Regulation of Extra-Pituitary Prolactin in Monocytes and Macrophages

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REGULATION OF EXTRA PITUITARY PROLACTIN IN MONOCYTES AND MACROPHAGES

by

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ABSTRACT

Recently it has been shown that leukocytes are capable of producing prolactin (PRL). Evidence of extra-pituitary PRL (ePRL) production is so far been limited to primates and is not shared across other mammal species such as mice and rats. While ePRL is characterized as an identical protein to traditional pituitary PRL, it is controlled under an alternative promoter and is thus regulated differently from pituitary PRL. Little is known about what regulates ePRL or its direct role in human physiology, but given that PRL has well over 300 described functions, it is likely that the autocrine and paracrine effects of this hormone could have far reaching implications in overall physiology. This work takes some of the first steps in understanding how leukocyte ePRL is regulated. Our results show that, adrenergic hormones are one key stimulus in ePRL expression in monocytes/macrophages. This is particularly intriguing considering the opposing role of these two signals in settings such as adipose tissue where adipose tissue macrophages are constantly exposed to pro-lipolytic adrenergic hormones that would in turn stimulate production of an anti-lipolytic hormone, PRL. Further, our work shows that the inflammatory phenotype of the leukocytes influences basal expression of PRL and overall ePRL expression increases significantly as monocytes differentiate into macrophages, as is a common occurrence in adipose tissue. The final portion of our work shows how monocytes/macrophages also respond to preadipocytes directly. These stem cell precursors to mature adipose cells release an unknown factor that stimulates ePRL production in

monocytes/macrophages. Analysis of our gene array shows many of the genes stimulated by adipose stem cells alongside PRL are important genes in tissue regeneration and remodeling, a possible role that fits well with known effects of PRL. Understanding such primate specific interactions between the immune system and major metabolic tissues such as adipose fills vital gaps in knowledge that may explain why so many treatments fail when transitioning from mouse models to humans.

Dedicated to my family, in particular, my father Lee, my mother Karen, my brother Dylan, and my grandmothers Reba and Brenda. Also in dedication of my late grandfathers Hiram Lee (H.L.) and James who were lovers of science themselves and helped foster my curiosity from my earliest age.

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LIST OF ABBREVIATIONS

AH – adrenergic hormones

Amp – amphipathic amino acids

Aro – aromatic amino acids

ASC – adipose stem cells

ATM – adipose tissue macrophages

BBB – blood brain barrier

BMI – body mass index

CD14 – Cluster of Differentiation 14

DA – dopamine

DC – Dendritic cells

DCM – DMEM:F12 preadipocyte conditioned medium

E – epinephrine

ECM – extracellular matrix

ED – extracellular domain

EDM – embryonically derived macrophage

ELISA – enzyme-linked immunosorbent assay

ePRL – extra-pituitary prolactin

GH – growth hormone

GLUT4 – glucose transporter type 4

HBSS – Hank's balanced salt solution

HCM – HBSS preadipocyte conditioned medium

HFD – high fat diet

HMW – high molecular weight fraction

HSL – hormone sensitive lipase

IC – intracellular domain

IL-6 – Interleukin-6

L-DOPA – L-3,4-dihydroxyphenylalanine

LMW – low molecular weight fraction

LPL – lipoprotein lipase

MA-CM – mature adipocyte conditioned medium

MCP-1 – monocyte chemoattractant protein-1

MDM – monocyte derived macrophage

MS – multiple sclerosis

NE – norepinephrine

Ox-LDL – oxidized low density lipoprotein

PA – preadipocytes

PA-CM – preadipocyte conditioned medium

PAI-1 – plasminogen activator inhibitor-1

PBMC – peripheral blood mononuclear cells

PL – placental lactogen

PMA – phorbol 12-myristate 13-acetate

PPARy - peroxisome proliferator-activated receptor gamma

pPRL – pituitary prolactin

PRL – prolactin

PRLR – prolactin receptor

SD – standard deviation

SDS – sodium dodecyl sulfate

SGBS – Simpson-Golabi-Behmel syndrome

SGBS-MA – Simpson-Golabi-Behmel syndrome mature adipocytes

SGBS-PA – Simpson-Golabi-Behmel syndrome preadipocytes

TAM – tumor associated macrophages

TCA – tricarboxylic

TM – transmembrane domain

TNFa – tumor necrosis factor alpha

TRH – thyrotropin-releasing hormone

VIP – vasoactive intestinal peptide

CHAPTER ONE: GENERAL INTRODUCTION

As the proposed studies describe the use of various cell types, a bird's eye view of the cell types is given below:

Monocytes

Monocytes are a type of leukocyte and a key component of the innate immune system. They are produced in the bone marrow from hematopoietic stem cells[1]. Originally defined by their morphology, they are now commonly identified by the cell surface marker Cluster of Differentiation 14 (CD14)[2]. CD14 plays an important role in both inflammatory responses where it acts as a co-receptor for TLRs recognizing LPS [3,4], and anti-inflammatory responses where it is an important mediator in recognizing and phagocytizing apoptotic cells.[5]

Monocyte subtypes can be further distinguished by the secondary marker CD16 and its ratio to CD14 expression. They are generally broken down into 3 subtypes: classical CD14++/CD16-, intermediate CD14++/CD16+, and non-classical CD14+/CD16++.[2] The classical subtype makes up the vast majority of monocytes in the blood, commonly constituting around 80-90% of monocytes, while the non-classical subtype typically constitutes around 10% of total monocytes and are more inflammatory in nature.[6,7] While there is some plasticity between the types, it is generally believed that there is a developmental relationship where classical monocytes give rise to non-classical monocytes with intermediate monocytes being a transitory

subtype.[7,8] All subtypes are capable of differentiating into macrophages although non-classical show a greater propensity for doing so, while classical monocytes are the only subset capable of differentiating into dendritic cells (DCs).[9]

Even within these subtypes monocytes are extremely plastic in their abilities. They help regulate both inflammatory and anti-inflammatory immune responses by releasing cytokines and antimicrobial factors[10], and travel to sites of infection by following chemoattractants such as monocyte chemoattractant protein-1 (MCP-1)[11,12] where their differentiation into DCs and macrophages extends their indirect roles in homeostasis even farther.

Macrophages

Macrophages are one of the primary professional phagocytes in the human body and a key component of the innate immune system. Originally it was thought that all macrophages were derived from monocytes, termed "monocyte derived macrophages" (MDM). However, it is now understood that the majority of the macrophage population actually stems from embryonic development.[13] Nonetheless, MDMs still play a significant role in the human body and fulfill vital roles in maintaining homeostasis and supporting other macrophage populations. MDM development begins when patrolling monocytes are targeted towards sites of inflammation where they then differentiate and migrate into the target tissue[14]. MDMs are most commonly recruited during periods of acute stress, such as in response to wounding, where a rapid increase in macrophage populations are needed to overcome pathogens or

return the tissue to homeostasis.[15,16] For the most part, there is a large overlap in the functions and abilities of MDM's and most embryonically derived macrophages (EDM), and both populations tend to work synergistically.

EDMs primarily include tissue resident macrophages that have functions highly specialized to their resident tissue, are capable of dividing in the tissue to increase numbers, and serve vital roles in homeostasis in the resident tissue[13]. Many tissues differ in the contribution of MDMs and EDMs to their macrophage populations. Microglial cells in the brain for instance are almost exclusively of embryonic origin[17] while Kupffer cells in the liver tend to be supplemented with higher levels of MDMs.[18] The focus on macrophages of the embryonic lineage has grown as studies have shown these populations have a much greater capacity for self-replication than previously thought. As a result, many tissue resident macrophages are able to maintain and expand populations when necessary and assistance from the MDM population is mostly required during times of acute stress and infection.[19]

Polarization

Macrophages are some of the most plastic cells within the human body and can be programmed for a variety of important functions. Traditionally, macrophage polarization has been viewed as a binary programming system designated by M1 polarization indicating pro-inflammatory profile and M2 polarization indicating an anti-inflammatory profile[20]. Many recent studies and more complex genetic analysis have shown that macrophage phenotypes

are far more complex and can be a blend of both M1 and M2 and can even include traits that fall entirely outside of the traditional M1/M2 spectrum.[21-23]

M1 inflammatory macrophages play particularly important roles in defending the body against invading pathogens by killing and phagocytizing bacteria or virus infected cells[21] . They are hallmarked by their production of inflammatory cytokines and are often defined by their increased expression of inflammatory cytokines such as tumor necrosis factor- alpha (TNFa) and interleukin-6 (IL-6). They also derive most of their energy via glycolysis using carbohydrates as a primary fuel source.[24]

M2 macrophages are designated by their production of anti-inflammatory cytokines such as MRC-1 and IL-10. They derive most of their energy via beta-oxidation of fatty acids.[24] These macrophages play an essential role in tissue repair and remodeling[25] but along the same lines, have also been shown to be permissive or even protective towards tumor cells as many of those repair and remodeling functions can be used to create a microenvironment more conducive to tumor growth.[26]

Adipose Tissue

Traditionally thought of as a simple lipid storage tissue, adipose tissue is starting to be recognized for the complex endocrine organ that it is. Adipose tissue is a complex milieu of not

only adipocytes, but also tissue matrix, nerve tissue, stromovascular cells, stem cells, and immune cells.[27] The major constituents of adipose tissue are discussed below.

Adipocytes

Clearly the stars of the adipose tissue are the cells from which it derives its name. Adipocytes function primarily as lipid storage cells. Their key feature is the development of a large lipid droplet that stores neutral lipids in the form of triglycerides.[28] These lipid droplets provide the densest form of energy storage in the human body, as fatty acids have large ATP production potential and storing energy in the form of lipids avoids the water retention that accompanies carbohydrate storage in forms like glycogen.[29]

The triglycerides within the adipocytes are formed from a variety of sources. Dietary fats are able to be stored directly through specialized uptake systems. Fats are absorbed in the intestine and transported to the liver as chylomicrons via the lymphatic system. Chylomicrons undergo lipolysis in the plasma, giving rise to “remnant” particles that are absorbed by the liver. Once in the liver, hepatocytes repackage the fats into lipoprotein particles, primarily VLDL using lipoprotein B as the primary organizing protein[30]. These particles are then able to more easily travel through the blood stream where they reach adipocytes and fats are mobilized out of the particle and into the adipocyte.

Lipid uptake is accomplished via lipases and esterases, such as lipoprotein lipase (LPL) that works to release free fatty acids and cholesterol from the particle, and receptors such as CD36 and FATP that allow uptake of FFA into the cell.[31] Once inside the adipocyte, free fatty acids are esterified to glycerol in groups of three forming neutral lipid triglycerides. Cholesterol is esterified to a free fatty acid. These two esterified lipid products make up the bulk of the lipid droplet in mature adipocytes.[28] Naturally, increase in adipocyte size is accompanied by the need to synthesize membrane components such as phospholipids.

Aside from direct uptake of lipids, adipocytes also have an enormous capacity for lipogenesis via carbohydrate metabolism.[32-34] Glucose is broken down to pyruvate that then enters the tricarboxylic (TCA) cycle. Citrate from the TCA cycle can then be converted to acetyl-CoA, and then malonyl-CoA which acts as an important starting molecule from which free fatty acids can be built[35]. After the formation of free fatty acids, they can then be incorporated into triglycerides just as FFA taken directly from the diet. This process is stimulated via insulin signaling which allows adipocytes to store carbohydrates as fat when blood sugar spikes.[35]

Preadipocytes and adipocyte differentiation.

Adipocytes are under constant turnover and replacement. Such turnover is possible thanks to a population of mesenchymal stem cells more specifically referred to as adipose stem cells (ASC) or preadipocytes (PA). While the total number of adipocytes in any individual's

adipose depots remains constant throughout their life, the population is replaced at a rate of about 10% per year and all adipocytes are replaced over about an 8-year cycle[36].

