E₂ at the same time for each different concentration. Data showed significance difference (*) for treatments 1 μ M [ARG2, CD163 and CD206], 100 nM [ARG2, CD163, CD 206 and IL-10], and for 10 nM [ARG2, CD206] using pair wise fixed reallocation randomization test N=3. At 10 nM ARG2, CD206 and IL-10 [p=0.001], 100 nM ARG2, CD163, CD206 and IL-10 [p=0.045], and 1 μ M ARG2, CD163, CD206 [p=0.001].

4.1 Quality of iloprost.

Recently studies have shown the short half-life of PGI₂. The modulator that was used was Iloprost. Iloprost is the drug analog of PGI₂. Because of the short half life time of PGI₂, an analysis to measure the quality of our reagent was performed before using it for cell treatment.

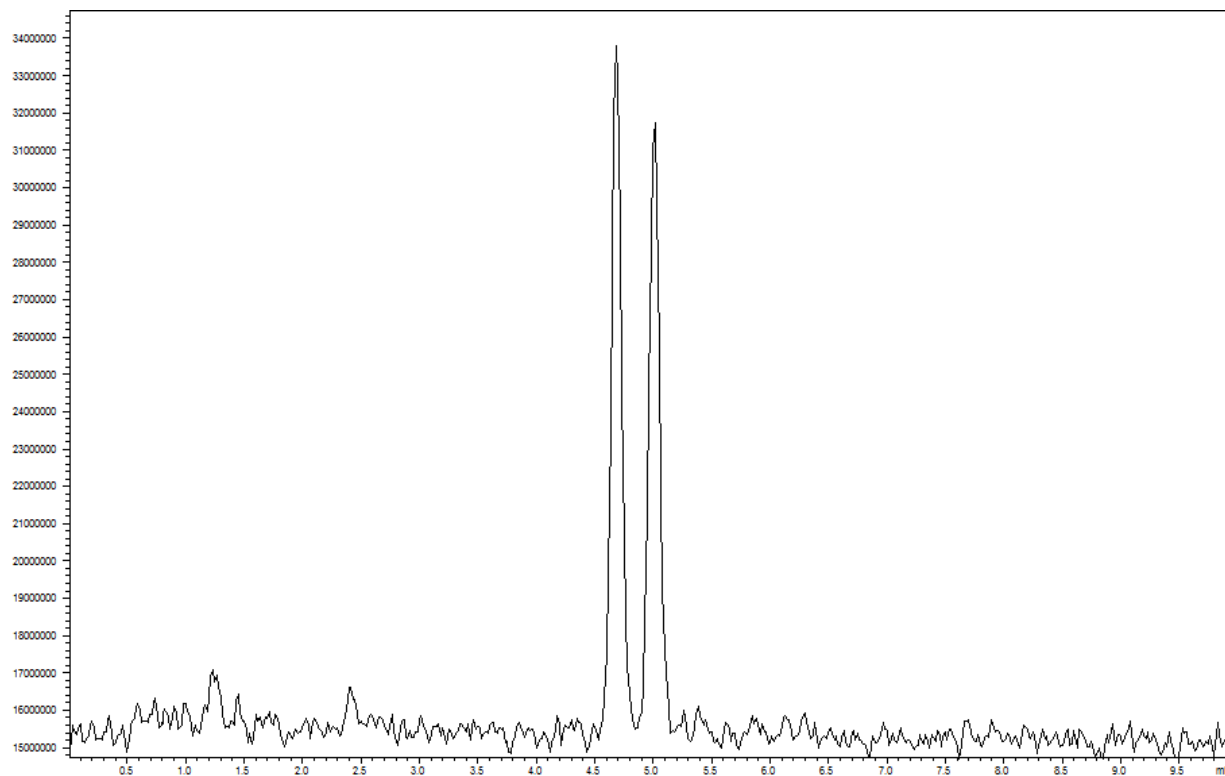


Figure 13: LC/MS analysis was performing on LCMS-8040 with an ESI ion source connected to a UPLC. As a solvent was used water and methanol, and each one contained 0.1% of formic acid. Iloprost was run an isocratic run with 65% MeOH/ 35% H₂O, 0.6 mL/min as a flow rate and 40°C as a column temperature.