ASC typically retain the ability to differentiate into a variety of cells. Differentiation into mature adipocytes specifically is achieved through activation of the master transcription factor peroxisome proliferator-activated receptor gamma (PPARy).[37] However, studies show that this "stemcellness" is reduced in ASC from obese patients and many of the ASC population is already committed towards adipocyte differentiation with a reduced ability to differentiate into other cell types.[38]

This differentiation process is essential to a healthy accumulation and storage of fat. Factors that inhibit differentiation of adipocytes, such as TNFa, plasminogen activator inhibitor-1 (PAI-1), and many other inflammatory cytokines, can cause undue burden on the remaining cell population to accumulate and store fat. This stress leads to metabolic dysregulation like insulin resistance, adipocyte cell death, further inflammation, and then reinforcement of many of the same signals that inhibit differentiation thus creating an inflammatory feedback loop.[39,40]

Factors that promote this differentiation process have been shown to aid in healthy fat accumulation. Bringing more adipocytes online allows the cell population to share the burden of fat accumulation and storage and thus lowers overall cellular stress. This allows an individual to store more energy without many of the other chronic issues that often accompany obesity.[41]

Adipose Tissue Macrophages

The next most common cells within the adipose tissue after adipocytes are adipose tissue macrophages (ATM). ATMs are resident within adipose but their overall numbers can fluctuate considerably under different conditions. In healthy, lean individuals ATMs comprise about 15% of the total adipose cell population; however, during excessive weight gain there is a large influx of macrophages into the adipose and numbers can climb to as high as 50-60% of total cells in obese individuals.[42,43] It is not well established as to why there is such a large expansion of the ATM population during obesity but many studies have shown that adipocytes are capable of secreting a number of immune chemoattractants, such as MCP-1 and CCL5, during obesity.[44,45]

It is still debated as to which factors have the greatest influence over macrophage infiltration and many of the roles they perform in the tissue are still being uncovered. So far, it is known that these ATMs play an important role in scavenging both FFAs leaked from overly-stressed adipocytes and the dying adipocytes themselves[46]. ATMs form "crown-like" structures as they encircle stressed and dying adipocytes[47,48]. ATMs have been shown to accumulate so many FFAs themselves that lipid droplets develop in the immune cells forming foam cells more commonly seen within atherosclerotic plaques.[47,49]

On top of this influx of macrophages, it has also been shown that the polarization of the macrophages also differs between lean and obese individuals. ATM's in lean adipose depots

tend towards an anti-inflammatory M2 polarization while ATMs from obese adipose depots tend towards an inflammatory M1 phenotype[50]. This has important implications in overall adipose physiology as the increased production of inflammatory cytokines characteristic of M1 macrophages can affect a variety of adipose functions such as differentiation mentioned above, and lipid metabolism.[51,52]

Prolactin and Prolactin Receptor

Pituitary PRL

Prolactin (PRL) is a 23kDa peptide hormone traditionally known for its role in coordinating milk production in mammals. It belongs to the cytokine class-1 receptor superfamily and more specifically the placental lactogens, which also includes growth hormone (GH) and placental lactogen (PL). PRL is primarily produced by lactotrophs in the anterior pituitary where it is released in a pulsatile fashion.[53]

In pituitary lactotrophs, PRL is under the control of the Pit-1 promoter[54]. The classical stimulators of pituitary PRL (pPRL) are suckling, stress, and ovarian steroids such as estrogen[53]. The most well defined inhibitor of pPRL expression is dopamine which negatively regulates PRLs secretion[55]. Pituitary PRL is also regulated through secretory granules that allow a buildup of PRL within the lactotrophs that can then be delivered in a much stronger burst than if simply

secreted as transcribed.[56] This is a key factor in regulating PRLs pulsatile release from the pituitary.

While PRL is traditionally thought of for its role in coordinating lactation in mammals, PRL itself is an evolutionarily ancient protein whose lineage reaches far enough back to be expressed in both birds and fish.[57] PRL is also exhibits extreme diversity in its bioactivity with well over 300 defined actions spanning multiple tissues and systems in the human body including the immune system, vasculature endothelial cells, and major metabolic tissues such as adipose.[58]

Extra-pituitary prolactin

More recently, it has been discovered that PRL production is not limited to the pituitary. In fact, a variety of tissues outside the pituitary have now been discovered produce PRL *de novo*. These tissues and systems include the decidua, immune system, brain, and myometrium, with more being discovered every year.[59] Studies show the ePRL protein is transcribed directly from the same gene as classical pPRL and indeed has an identical protein structure.[60] Where these two sources of PRL differ is in their regulation. ePRL falls under the control of an alternative promoter which results in differential regulation and the addition of a 150bp promoter to the ePRL mRNA.[60] As a result, much of what is known about PRL regulation in the pituitary does not translate to ePRL regulation. Classical regulators of pituitary PRL such as

dopamine, thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide (VIP), and estrogen, show no significant regulatory effect on ePRL in cell culture.[59]

On the other hand, specific regulators of ePRL expression are far less well defined and can differ slightly depending on the tissue.[61] So far, cAMP activation has been shown to be effective in stimulating ePRL expression in the decidua[62] and lymphoid cells [63,64] ENREF 63 although these results have not always held up across all tissue types.[61] Work done in our lab is in agreement with this concept though, showing that adrenergic hormones, which signal through cAMP, are able to stimulate ePRL in monocytes and MDMs.[65] Further, these extra-pituitary sites lack the storage granules present in pituitary lactotrophs and as a result, any PRL synthesized is likely immediately excreted into the surrounding space[59]. Far more work is needed however to uncover the regulational complexities of ePRL across various cell types.

The concept that ePRL is an identical protein that is regulated differently to pituitary PRL is a particularly important concept to consider when looking to apply previous studies to current work. As a signaling hormone, much of the previous work on PRLs bioactivity and characteristics would be largely applicable to better understanding the functions of ePRL, and indeed many studies have shown that the autocrine and paracrine effects of ePRL mirror those of pPRL.[66] Nonetheless, autocrine and paracrine PRL signaling could have vastly different effects on whole body physiology thanks to its unique regulation and delivery.

Vasoinhibins (PRL fragments)

PRL can undergo a variety of post-translational modifications including dimerization, polymerization, phosphorylation, glycosylation, and proteolytic cleavage.[53] In general, most of these post-translational modifications tend to lower PRLs biological activity with the exception of proteolytic cleavage.

Cleavage of the PRL protein results in a 16kDa N-terminal protein fragment called vasoinhibin that no longer binds the PRLR but instead shows entirely separate biological activity[67]. A number of different proteases have been shown to cleave PRL into this bioactive fragment including multiple matrix metalloproteases [68,69] and cathepsin-D[67,70]. The reason that such a wide variety of proteases are able to create these peptides is that bioactivity is maintained in the fragments across cleavages at various sites and in fact, N-terminal fragments from 12-17kDa have been shown to all be bioactive as vasoinhibins.[68] Further, due to sequence homology, similar cleavage of both GH and PL leads to fragments of the same size and bioactivity, lending credence to the idea that vasoinhibins are actually a family of peptides and not a singular protein.[71,72]

The most interesting aspect of vasoinhibins is that their bioactivity seems to be in opposition to that of whole PRL. While whole PRL has been shown to promote angiogenesis by driving endothelial cell survival, proliferation, and migration,[73] vasoinhibins most well documented physiological effects are on endothelial cells include inhibiting vasodilation[74],

being potently anti-angiogenic.[75,76] and promoting apoptosis of endothelial cells[72] While the mechanistic actions of vasoinhibins are not yet as well understood, recent work shows that they are able to exert much of these effects through binding and inactivation of PAI-1 resulting in anti-angiongenic and profibrinolytic effects.[77]

Vasoinhibins in general are extremely important to consider when studying PRL. Other isoforms of PRL such as macroprolactin have been shown to cause false diagnoses of hyperprolactinemia as it is recognized as PRL in many assays clinically used, but lacks much of the biologic activity.[78] Vasoinhibins could pose a similar risk as there is not clear evidence as to the specificity of different detection methods and whether or not they are able to discern between the two as the two protein types may share common epitopes. And given that vasoinhibins have vastly different bioactivity, such oversights could have much larger physiological consequences clinically.

Prolactin Receptor (PRLR)

The primary receptor through which monomeric PRL exerts its effects is the PRLR. This receptor belongs to the hematopoietic receptor family and functions through a variety of secondary messenger cascades, most notably the Jak-STAT pathway. The receptor has an extracellular domain (ED), a transmembrane domain (TM), and an intracellular domain (IC)[79]. The receptor can be modified in a variety of ways including alternative splicing of the mRNA as

well as post translational cleavage. These processes result in 4 primary forms of the PRLR: the long form, intermediate, and two short forms.[80]

The primary form of the receptor of physiological significance is the long form. However, cells frequently express multiple forms of PRLR and can even heterodimerize two different forms upon ligand binding resulting in an inactive complex[81]. It is thought that these inactive complexes could also have physiological significance and provide a mechanism for dampening PRL signaling, possibly helping to regulate which tissues respond to the signal and which tissues don't.[81]

Aside from such isoform complexity, the PRLR can also bind ligands other than PRL. GH and PL can both bind the PRLR thanks to strong sequence similarities. While they bind to the receptor with lesser affinity, it is believed to deliver the same signal.[82]

Role of Prolactin

In Immune Cells

The PRLR is expressed on all leukocytes.[83] One of the most prevalent effects of PRL treatment on the immune system is immunoproliferation. This proliferative action has been most clearly demonstrated in the Nb2 T lymphoma cell line where a variety of cell growth related genes are upregulated upon PRL treatment.[84] Also administration of anti-PRL antibodies is enough to inhibit lymphocyte proliferation in response to T and B cell mitogens in both

mouse and human in vitro.[85] It is important to mention however that many PRLR-/- models have shown limited variation in immune profiles when compared to wild type[86] and more recent work has targeted PRL more so as a co-mitogen for proliferative factors such as IL-2 rather than exerting proliferative properties on its own.[87,88]

PRL also shows immune activating properties that follow a similar profile to their pro-proliferative properties where PRL is believed to act in concert with other signals rather than in isolation, the effect often being stimulus dependent. The complexity of PRL signaling in the immune system is evident in studies using whole blood where PRL treatment alone increased levels of IL-10 and IL-12, PRL/LPS/PHA treatment increased IFNy and IL-12, but PRL/LPS treatment increased levels of only Il-10.[89] Such complexity can often make it difficult to predict the systemic effects of PRL based on observed responses in isolated cells.

Finally, PRL has also shown anti-apoptotic effects in immune cells, the physiological consequences of which are most evident in autoimmune diseases. Serum PRL levels have been shown to be unusually high in a number of autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (MS).[90] Studies in MS in particular have shown that throughout pregnancy and post-partum, relapse rates closely correlate with serum PRL levels.[91] A more direct link between PRL and autoimmune diseases has been made by showing that PRL interferes with tolerance induction in B cells, driving more autoreactive B cells away from apoptosis, allowing them to survive into clonal expansion.[90,92]

In adipose tissue

It was well established early on that significant changes in adipose tissue take place as PRL levels fluctuate throughout pregnancy[93]. Even in isolation PRL appears tied to weight gain as patients with hyperprolactinemia have increased body mass index (BMI), and the condition can be corrected by administering the dopamine agonist bromocriptine that lowers PRL levels back to normal.[94]

PRL has also been shown to act directly on adipocytes. This work began with the discovery that adipocytes themselves express PRL receptors and can respond directly to the hormone.[95] In fact, PRLR expression increases up to 90 fold as preadipocytes differentiate into mature adipocytes.[95,96] Many subsequent studies highlight PRL as an adipogenesis promoting hormone that acts by enhancing transcriptional regulators of adipogenesis such as PPARγ[97] and results in adipocytes with increased lipoprotein lipase expression and triacylglycerol content.[98]

Many of these findings are in accordance with in vivo studies where mice on high fat diets (HFD) and PRL infusion show increased fat mass as a result of hyperplasia (increased number of adipocytes), which is a metabolically healthier form of weight gain than hypertrophy (increased size of individual adipocytes). PRL infused animals also showed a healthier metabolic profile with increased adiponectin levels in greater insulin sensitivity despite the HFD.[41]

CHAPTER TWO: ADRENERGIC HORMONES INDUCE EXTRAPITUITARY PROLACTIN GENE EXPRESSION IN LEUKOCYTES – POTENTIAL IMPLICATIONS IN OBESITY

Barrett, R., Narasimhulu, C. A. & Parthasarathy, S. Adrenergic hormones induce extrapituitary prolactin gene expression in leukocytes-potential implications in obesity. *Sci Rep* **8**, 1936, doi:10.1038/s41598-018-20378-1 (2018).