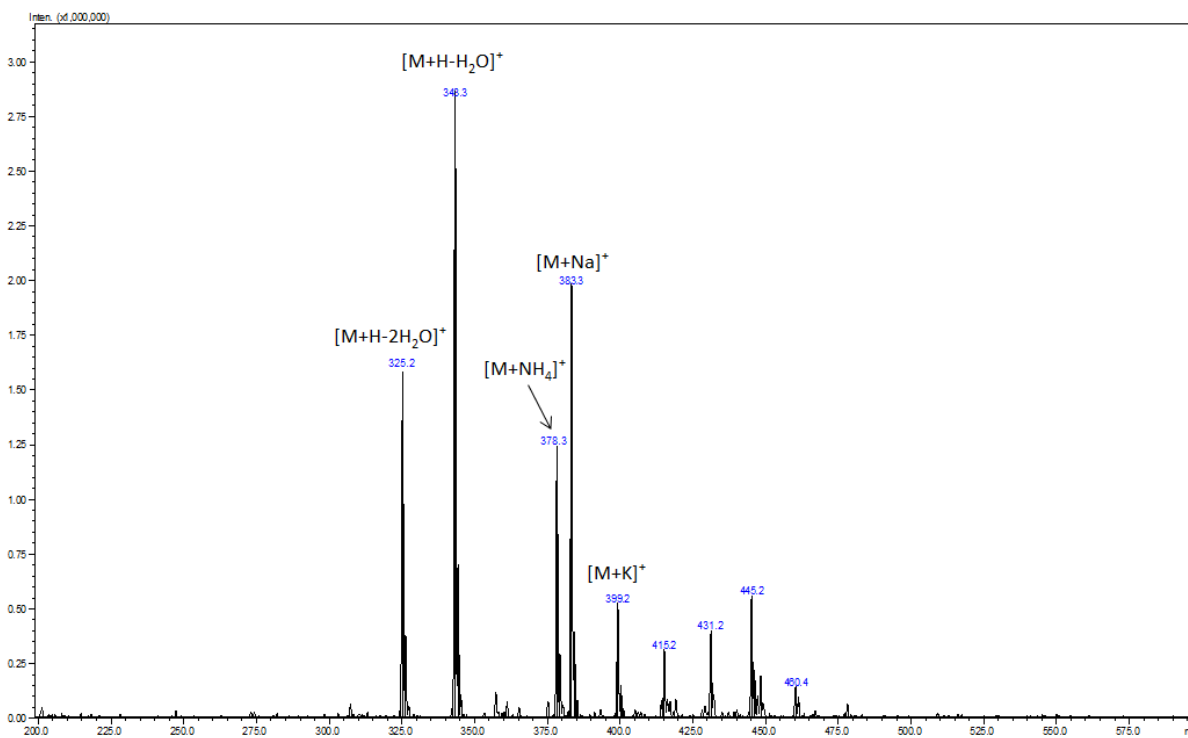


Figure 14: Using a selected ion monitoring in the MS, 325.3 [M+ H⁻ 2H₂O]⁺, 343.3 [M+ H⁻ H₂O]⁺, 378.3 [M+ NH₄]⁺ and 383.3 [M⁺ Na]⁺ m/z was followed. These ions are presented in iloprost, respectively.

In the LC data, it was observed that iloprost is an diastereomers (Schermuly et al., 2002). An diastereomers are a type of stereoisomer that with two or more stereocenter. These diastereomers are represented by chromatographic peaks that was shown in the standard. In addition to Figure 13, Figure 14 is the representation of m/z of first peak. As in Figure 14, iloprost is not showing degradation products. Finally, Figures 13 and 14 shown that iloprost is not degradaing into unknown molecules, and that our standard could be use as a stock solution in the treatment of NR8383 cells.

4.2 Secretion of anti and pro-inflammatory cytokines and gene expression in NR8383 cells treated with iloprost.

For this experiment, NR8383 cells were treated with iloprost at [1 μ M, 100 nM and 10 nM]. In Figure 15 the graph shows, the three different concentrations.

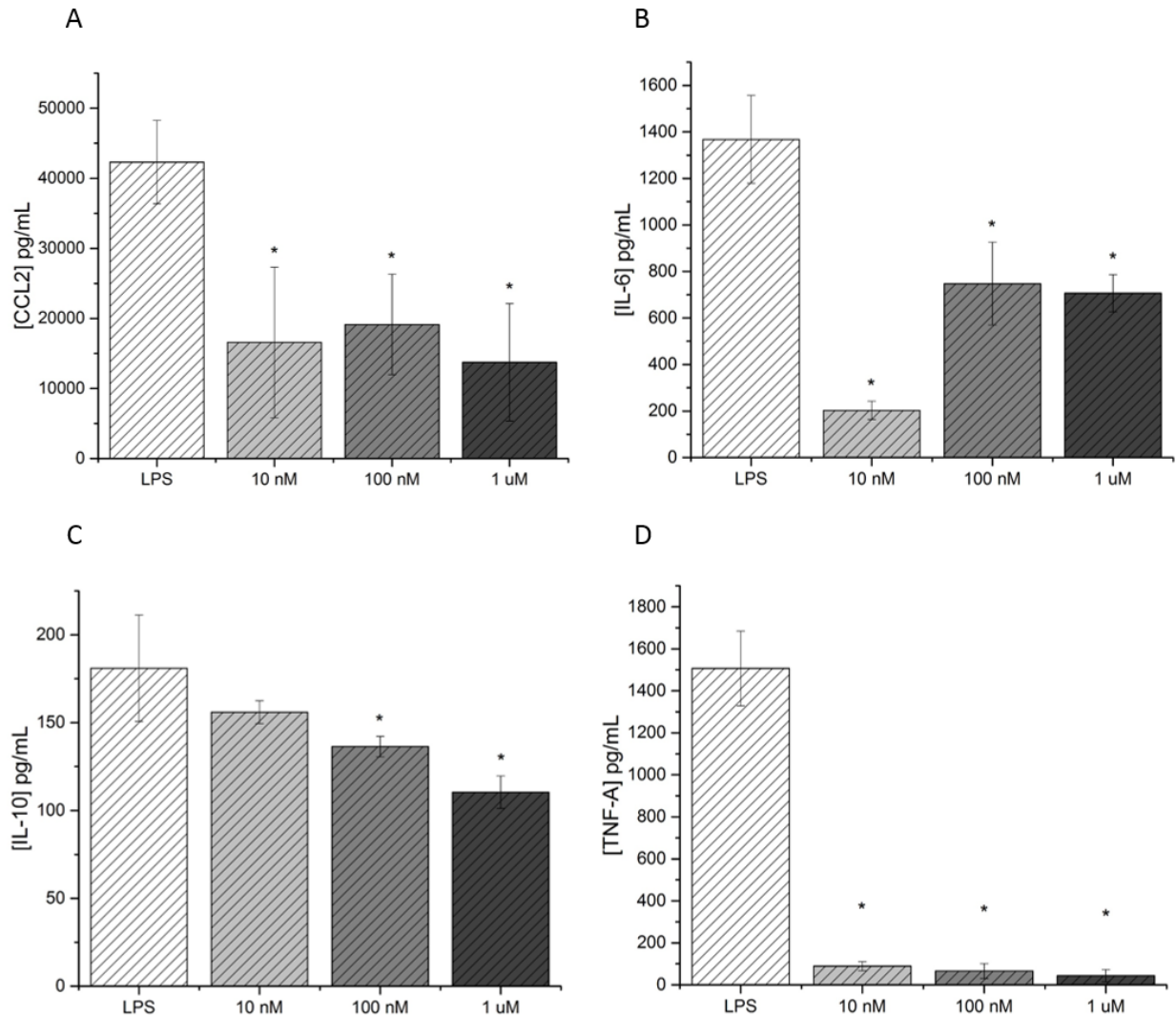


Figure 15: NR8383 induced with LPS and treated with iloprost following treatment Table 1. A) Concentration of CCL2 (B) Concentration of IL-6 (C) Concentration of IL-10 (D) Concentration of TNF- α . Data showed significance (*) compare with LPS determined by using ANOVA and Tukey as a post-test ($p=0.01$ (A) and $p=0.05$ (B, C and D)) $N=3$.

Figure 15 shows a significance decrease of protein cytokine secretion. CCL2, IL-6, IL-10 and TNF- α show a decrease treated cells with 100 nM and 1 μ M of iloprost compared with cells induced with LPS. Thus, the protein secretion of pro-inflammatory cytokines at 100 nM and 1 μ M of iloprost decrease with in cells that were induced with LPS.

4.3 Gene expression from NR8383 cells treated with iloprost.

In the gene expression patterns after iloprost treatments that are shown in Figure 16 revealed that gene expression at 10 nM of iloprost was significantly up-regulated in CD163, CD206 and IL-6. In cells treated with 100 nM of iloprost showed a significant up-regulate CD206 and IL-6; however, cells induced with 1 μ M showed a significant up-regulation for IL-6. Thus, these three different concentrations of iloprost revealed an up-regulation of CD163 and CD206. Thus, iloprost is an anti-inflammatory modulator that decreases significantly the secretion of CCL2 and TNF α , and also up-regulates CD206, and CD163 in the gene expression.