Introduction to adrenergic hormones

Catecholamines are widely utilized signaling molecules throughout the body. Biosynthesis of the catecholamines begins with the amino acid tyrosine which is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) via the rate limiting enzyme in catecholamine synthesis, tyrosine hydrozylase. L-DOPA can then be converted to dopamine (DA), DA to norepinephrine (NE), and finally NE to epinephrine (E), each step facilitated by its corresponding enzyme.[99]

Each catecholamine fulfills multiple signaling roles throughout the human body. Dopamine is primarily considered for its role as a neurotransmitter and plays an important role in a number of neurological diseases such as Parkinson's disease where the death of dopaminergic neurons leads to low DA signaling. [100] DA also plays a role in hormone regulation, as mentioned earlier DA signaling in the anterior pituitary sharply inhibits PRL gene expression and release from pituitary lactotrophs.[101]

Diseases like Parkinson's and hyperprolactinemia can both be treated by increasing DA signaling to the tissues of interest such as the brain. However, DA is not particularly stable in the human blood stream with a half-life on the order of a few minutes, so intravenous injections have little benefit.[102] Further DA is unable to cross the blood brain barrier (BBB) so even if successfully delivered to the bloodstream it would be unable to reach its target sites.[103] As a result, L-DOPA has become a treatment of choice as it has both a longer half-life and is capable of crossing the BBB. Being an important precursor for DA, increasing L-DOPA concentrations result in increased DA levels in dopaminergic neurons.[103]

NE and E are collectively referred to as adrenergic hormones (AH). AH also play important roles in neurological signaling, most notably in the classic "fight or flight" response where they are quickly released from the peripheral nervous system and adrenal glands. In congruence with such a role, many of their well-defined actions deal with energy redistribution. One of their most potent signals is in the activation of hormone sensitive lipase (HSL) in adipocytes. [104] Activation of HSL in adipocytes leads to an immediate cleavage of FFA from triglycerides which can then be mobilized out of the cell and into the blood stream as an energy source for other tissues.[104] Proper signaling of AH is important in controlling overall lipid metabolism whether small amounts of fat are mobilized for day-to-day energetic needs, or whether large amounts of energy are mobilized at once as is needed in a "fight or flight" response.[104,105]

Materials and methods

Reagents

TRIzol™, primers, RPMI-1640, FBS, penicillin-streptomycin, and 1× PBS, were purchased from Life Technologies (Carlsbad, CA). Superscript III cDNA kit and Sybr green were ordered from Invitrogen (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA), Sodium dodecyl sulfate (SDS), urea, lipopolysaccharide from *E. coli*, L-DOPA, norepinephrine with bitartrate salt, and epinephrine with bitartrate salt were purchased from Sigma-Aldrich (St. Louis, MO). IL-4 was purchased from Promega (Madison, WI). Ficoll-Paque PREMIUM density gradient media 1.073g/mL was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Human prolactin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Boster Biological Technology Co., LTD (Pleasanton, CA)

Cell Culture

Monocytes and macrophages were modeled using the THP-1 human monocyte cell line. Cells were maintained in RPMI-1640 along with 10% FBS, 1% penicillin-streptomycin. THP-1 monocytes were differentiated to “naive” macrophages by supplementing media with 50 ng/mL PMA for 72 hours. PMA was then rinsed away and “naive” macrophages were polarized to either inflammatory “M1” macrophages with 100 ng/mL LPS for 24 hours, anti-inflammatory

"M2" macrophages with 20 ng/mL IL-4 for 24 hours, or maintained in the basal medium for an additional 24 hours to reduce the influence of PMA.

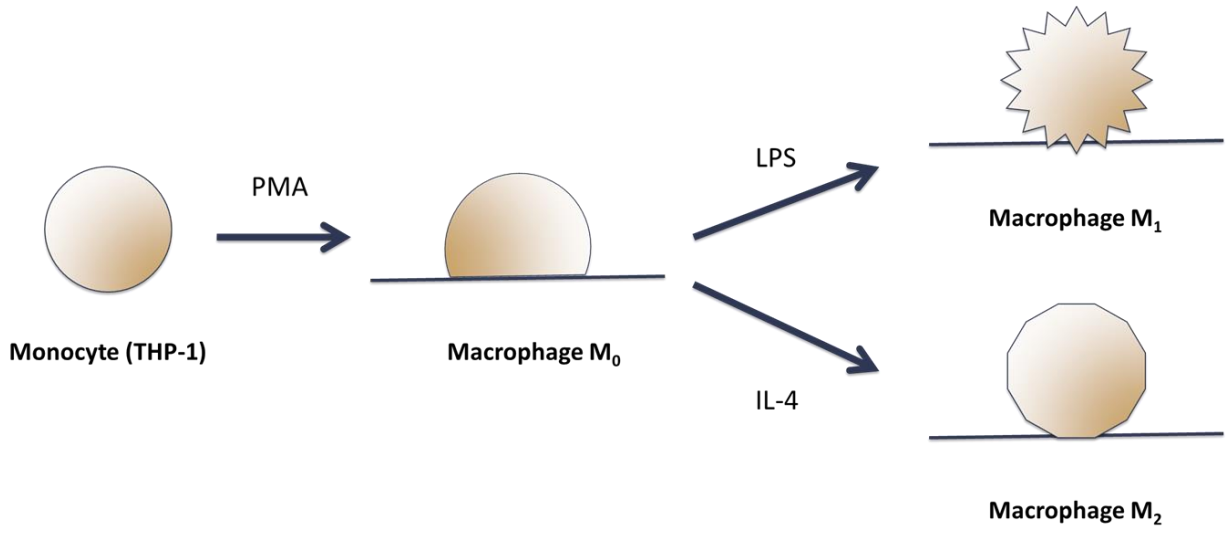


Figure 1: In vitro differentiation and polarization of THP-1 cell line

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMC's were isolated using Ficoll-Paque of a 1.073g/mL density, specifically formulated for monocyte recovery from buffy coat and whole blood. Human buffy coat samples were provided by One Blood (Orlando, FL). (Figure 2)

Figure 2: Human buffy coat as received from One Blood Orlando

First, 50mL of buffy coat was diluted using 50mL RPMI. 20mL Ficoll-Paque PREMIUM was then pipetted into a 50mL Falcon tube and 20mL of diluted human buffy coat was carefully layered on top, with care to not allow mixing between the two layers. The tube was then centrifuged at 400g for 40 minutes resulting in the formation of 4 layers: plasma, PBMCs, Ficoll-Paque, and red blood cells along with other immune cells denser than 1.073g/mL. (Figure 3)

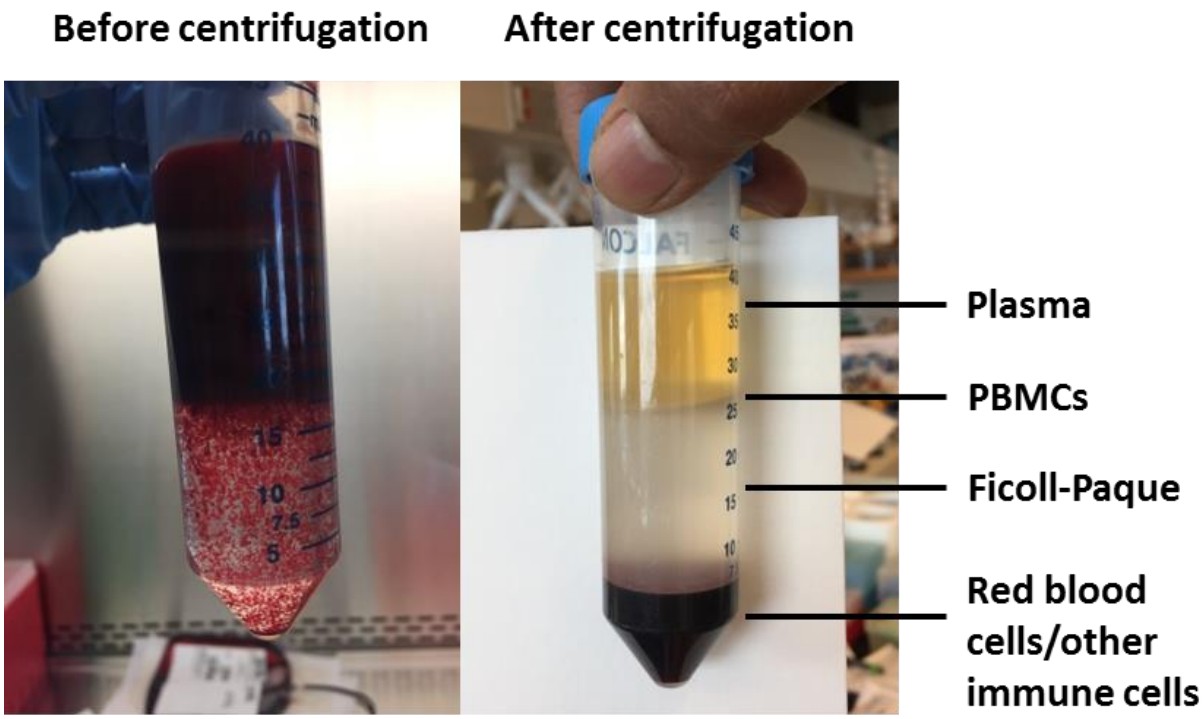


Figure 3: Human buffy coat and Ficoll-Paque separation

After separation, the top layer of plasma was drawn off and PBMC's were carefully pipetted into another 50mL Falcon tube. The cells were then washed 4-6 times using RPMI to remove platelet contamination and plated in RPMI supplemented with FBS. Cells were incubated for 2 hours and any nonadherent cells were rinsed away. The remaining PBMC's were used for experimentation.

Quantitative real-time PCR

Cells were dissolved in TRIzol™ reagent and RNA was isolated according to the manufacturer's instructions; cDNA synthesis was performed using 1 µg of total RNA. Quantitative real-time PCR was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green as the detection dye. mRNA expression levels of target genes were normalized against GAPDH.

ELISA

Medium was collected from experimental conditions and analyzed via ELISA. Sensitivity of ELISA used was <1.0pg/mL with a suggested work range of 156pg/ml-10,000pg/ml PRL. Protocols were according to manufacturer's instructions. Readings were taken using a 96-well plate reader (Benchmark Plus Microplate Spectrophotometer System, Bio-Rad, Hercules, CA).

Statistical Analysis

Values are presented as a mean ± standard deviation (SD), and statistical analyses were performed using student's t-test, with $P < 0.05$ as the cutoff level of significance.

Results

Not all catecholamines stimulate PRL in THP-1s

Drawing from the knowledge that dopamine is the primary negative regulator of PRL expression in the pituitary and that L-DOPA is the most commonly used drug to raise dopamine levels in humans, we first tested high levels of L-DOPA in cell culture to see if it could also influence ePRL expression in monocytes. Monocytes have been shown to express L-DOPA decarboxylase, the enzyme necessary to convert L-DOPA to DA.[106] However, no significant change in PRL expression was observed in THP-1 monocytes except for a slight, insignificant decrease in gene expression at 200uM, a concentration well beyond physiological levels. (Figure 4).

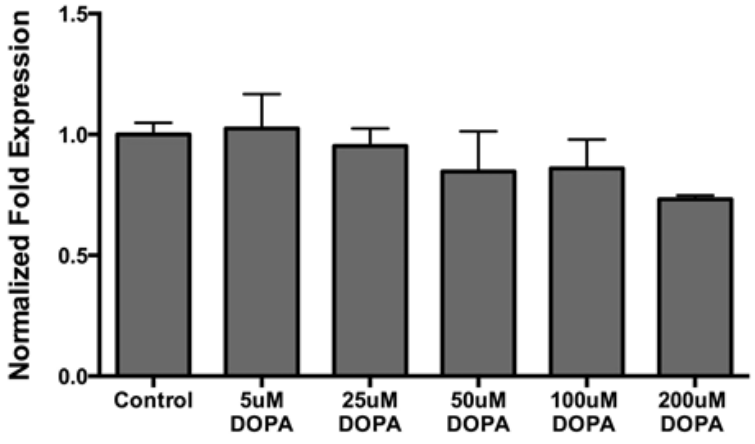


Figure 4: PRL expression in monocytes treated with L-DOPA

Adrenergic hormones stimulate PRL expression

After observing no response with L-DOPA or dopamine, we moved on to the adrenergic hormones NE and E. At the same supraphysiological concentrations originally tested with L-DOPA a huge increase in PRL expression was observed; however, this response appeared saturated and was not concentration dependent at these levels (Figure 5).