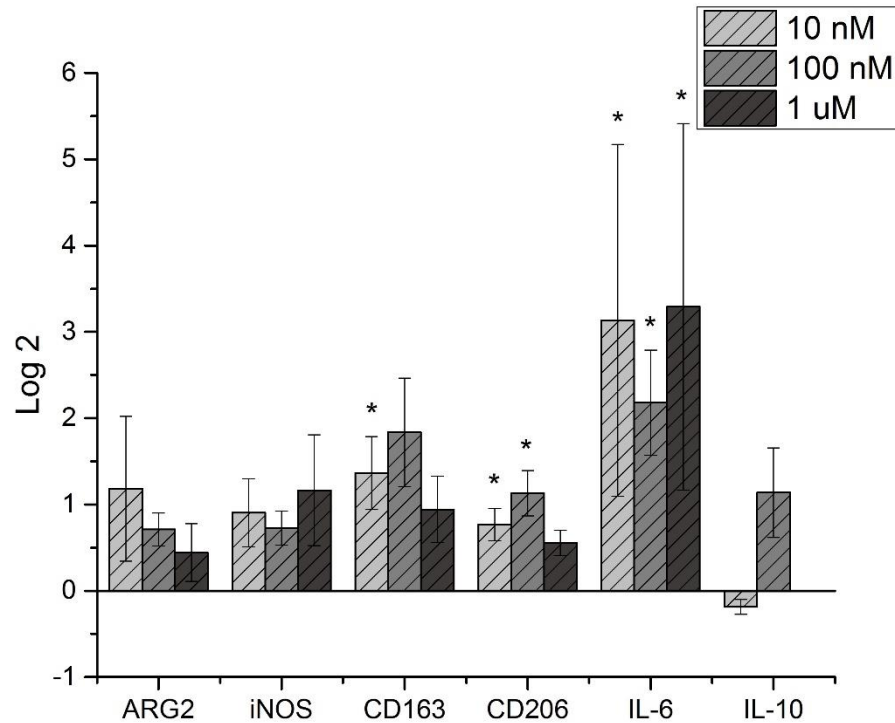


Figure 16: NR8383 induced with LPS and treated with iloprost at three concentration 10 nM, 100 nM and 1 μ M. Cells were induced with LPS and treated with iloprost at the same time for each different concentration. Data showed significance difference (*) for treatments 10 nM [CD163, CD206 and IL-6], 100 nM [CD163, and CD 206], and for 1 μ M [IL-6] using pair wise fixed reallocation randomization test N=3. At 10 nM CD163 ($p \leq 0.02$), CD206 ($p \leq 0.05$) IL-6 ($p = 0.001$); 100 nM CD206 ($p = 0.05$) IL-6 ($p = 0.001$) and 1 μ M IL-6 ($p = 0.05$).

5.1 Secretion of anti and pro-inflammatory cytokines and gene expression in NR8383 cells treated with resolvin D₁ (RvD₁).

For this experiment, NR8383 cells were treated with RvD₁. These cells were treated using three different concentrations of RvD₁ [1 μ M, 100 nM and 10 nM]. In Figure 17, the graph shows four cytokines that were analyzed CCL2, IL-6, IL-10 and TNF- α .

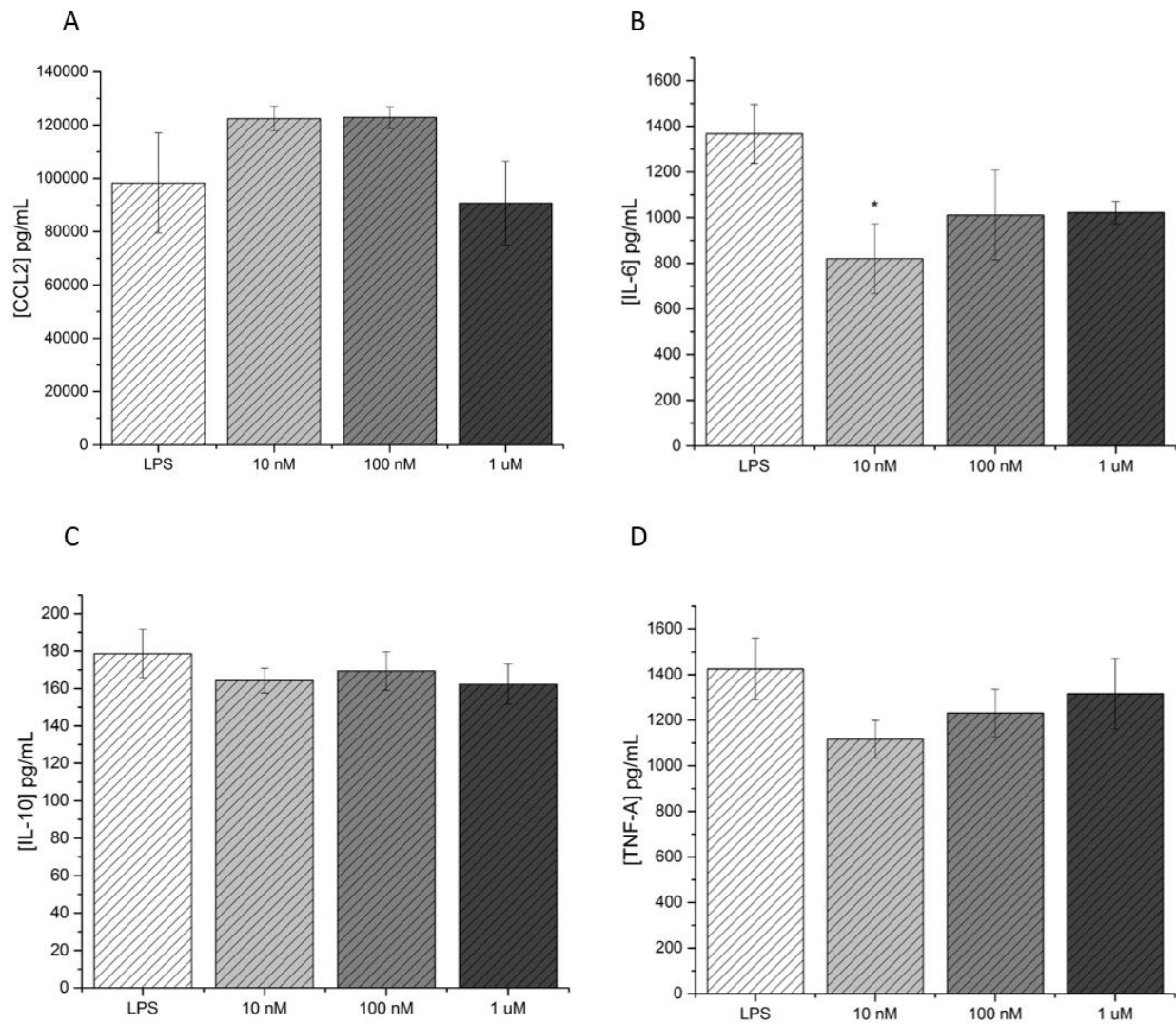


Figure 17: NR8383 induced with LPS and treated with RvD₁ following treatment from Table 1. A) Concentration of CCL2 (B) Concentration of IL-6 (C) Concentration of IL-10 (D) Concentration of TNF- α . Data showed a significance (*) compare with LPS as determined by using ANOVA and Tukey as a post-test ($p=0.05$) $N=3$.

Figure 17 showed a significant decrease in [IL-6] in cells induced with LPS and then treated with 10 nM of RvD₁. Thus, RvD₁ does not significantly decrease the secretion of pro-inflammatory cytokines such as TNF- α , and CCL2. Moreover, RvD₁ also does not significantly increase anti-inflammatory cytokines such as IL-10. However, RvD₁ significantly decreases the concentration of IL-6 at 10 nM of treatment.

5.2 Gene expression from NR8383 cells treated with resolvin D₁ (RvD₁).

In the gene expression patterns after RvD₁ treatments that are shown in Figure 18 revealed that only cells treated with 100 nM of RvD₁ were significantly up-regulated in IL-10. Thus, at 10 nM and 1 μ M concentrations of RvD₁ does not reveal neither an up-regulation nor down-regulation of ARG2, iNOS, CD206, CD163, IL6 and IL-10.