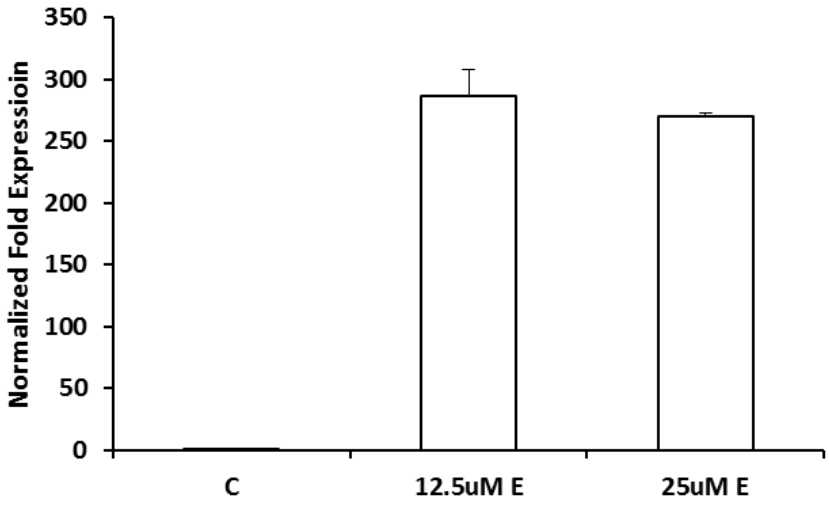


Figure 5: PRL gene expression in monocytes treated with E

We then tested concentrations reflecting physiological levels and observed a dose dependent increase in PRL expression for both NE and E that was significant even at very low concentrations (Figure 6).

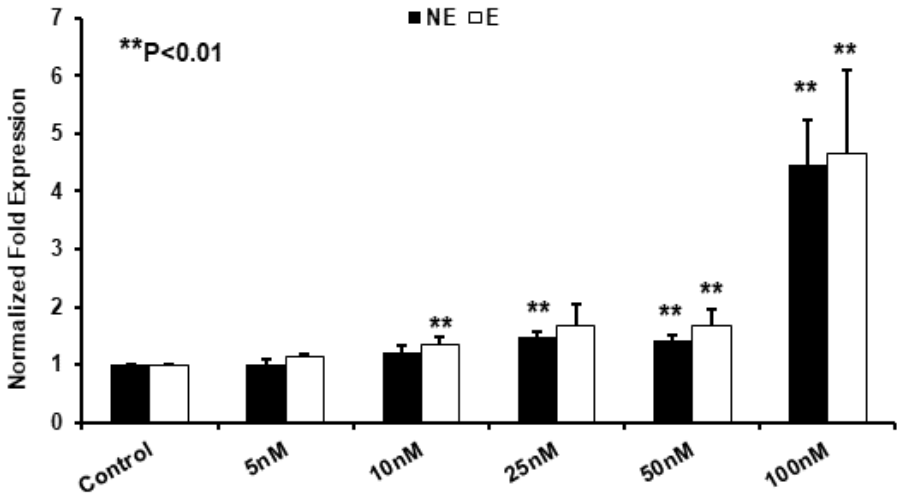


Figure 6: PRL gene expression in monocytes treated with physiological concentrations of NE and E

Next we wanted to see if these changes in PRL gene expression corresponded to actual increases in PRL produced by these cells. As mentioned earlier, monocytes are not capable of storing PRL intracellularly as pituitary lactotrophs are, so all PRL produced by the cells is thought to be directly excreted into the surrounding medium. With this in mind, we exposed monocytes to varying concentrations of NE and E for 24 hours and measured the concentrations of PRL protein in the medium via ELISA (Figure 7). PRL protein production

closely followed genetic expression in monocytes treated with NE and E. Western blot analysis was also performed on protein samples but was not used for analysis because of reoccurring issues with antibody and low resolution.

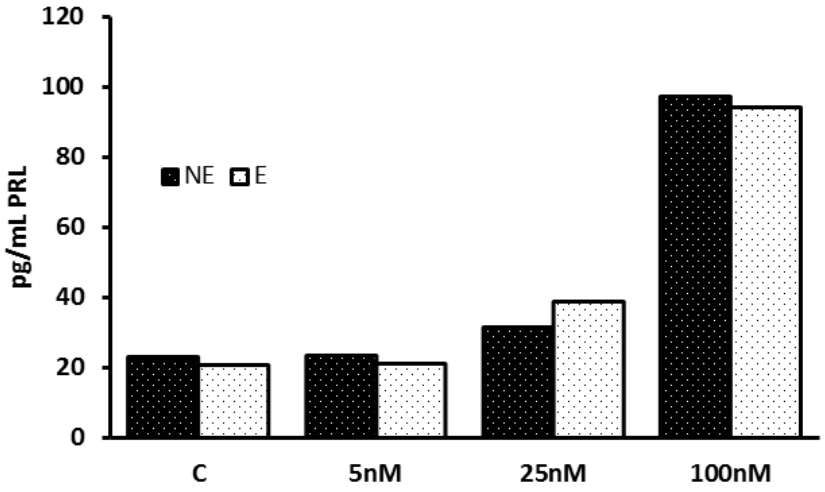


Figure 7: Total PRL secreted into medium of monocytes treated for 24 hours

Effect of NE and E on freshly isolated PBMCs.

To determine whether this response is one specific to THP-1 cells alone or if could be repeated using freshly isolated monocytes, we purchased buffy coat from One Blood Orlando and isolated PBMC's via the protocol detailed above. The results were somewhat muted in the fresh PBMC population in response to NE and E and not much induction of ePRL was observed.

However, the raw qRT-PCR data did show rather large error bars indicating variance between the duplicate samples analyzed, suggesting that the working PBMC population may have been somewhat distressed and as a result normal signaling may have been disturbed. As a result, we cannot draw any confident conclusions from this data and further optimization of the protocols will be necessary before this question can be answered. These results also leave open the possibility that ePRL stimulation by adrenergic hormones could be a response specific to THP-1 monocytes as cell line biology can often drift from biology observed in the primary cells they model.

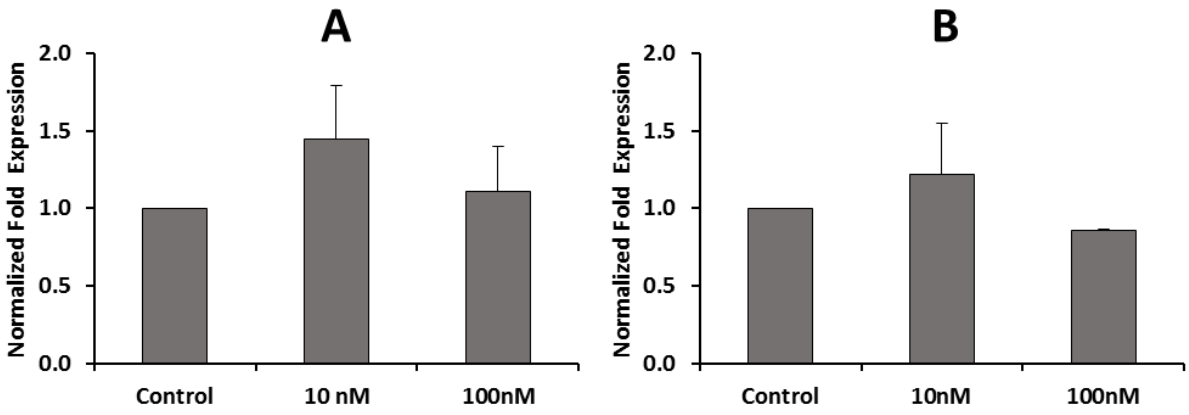


Figure 8: PRL gene expression in PBMCs treated with A) NE and B) E for 24 hours

Adrenergic stimulation occurs quickly with increases in gene expression evident within 2 hours and peaking at around 4-8 hours

In order to better understand the time course of adrenergic stimulation, THP-1 monocytes were exposed to 100nM NE and collected at different timepoints. Even at the earliest timepoint of 2 hours an increase in PRL gene expression is evident. After about 4 hours the cells seem to be responding fully with little change in expression there after. Sometime after 8 hours the increase in PRL gene expression begins to return to baseline though the response is still clearly observable at 24 hours (Figure 9).

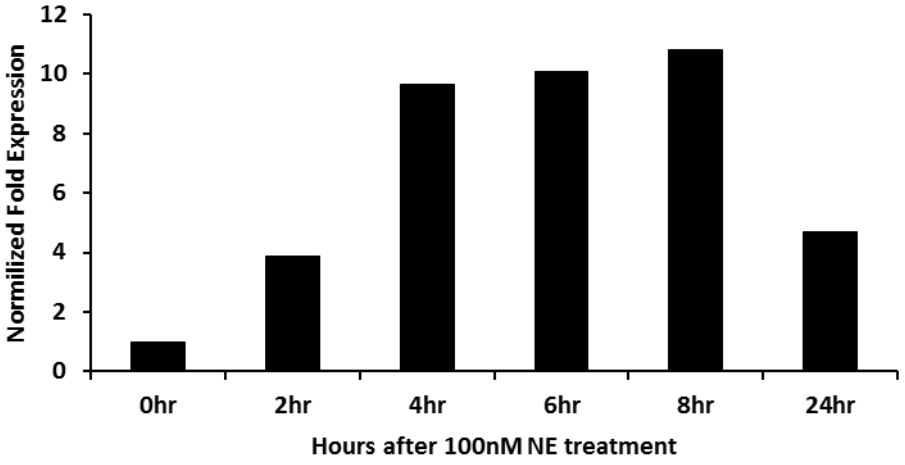


Figure 9: PRL gene expression at different timepoints in monocytes treated with 100nM NE

Differentiation and polarization of monocytes and macrophages influences basal PRL expression

THP-1 monocytes were differentiated via the protocol above. Naïve macrophages showed about a 6 fold increase in basal PRL expression over monocytes (Figure 10).

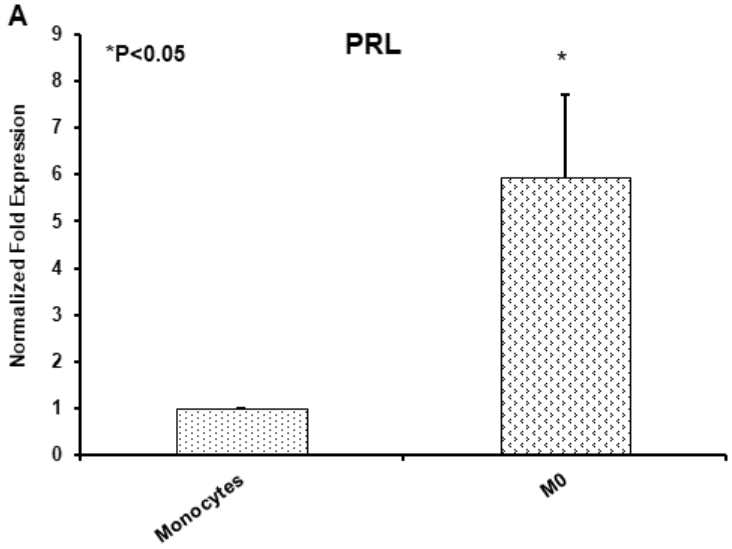


Figure 10: Basal PRL expression in monocytes and MDM

After an increase in gene expression was observed, we measured total PRL protein in the medium of the samples after 24 hours via ELISA. PRL protein production through monocyte differentiation closely mirrors the increase in gene expression with about a 10 fold increase in PRL protein excreted into the medium. (Figure 11).