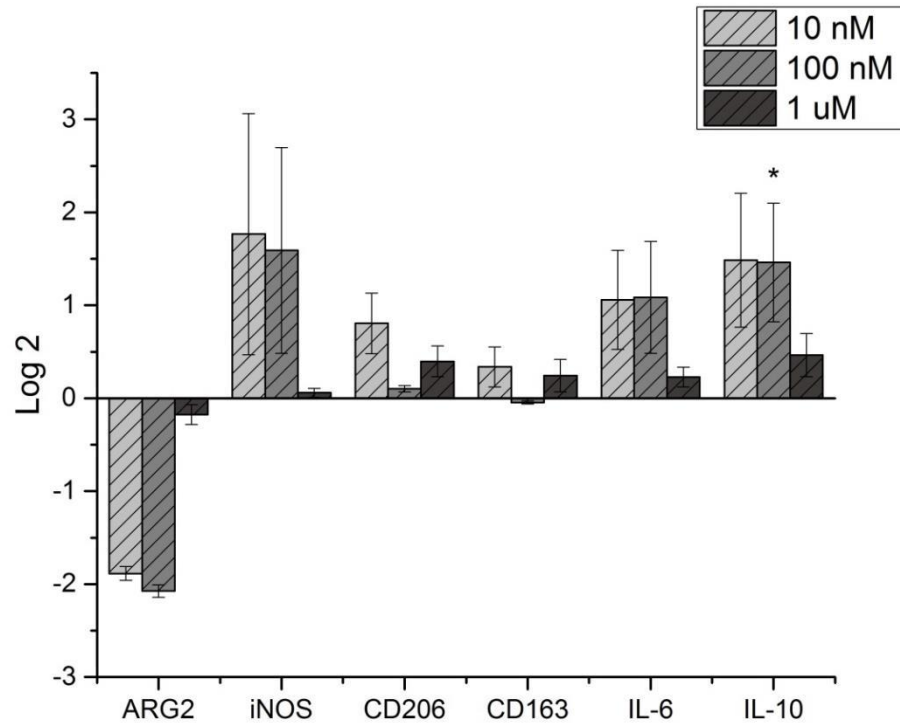


Figure 18: NR8383 induced with LPS and treated with resolvin D₁ at three concentration 10 nM, 100 nM and 1 μM. Cells were induced with LPS and treated with RvD₁ at the same time for each different concentration. Data showed significance difference (*) for treatments 10 nM [IL-10] using pair wise fixed reallocation randomization test (p=0.001) N=3.

DISCUSSION

Our study presents five different modulators that are known to shift the microenvironment from pro-inflammation, which can be induced with LPS, to a wound healing. The five modulators, that our study measured, were acetylsalicylic acid (ASA), dexamethasone (DEX), prostaglandin E₂ (PGE₂), iloprost and resolvin D₁ (RvD₁). Moreover, the cytokines responses and macrophage activation of the NR8383 cell was measured by inducing the cell with LPS and then treating them with each one of these modulators. Then, our study concludes with the importance in the selection of a modulator that can be used as a powerful guidance for *in vivo* modulator studies.

1. NR8383 cells treated with Acetylsalicylic acid (ASA).

Macrophages are known to secrete pro-inflammatory cytokines and regulate the activation of pro-inflammatory gene which can be regulated by the activation of NF- κ B. The activation of NF- κ B leads to the secretion of inflammatory cytokines: TNF- α , IL-1, IL-6, IL-8, IFN- β and other cytokines (Kopp and Ghosh, 1994). ASA had demonstrated to be an anti-inflammatory agent due to the partial inhibition (approximate 75%) of the endogenous IKK kinase activity (Yin et al., 1998). Moreover, aspirin, also known as an acetylsalicylic acid, significantly can inhibit cells that were induced with LPS, and also suppressed mRNA accumulation and secretion of TNF- α at 0.1 mM concentration (Shackelford et al., 1997).

Our data showed a spike of IL-10, TNF- α , CCL2 and IL-6 inducing with LPS at 24 hours of incubation; however cells treated with ASA, our data showed a significant decrease of cytokines levels. Similar response were observed by the knock-down macrophage migration inhibitors

factors (MIF) in murine ovarian cancer cells (Hagemann et al., 2007). Moreover at 10 mM of ASA, our data revealed a decrease of TNF- α , CCL-2 and IL-6 which are known to be pro-inflammatory. In addition to the cytokine levels, cells treated at 1 mM of ASA, our data did not show different regulation. However for cells treated at 10 mM of ASA (qRT-PCR data), our data showed up-regulation of IL-10 in treatment B (LPS, and then 80 minutes later the modulator), and also CD206, and IL-6 for treatment C (LPS and the modulator at the same time). Moreover for 0.1 mM, our data showed an up-regulation of CD163 in treatment B. Thus, the protein secretion shows a decrease of pro-inflammatory cytokines in the protein secretion; however, IL-10 which is an anti-inflammatory cytokines shows a significant decrease in cells treated with ASA. Also, the gene expression show an up-regulation IL-10, CD206 and CD163 which are known to be up-regulation in anti-inflammatory microenvironment or wound healing response.

2. *NR8383 cells treated with dexamethasone (DEX).*

Dexamethasone is also known to be an anti-inflammatory glucocorticoid due to inhibition of NF- κ B and activator protein-1 (AP-1) pathway. In addition, NF- κ B and AP-1 inhibition, dexamethasone is known to reduce pro-inflammatory cytokine: TNF- α , IL-8, and IL-6. Similar to others studies, our data showed a decrease of IL-6, TNF- α , and IL-10. To illustrate, the anti-inflammatory response of the dexamethasone, it can be display by the lowering of cytokine levels that are known to be pro-inflammatory. However, our data also showed a decrease of IL-10 which is a known to be an anti-inflammatory cytokine. In addition, dexamethasone did not significantly affect CCL2 in treatments A and B. However, cells, that were treated with dexamethasone and at the same time induced with LPS (treatment C), our data shown a significant decrease of CCL2

levels. To recall, CCL2 is a chemokines that is known to recruit of cells in to the site of inflammation.