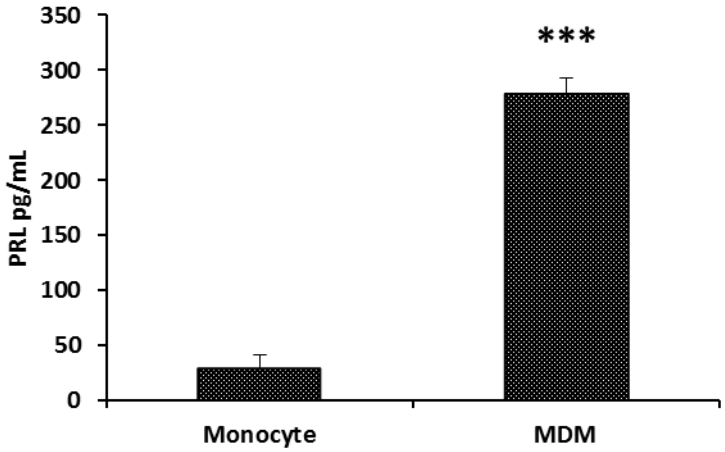


Figure 11: Total PRL protein in medium of untreated monocytes and MDMs after 24 hours

After differentiation, MDMs were polarized via the protocol above. We measured basal PRL gene expression between naïve (M0), inflammatory (M1), and anti-inflammatory (M2). Successful polarization was verified using traditional genetic markers. The M1 population showed a significant increase in TNFa and IL-6 expression confirming M1 polarization. The M2 population showed a significant increase in MRC-1 expression confirming M2 polarization. M2 polarized macrophages showed no significant difference in PRL gene expression compared to M0. M1 macrophages however, showed an almost 5 fold increase in expression (Figure 12).

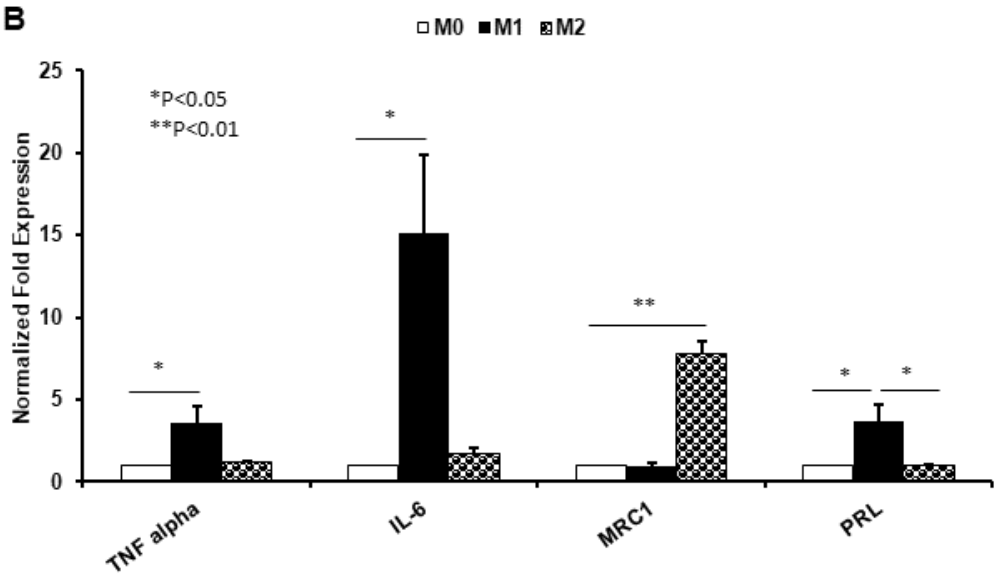


Figure 12: Genetic expression of polarization markers and PRL in M0, M1, and M2 MDMs

To our surprise however, PRL protein production did not mirror PRL gene expression across macrophage types. While M0 and M2 macrophages did show similar levels of protein production as would be expected from genetic expression levels, M1 macrophages actually showed a significantly reduced concentration of PRL in medium as compared to M0 and M2 macrophages. This was in direct opposition to the increase in PRL gene expression measured.

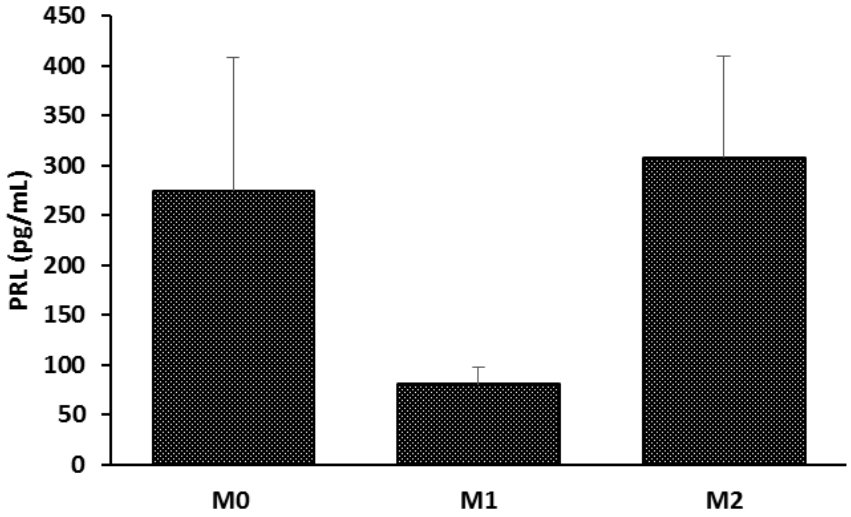


Figure 13: Total PRL secreted into medium of macrophage types after 24 hours

Polarized Macrophages can be further stimulated with adrenergic hormones despite increased basal expression of PRL

After observing that differentiation and polarization could increase basal PRL expression in macrophages we set out to see if PRL expression could be further stimulated with adrenergic hormones. M0, M1, and M2 macrophages were treated with 100nM NE and E for 24 hours. All macrophage types could be further stimulated by both NE and E resulting in even higher ePRL expression than the already elevated basal levels. (Figure 14)

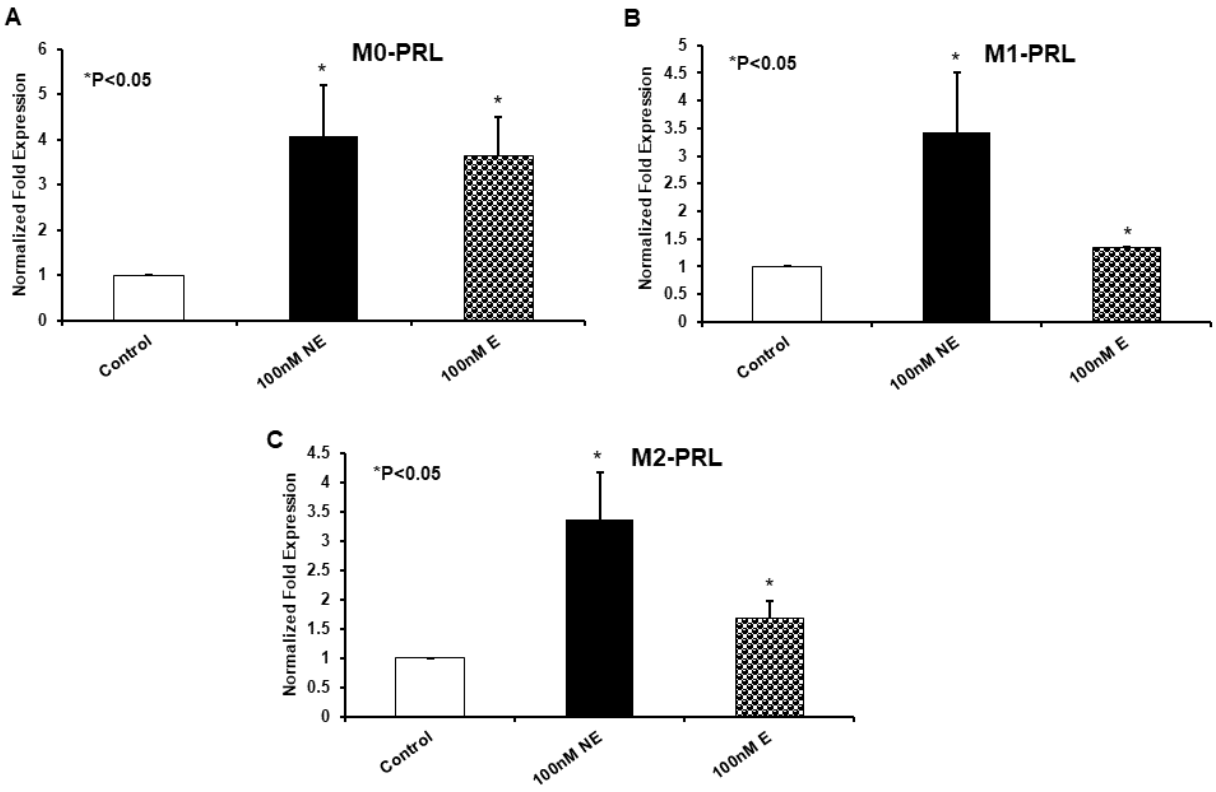


Figure 14: A) PRL gene expression in M0 MDMs in response to NE and E. B) PRL gene expression in M1 MDMs in response to NE and E. C) PRL gene expression in M2 MDMs in response to NE and E.

Upon measuring the total PRL protein released by MDMs stimulated with adrenergic hormones, we noticed that the significant increase in expression observed from NE and E treatment only amounted to a small, increase in total protein. Although an increase in protein

was observed for almost all treatments, none of the results were significant although this could likely be due to the smaller sample number. (Figure 15)

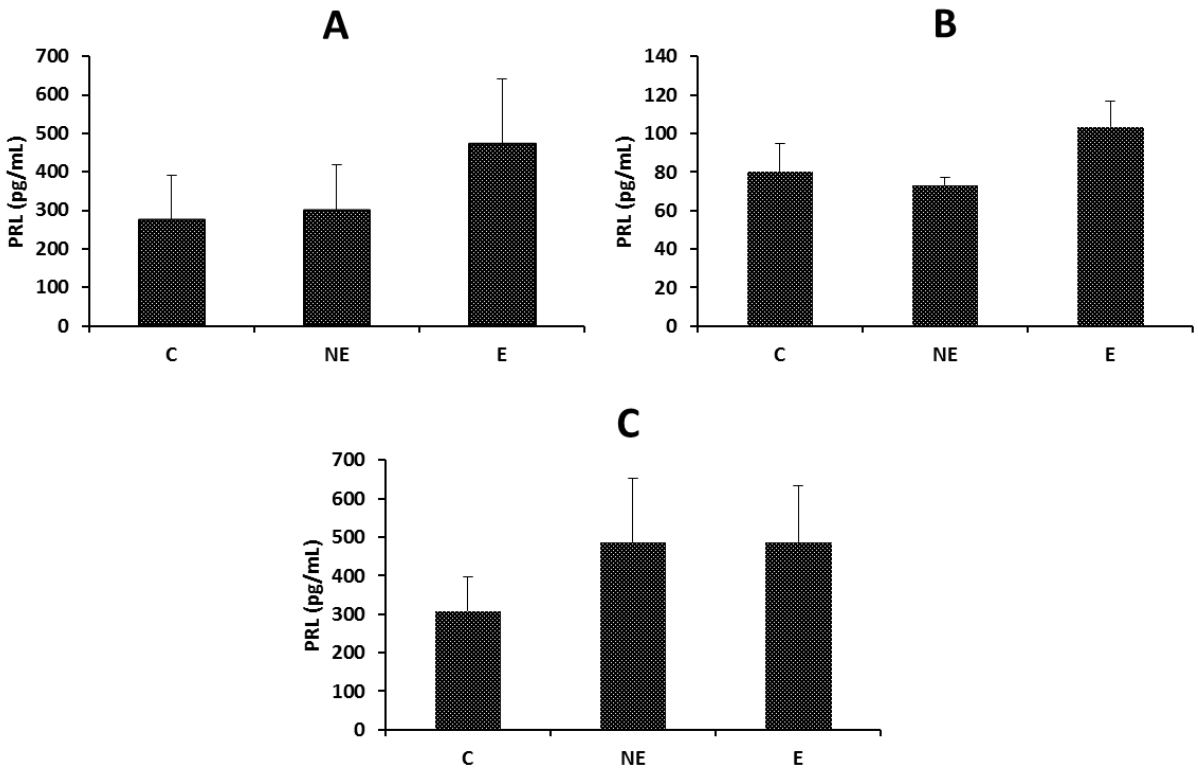


Figure 15: Total PRL measured in medium after 24 hour stimulation with 100nM NE or E in A) M0 MDMs B) M1 MDMs and C) M2 MDMs

Much of the variance noted in the total PRL protein from the Figure 15 results actually took place at a basal level in the controls. We observed rather large differences in the basal PRL output from trial to trial that could have been due to a number of factors including different

numbers of MDM's originally seeded on the plate and natural variations in FBS used to culture the MDMs. Because of this, fold increase in PRL protein output showed more consistent data as, regardless of the basal level observed in the controls, the increase from adrenergic hormone treatment was rather consistent (Figure 16). Such analysis brought many of the data points much closer to significance although the low sample number still made the benchmark of p-value 0.05 difficult to obtain.

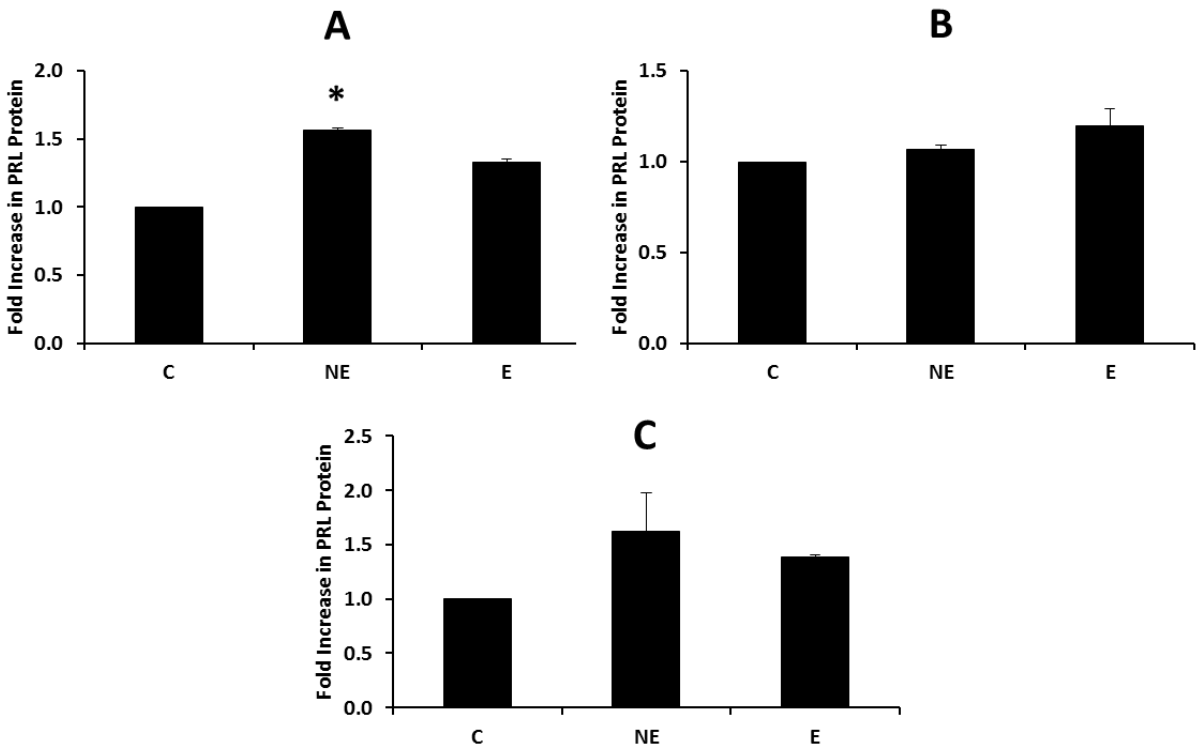


Figure 16: Fold increase in PRL measured in medium after 24 hour stimulation with 100nM NE or E in A) M0 MDMs B) M1 MDMs and C) M2 MDMs

Other factors that affect the PRL response

During our experimentation, treatment with adrenergic hormones consistently resulted in an increase in PRL expression; however, the degree to which expression increased varied considerably. Some trials increased as little as 1.1 fold while other responded with over 40 fold increases in expression. Seeing as this is a newly established response, we wanted to investigate whether any of our culture or handling conditions could have an effect on the PRL response. Altering many culture conditions such as temperature, FBS lots, confluence at time of experiment, and passage number presented no clear and repeatable effects on the PRL gene or the response to adrenergic hormones. However, the presence of serum greatly affected the degree to which monocytes responded to adrenergic hormones. Although cells would respond under serum free conditions, running the same experiment without starving cells resulted in an almost 4 fold greater induction of the PRL gene at 6 hours. (Figure 17)

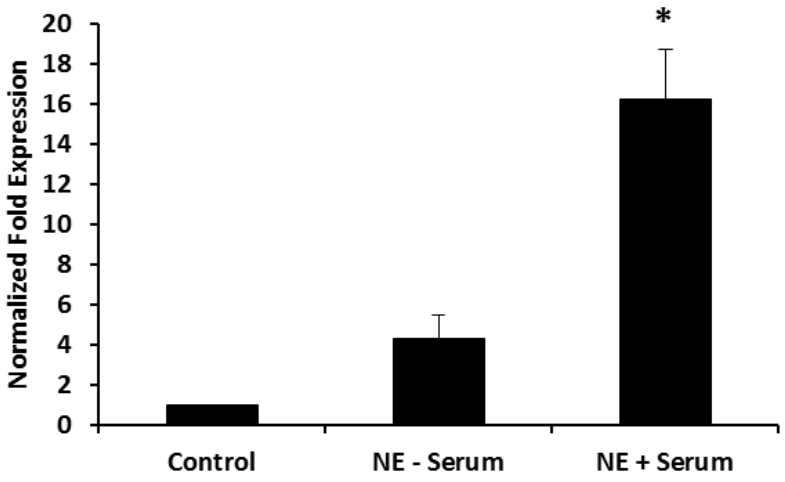


Figure 17: PRL gene expression in THP-1 monocytes at 6 hours with and without serum

Discussion

Overall, our results clearly show that ePRL expression increases as monocytes differentiate into MDMs and that adrenergic hormones are able to increase ePRL expression to some degree in both. These two findings alone could have important implications in how macrophages can influence overall adipose tissue fitness and function.

As previously mentioned, while the specific actions of PRL on adipocytes have been somewhat muddled and at times conflicting depending on the methods used, there is a clear consensus that PRL does indeed influence adipocyte biology. Some of this confusion could be

brought about by the fact that much of the work up till now has only considered PRL as an endocrine hormone derived entirely from the pituitary. While this assumption may hold true in mice, it neglects an important source of the hormone in humans.

This fact is particularly important when considering our data showing a large increase in PRL expression as monocytes differentiate into macrophages. An influx of MDMs into the adipose tissue is a hallmark of obesity. Even when not accounting for other stimulatory factors, this increase in basal PRL production alone could cause drastic changes in adipose physiology via paracrine signaling. Further, it is likely this signaling could be overlooked entirely if only monitoring serum levels of the hormone. Human PRL has been shown to have heparin binding properties[107] and seeing as adipose tissue is comprised of large amounts of heparin sulfate proteoglycan rich connective tissue, which has been shown to trap growth factors with such properties[108], it is most likely that PRL produced at such sites would accumulate there with only a smaller percentage leaking into circulation.[109] Thus, macrophages ePRL could be exerting far greater effects on adipose tissue than what is reflected in serum levels.

Secondly, our results show that ePRL expression is increased in the presence of adrenergic hormones. Seeing as adrenergic hormones are primary signaling molecules for lipolysis in adipose tissue and that PRL has been shown to exert anti-lipolytic effects on adipocytes, these results suggest a possible opposing role between the two signals. ePRL released in response to normal adrenergic signaling could result in a dampening of the signal.

This could lead to less efficient lipid metabolism in the human system overall as lipids are able to be stored in adipocytes but are not able to be effectively mobilized into the bloodstream during times of high energetic demand.

Our results on how macrophage polarization effects ePRL expression are less clear. While M0 and M2 macrophages appear to have generally similar ePRL regulation, M1 macrophages show significantly higher basal gene expression while at the same time showing lower production of the actual protein. This could be for a variety of reasons. For one, mRNA levels do not always accurately reflect protein production and although M1 macrophages show higher levels of transcription of the gene, translation of the mRNA to a protein could be stalled by another mechanism. Secondly, consumption of PRL could be increased in M1 macrophages to the point where it overtakes an increased production rate. And thirdly, it is possible that M1 macrophages have mechanisms to degrade PRL. As mentioned earlier, cleavage of PRL results in a bioactive 16kDa fragment referred to as vasoinhibin. It is possible that M1 macrophages express one of the proteases necessary to cleave PRL at high levels into vasoinhibin or other fragments. Because ELISAs rely on antibody recognition of a specific epitope in the protein, such alterations could render PRL unrecognizable by the assay, thus reporting much reduced levels. This second possibility in particular could have important biological implications in itself and is worthy of further exploration.

It is also interesting to note that the presence of serum played a significant role in the magnitude of the ePRL response to adrenergic hormones. It is unknown whether an important cofactor in the signaling process is present in the medium. It is also possible that the nutritional status of the monocytes and MDM's may play a large role in the magnitude of the response and that the fasting state could downregulate this signaling.

For now, these conclusions are speculative and further work will need to be done to determine if these signaling responses play out in vivo and to what degree such responses influence human physiology. If so, this could open up an avenue of new diagnostics and potential treatment targets. Understanding how hormones are regulated within the very tissues they exert their effects is of vital importance when treating patients suffering from such hormone imbalances and understanding such complex signaling is a necessary first step in developing drugs and therapeutics that can help alleviate such imbalances.

CHAPTER THREE: REGULATION OF PRL IN MONOCYTES AND MACROPHAGES BY ADIPOSE STEM CELLS

Materials and Methods

Reagents

Transwell polyester membrane cell culture inserts of 12mm diameter and 0.3 µm pore size were purchased from Corning Inc. (Corning, NY). DMEM:F12 and Hank's balanced salt solution (HBSS) were purchase from Life Technologies (Carlsbad, CA). Amicon ® Ultracel 3kDa 2mL Centrifugal Filters were purchased from Millipore Sigma (Burlington, MA). All other materials are same as mentioned in Materials and Methods from Chapter 2.

Cell Culture

Adipocytes were modeled using the Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocyte cell line kindly provided by the Dr. Martin Wabitsch lab at the University Clinic for Paediatrics Ulm. Cells were maintained in DMEM:F12 and differentiated via the protocol provided by Dr. Wabitsch based on his previous publications.[110,111] The mediums referred to are detailed in Table 1.

Table 1: Assorted SGBS mediums and their components

Ingredient	Final Amount/Concentration
0F medium (serum-free basal medium)	
DMEM:F12	To 500mL
Pantethenate	17uM
Biotin	33uM
P/S	5mL
Serum-containing medium (0F + 10% FBS)	
0F medium	To 500mL
FCS	50mL
Freezing Medium	
0F medium	8mL
FBS	1mL
Glycerol	1mL
3FC Medium	
0F Medium	To 500mL
Transferrin	0.01 mg/mL
Insulin	20nM
Cortisol	100nM
T3	0.2nM
Quick-Diff Medium	
0F Medium	To 500mL
Transferrin	0.01 mg/mL
Insulin	20nM
Cortisol	100nM
T3	0.2nM
Dexamethasone	25nM
IBMX	250uM
Rosiglitazone	2uM

SGBS preadipocytes (SGBS-PA) were maintained in serum-containing medium for routine growth and maintenance. For differentiation protocol, SGBS-PA were handled as follows:

Cells were allowed to grow to near confluence (~80-90%) in 12 well plates. Cells were then washed with PBS and Quick-Diff medium was added. Cells incubated in Quick-Diff medium for 4 days. At day 4, Quick-Diff medium is removed and replaced with 3FC medium (cells are not washed between mediums). Cells are then cultured in 3FC medium for at least 8, and no more than 12 days with 3FC medium changed every 4 days. In most cases, differentiation took place over exactly 12 days.