Gene expression of DEX at 100 nM, for treatments A and C showed similar regulation. In treatments A and C, iNOS and CCL2 were down-regulated. To illustrate, iNOS and CCL2 down-regulation shows the inhibition effect of DEX. On the other hand, treatment B has shown a different regulation compare to treatments A and C. To recall, treatment B (LPS, and then 80 minutes later the modulator) were cells that were induced with LPS, and then 80 min later were treated with DEX. Cells treated using treatment B shows an up-regulation of CD163, CD206 and IL-10. Thus, treatment B illustrated the presence of M2 macrophage which were not showed in treatment A and C. Also, in treatment B has shown an up-regulation CD163 which is a scavenger receptor which is known to be expressed by cells of the M2c phenotype, and also an up-regulation of IL-10 which is a known cytokine which is known to drive macrophage to the M2 phenotype. Even though, IL-10 show a decrease in the protein levels, in the gene expression showed the up-regulation of these anti-inflammatory cytokine. Finally, dexamethasone has showed a decrease of pro-inflammatory cytokines and an up-regulation of M2 phenotype; on the other hand, one side of effect of dexamethasone has shown to slowdown: wound healing at 1 mg/kg in patients, ulcer healing in rats, and muscle proteins in healthy patients (Luo et al., 2004; Minetto et al., 2010; Schermuly et al., 2002).

3. *NR8383 cells treated with prostaglandin E₂ (PGE₂).*

Prostaglandin E₂ is a known lipid mediator that is derived from arachidonic acid (AA). Moreover, PGE₂ is also known to be an anti-inflammatory lipid mediator. In the process of reestablishment homeostasis, PGE₂ and prostaglandin D₂ triggers a changing from the generation of leukotriene B₄ to the production of lipoxin A₂. A shift from inflammation into homeostasis is mediated by a switch of an increase of the secretion of pro-resolution lipid mediators such as E and D-series of resolvins and protectins (Serhan et al., 2008). Our data showed that PGE₂ at 10, 100 nM and 1 μM decreased the concentration of TNF-α. Moreover, the decrease concentration of TNF-α can be represented by homeostasis response of PGE₂. PGE₂, also, has showed to increase IL-6 in cells treated at 1 μM. However, IL-6 is a known signaling molecule that is secreted as a pro-inflammatory response, but also IL-6 can be expressed as an inhibitory effect of TNF-α and IL-1 (Ataie-Kachoie et al., 2014). Our data did show a significant increase of IL-6. This is consistent with recent studies showed by R. M. Hinson, J. A. Williams and E. Shacter, PGE₂ can cause an increase in IL-6 proteins levels. The expression of COX-2 may mediate the higher expression of IL-6 in murine model (Hinson et al., 1996). However, our data did not shown a significantly change in the secretion of IL-10 and CCL2.

For PGE₂, our data shows an up-regulation of ARG2, and CD206 genes at 10 nM. For 100 nM, our data shows an up-regulation of ARG2, CD163, CD206 and IL-10 genes, and for 1 μM, an up-regulation of ARG2, CD163 and CD206 genes. Our data shows the expression of M2 phenotype in our treated cells; however, only at 1 μM and 100 nM is expressed the wound healing macrophage which is the represented by the marker CD163 (M2c phenotype). In addition, at 100 nM is

expressed the up-regulation of IL-10. Finally, PGE₂ has showed the anti-inflammatory effect by decreasing TNF- α protein secretion, and also by the up-regulation of CD163, CD206 and IL-10.

4. *NR8383 cells treated with iloprost.*

Recently studies have shown that the half-life of PGI₂ is approximate 14.5 min at 4 °C in aqueous solution (Cho and Allen, 1978). The analog of PGI₂ that was used in our experiment was iloprost. Iloprost is a drug that has been used in the treatment of pulmonary arterial hypertension (PAH), scleroderma, and others types of diseases. Moreover, iloprost is known to be a potent anti-inflammatory drug (Al-Shorbagy et al., 2012). Our data shown, the cells treated with iloprost the concentration of TNF- α , IL-6, CCL2 and IL-10 decrease compare to the cells that were only induce with LPS. The anti-inflammatory response of iloprost is presented by the decrease of pro-inflammatory cytokine such as CCL2, IL-6 and TNF- α . Finally, iloprost is a drug that is represented as an analog of PGI₂. Iloprost showed to be a potent anti-inflammatory drug in cells induced and then treated with it.

For the gene expression of iloprost showed an up-regulation at 10 nM of CD163, CD206 and IL-6. At 100 nM showed an up-regulation of CD206 and IL-6 and in cells treated with 1 μ M showed an up-regulation of IL-6. At lowered concentration is possible to observe the presence of M2 and M2c phenotype which is known to be a characteristic of anti-inflammatory microenvironment. Thus, iloprost presented an anti-inflammatory drug because of the decrease of cytokines such as TNF- α , CCL2, and IL-6 and also showed the up-regulation of gene such as CD206, CD163 and IL-6.