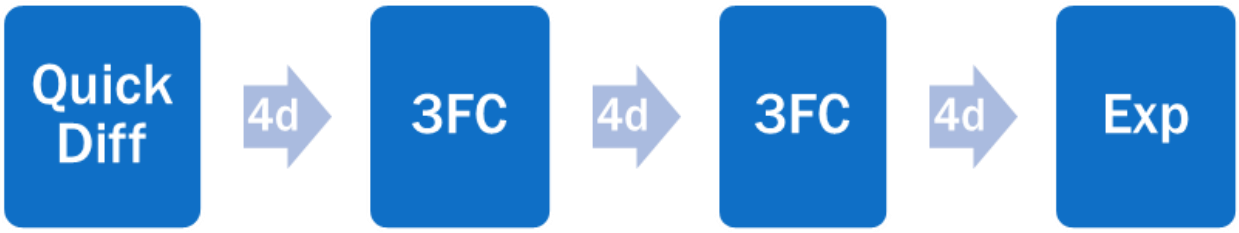
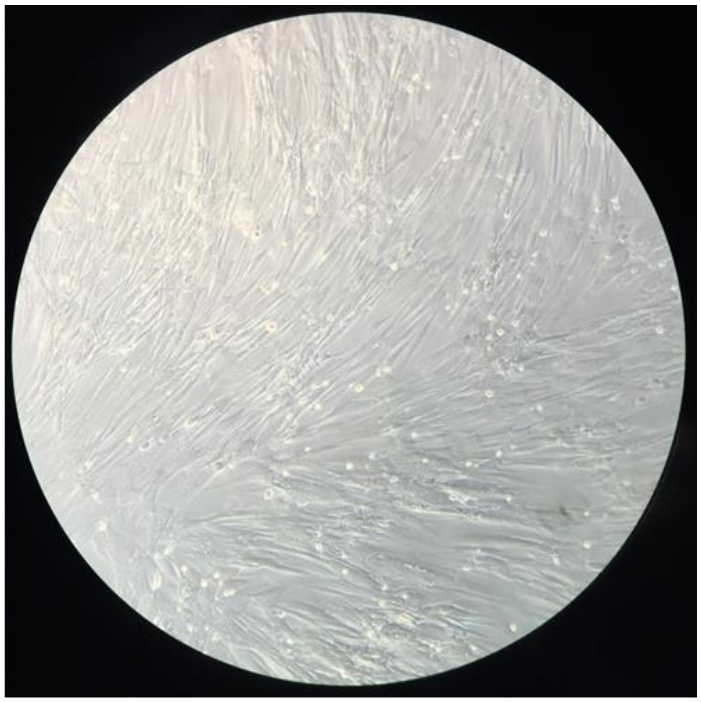


Figure 18: Flow chart of adipocyte differentiation protocol

Differentiation of SGBS-PA to mature adipocytes (SGBS-MA) is a clearly visible process as lipid droplet formation is a key step in adipogenesis and the lipid droplets are clearly visible by light microscopy, even without the use of stains.

A



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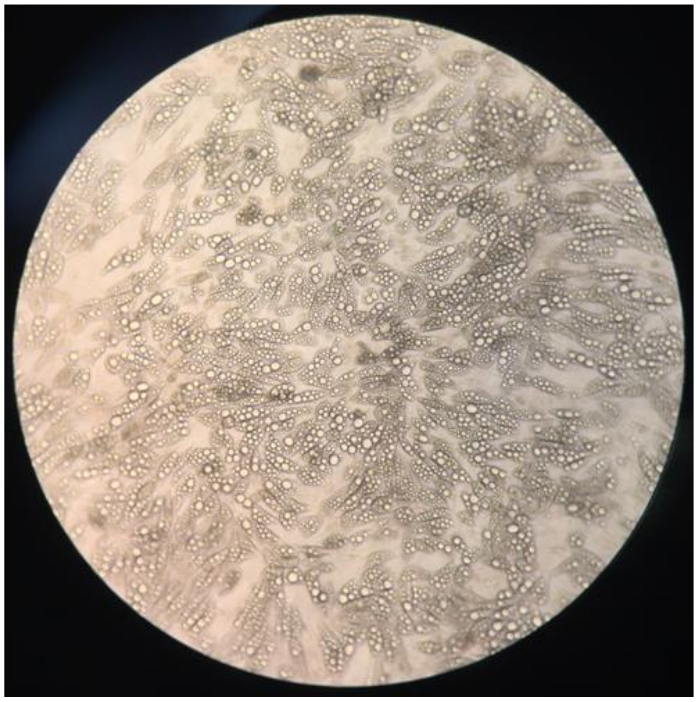

Figure 19: A) SGBS-PA B) SGBS-MA

While lipid droplets are easily identifiable under normal unstained conditions, we also performed Oil Red O staining to confirm that they were in fact lipid droplets consisting of neutral lipids (Figure 20.) In this particular experiment, there was a lower level of differentiation so undifferentiated SGBS-PA are clearly visible at the top, unstained by the Oil Red O.

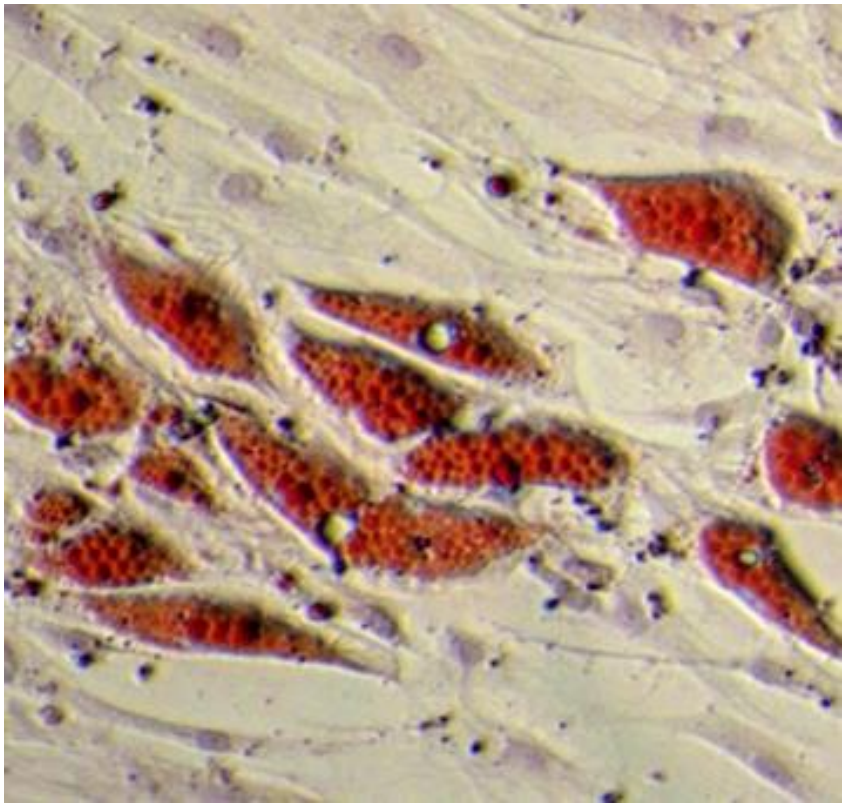

Figure 20: Oil Red O stain of differentiated SGBS adipocytes

Lastly, although differentiation appeared clearly visible based on changes in morphology alone, we measured expression of key genes associated with adipocyte differentiation.

Adiponectin, glucose transporter type 4 (GLUT4), and LPL all showed substantial increases in expression, in line with complete adipocyte differentiation. (Figure 21)

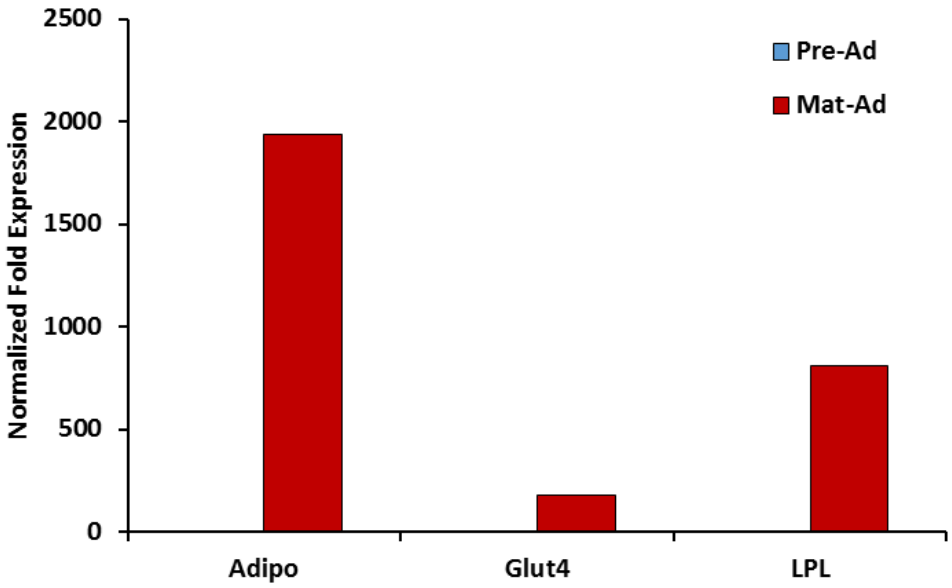

Figure 21: Primary markers of adipocyte differentiation

We also measured other genes typically associated with adipocyte differentiation which all showed increases in expression through differentiation. Two housekeeping genes showed no significant change in expression. GAPDH was used to normalize genetic expression. (Figure 22)

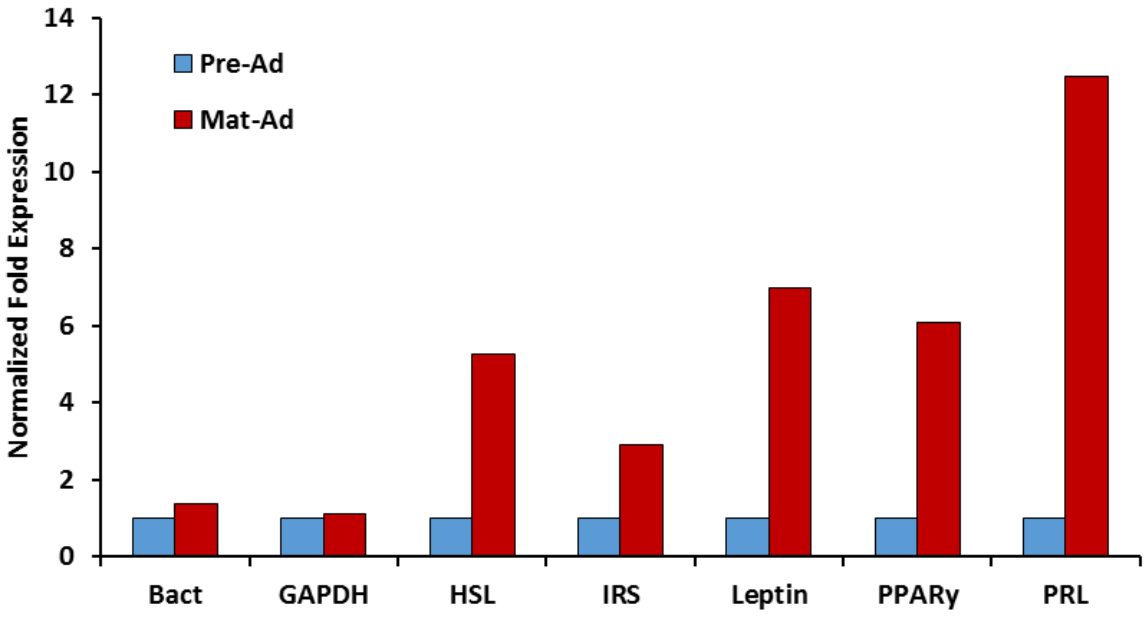

Figure 22: Genes relevant to adipocyte differentiation

Coculture methods

Cocultures were performed in two ways. The primary coculture method used 12-well plate transwell chamber inserts. SGBS-PA and SGBS-MA were cultured in standard 12-well plates for experimentation. THP-1 cells were then loaded into the upper chamber, inserted into the appropriate well, and allowed to interact for 24 hours. In the case of coculture with macrophages, THP-1 monocytes were differentiated onto the transwell chamber inserts using the aforementioned protocol and then placed into coculture (Figure 23).