5. *NR8383 cells treated with Resolvin D₁ (RvD₁).*

Recently studies have shown that administrated a high dose (from milligrams to grams) of omega-3 can be beneficial to many inflammatory disease (Serhan et al., 2008). Resolvins are derived for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) the two from E-series and D-series, respectively. Resolvin D₁ has shown in other studies to stops the recruitment of neutrophil and protect against neovascularization in mouse (Serhan et al., 2008). Moreover, DHA can derived to group referred to as 17*S* and 17*R* D- series, and also both has shown to block the production of TNF- α in the treatment of neural injury (Serhan et al., 2008). Our study shows that cells treated with RvD₁ did not shown a significantly change the concentration of cytokines such as CCL2, IL-10 and TNF- α . However, IL-6 showed a significantly decrease at 10 nM of RvD₁.

In the gene expression for 10 nM and 1 μ M, our data did not show a significantly change compare to LPS. On the other hand, cells that were treated with RvD₁ at 100 nM showed an up-regulation of IL-10. Thus, RvD₁ has shown to be an anti-inflammatory modulator because of the up-regulation of IL-10 in the gene expression.

CONCLUSION

Our data has showed that ASA, DEX, iloprost, and PGE₂ are an anti-inflammatory modulator in macrophage (NR8383 cells). Each modulator has presented to be able to decrease the concentration of pro-inflammatory cytokines response; however, not all of them have shown to increase anti-inflammatory cytokines levels. Each modulator has shown to act differently to reach homeostasis. For that reason each modulator can be used depending on the solution that is looking. To illustrate, RvD₁ is known to come from a family that can be administrated high dose in the treatment of inflammation. On the other hand, DEX has showed that is limited by their long therapeutic because of the inhibition of AP-1 pathway (Vayssiere et al., 1997). AP-1 is a transcription factor which is known to control the difference cellular process such as differentiation, proliferation and transformation (Angel and Karin, 1991). Moreover, ASA is an anti-inflammatory modulator; however, high dose is needed it to obtain the anti-inflammatory response. Iloprost and PGE₂ are lipid mediator that are derived for omega-6, and our study shown has shown the anti-inflammatory response of these two modulators. In addition, iloprost and PGE₂ can be presented as an anti-inflammatory modulator, but more work is needed it. Thus, all five modulator has shown that can be used to decrease the pro-inflammatory response. Depending on the work that it is needed, it is possible preference the modulator that can effectively be used depending off each situation. Future work, further analysis will be necessary to determine any functional of these modulator in *in vivo* starting with PGE₂. For this reason is needed the analysis of using one of each modulator using peritoneal macrophage and/or spleen macrophage.

Table 3: Summary of data

<u>Modulator</u>	<u>Concentration</u>	<u>Increase protein secretion</u>	<u>Decrease protein secretion</u>	<u>Up-regulation gene expression</u>	<u>Down-regulation gene expression</u>
<u>Acetylsalicylic acid (ASA)</u>	0.1 mM	N/A	IL-6, IL-10 (C) TNF- α	CD163 IL-10 (C)	N/A
	1 mM	N/A	CCL2, IL-6, IL-10(A), IL-10(C), TNF- α	N/A	N/A
	10 mM	N/A	CCL2, IL-6, IL-10, TNF- α	IL-10 (B) CD206 (C)	N/A
<u>Dexamethasone (DEX)</u>	100 nM (A)	N/A	IL-6, IL-10, TNF- α	N/A	iNOS CCL2
	100 nM (B)	N/A	IL-6, IL-10, TNF- α	CD-163, CD-206 IL-10	N/A
	100 nM (C)	N/A	CCL2, IL-6, IL-10, TNF- α	N/A	iNOS CCL2

<u>Prostaglandin E₂ (PGE₂)</u>	10 nM	N/A	TNF- α	ARG2, CD-206	N/A
	100 nM	N/A	TNF- α	ARG2, CD163, CD206 IL-10	N/A
	1 μ M	IL-6	TNF- α	ARG2, CD163, CD206	N/A
<u>Iloprost</u>	10 nM	N/A	CCL2, IL-6, TNF- α	CD206, IL-6	N/A
	100 nM	N/A	CCL2, IL-6, IL-10 TNF- α	CD206 IL-6	N/A
	1 μ M	N/A	CCL2, IL-6, IL-10 TNF- α	IL-6	N/A
<u>Resolvin D₁ (RvD₁)</u>	10 nM	N/A	IL-6	N/A	N/A
	100 nM	N/A	N/A	IL-6	N/A
	1 μ M	N/A	N/A	N/A	N/A

Table 3: Data of NR8383, cells were induced with LPS and treated with a modulator.

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