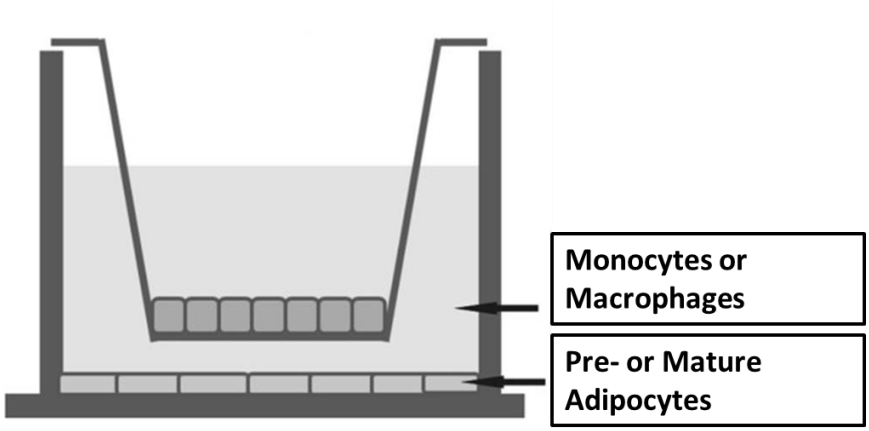

Figure 23: Depiction of transwell coculture system

In the case of SGBS-PA/monocyte coculture, a direct coculture was also possible. SGBS-PA were cultured to confluency in a 12-well plate and then monocytes were added for 24 hours. Monocytes are a suspension cell and were easily washed off of the firmly adhered SGBS-PA and taken for analysis. SGBS cells from these experiments were not analyzed do to difficulty of removing all THP-1 contaminants. This method of coculture was only used to see if direct contact was necessary for signaling, once it was confirmed that direct contact was not necessary, direct coculture was no longer used as it was not directly comparable across all cell to cell interactions.

Conditioned medium was prepared by rinsing SGBS cells with serum free DMEM:F12 two times and then culturing cells with serum free DMEM:F12 for 24 hours. Starvation of SGBS cells was performed by continuing this serum free culturing for the indicated length of time,

changing medium every 4 days. Starved SGBS conditioned medium was obtained by applying fresh medium for 24 hours at the indicated number of days.

Results

SGBS pre-adipocytes stimulate PRL expression in THP-1 monocytes under normal culture conditions

Once the SGBS cell line was established in our laboratory, the first set of experiments performed were direct coculture using THP-1 monocytes and SGBS-PA. After a 24-hour coculture, monocytes were removed and ePRL expression was measured via qRT-PCR. Monocytes from coculture consistently showed about a 30-fold increase in PRL expression over controls. This increase in PRL expression did not appear greatly affected by the number of SGBS cells in coculture as SGBS confluency from 50%-100% was tested with little difference in the increase in PRL expression at 24 hours. (Figure 24)

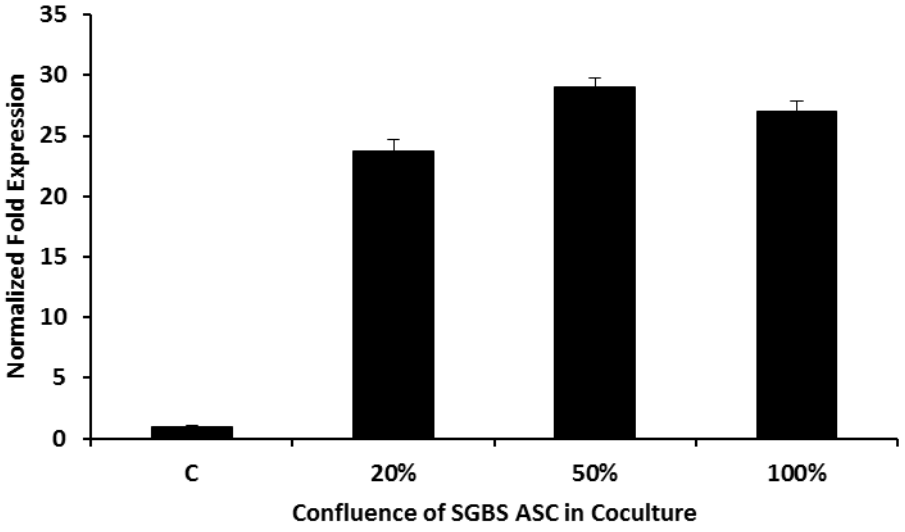

Figure 24: PRL gene expression in monocytes cocultured with SGBS-PA

SGBS cells also express the PRL gene although at far lower basal levels than THP-1 monocytes. Originally, it appeared as if coculture with monocytes also increased PRL expression in SGBS pre-adipocytes; however, upon further experimentation this appeared to be due to monocyte contamination in the SGBS sample. While monocytes can be cleanly removed from the SGBS cells adhered to the bottom with gentle pipetting, many stay behind contaminating the SGBS sample. For this reason, only monocytes were analyzed in direct coculture experiments.

SGBS-PA stimulate PRL in THP-1 monocytes in transwell coculture and with SGBS conditioned medium

To test whether PRL is being stimulated through a contact dependent mechanism we proceeded to indirect cocultures via transwell inserts as described above. This allows cells to signal back and forth through a porous membrane, but not to come into direct contact with each other. After a 24 hour coculture we again observed an almost identical increase in PRL expression in monocytes lending us to believe that the response is likely triggered by a factor or factors released into the medium. (Figure 25)

Lastly, we wanted to investigate whether continuous crosstalk between monocytes and SGBS-PA was necessary for the ePRL stimulating response or whether unstimulated pre-adipocytes constitutively altered the media in a way that caused such a response. This was done by allowing SGBS-PA to condition serum free medium for 24 hours and then subsequently exposing THP-1 monocytes to this "conditioned medium" for 24 hours. SGBS-PA conditioned medium (PA-CM) was able to significantly increase PRL expression in THP-1 monocytes and almost identical increases in PRL expression were observed regardless of the method used. These results show that whatever factor increases PRL expression in the monocytes is constitutively expressed by preadipocytes and does not require interaction with the monocytes on the preadipocytes side.

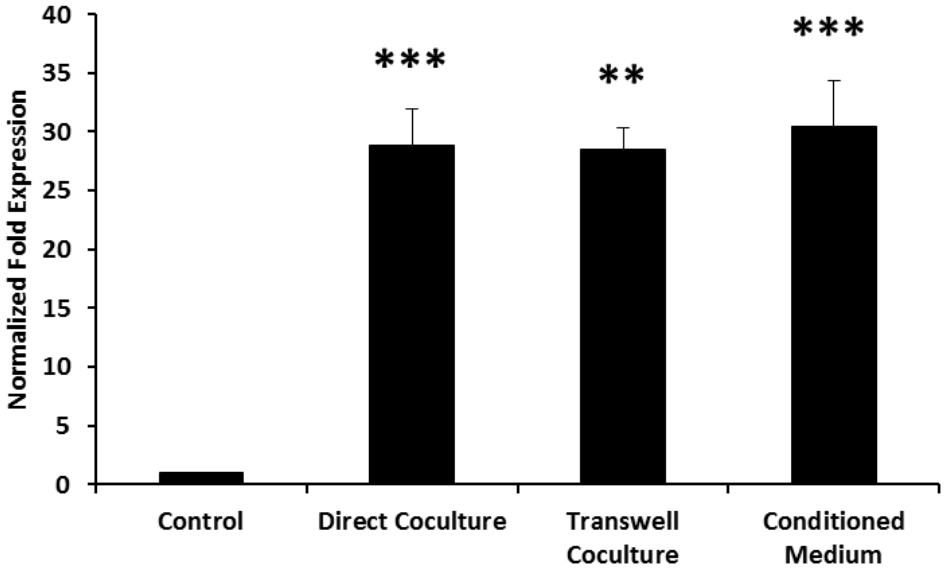

Figure 25: PRL gene expression in monocytes exposed to SGBS-PA through different methods

We were also able to determine a timeline for PRL gene response. PA-CM treatment showed the greatest increase in PRL gene expression early on at 6 hours (Figure 26). Such a robust response within 6 hours leads us to believe that PRL is directly stimulated by PA-CM and not an eventual target of some downstream cascade. Increases in PRL gene expression were noted as early as 2 hours. (Data not shown)

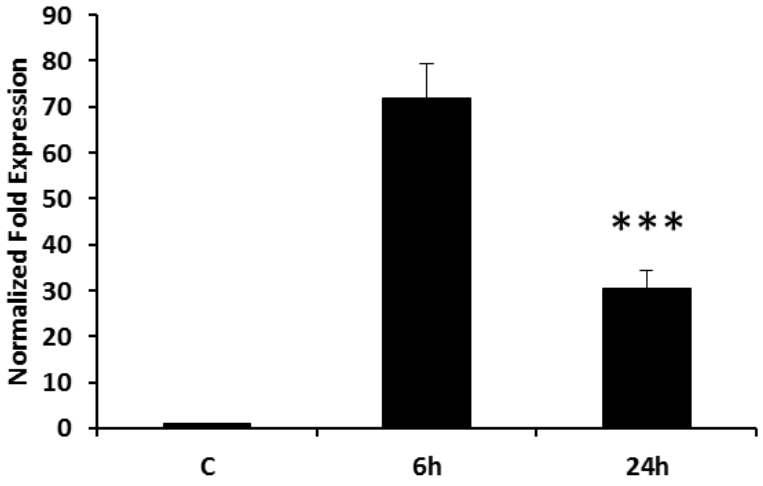

Figure 26: PRL gene expression in monocytes exposed to PA-CM at different time points

A decrease in TNFa expression is observed alongside PRL stimulation from CM

There have been many reports showing that ASC have capacity to suppress inflammation. While undifferentiated SGBS cells are typically referred to as "pre-adipocytes", they also fall under the category of ASC and have been used as a model in studies investigating ASC secretomes.[112] With this in mind we decided to investigate whether TNFa expression, a classical marker of inflammation, had also been effected by our PA-CM treatments. We found that in all cases where PRL had been upregulated in both monocytes and macrophages TNFa was downregulated. Further, the degree to which TNFa was downregulated seemed to correlate with the degree to which PRL was upregulated. Experiments with higher induction of PRL also saw greater downregulation of the TNFa gene.

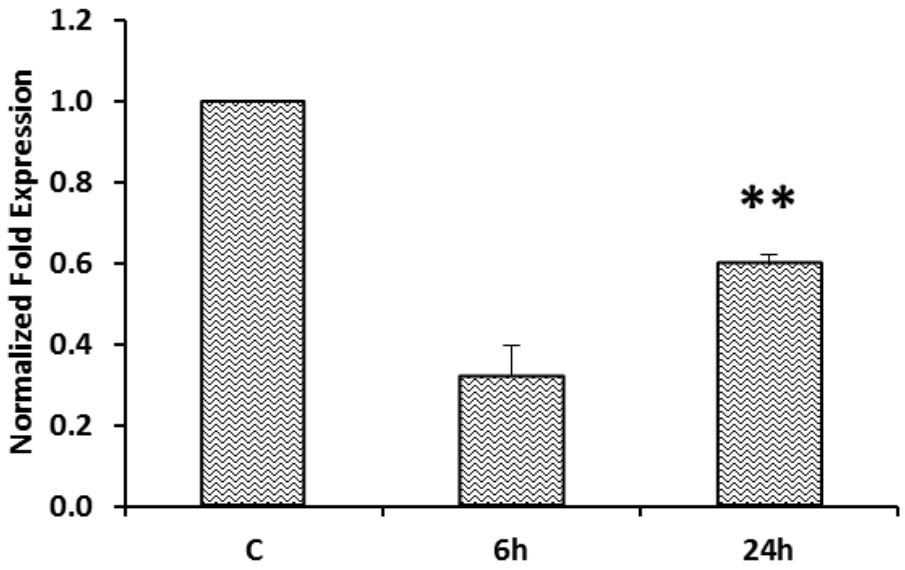

Figure 27: TNFa gene expression in monocytes treated with PA-CM

MDMs treated with PA-CM show increase in ePRL expression and decrease in TNFa expression

Our previous work showed that macrophages have an increased basal level of PRL. In order to investigate whether SGBS-PA were able to influence this already increased basal PRL expression, the above experiments were performed using THP-1 MDM differentiated via the protocol outlined in Chapter 2. Here we see that ePRL gene expression does increase in MDM's although only about a third of the fold expression increase seen in monocytes.

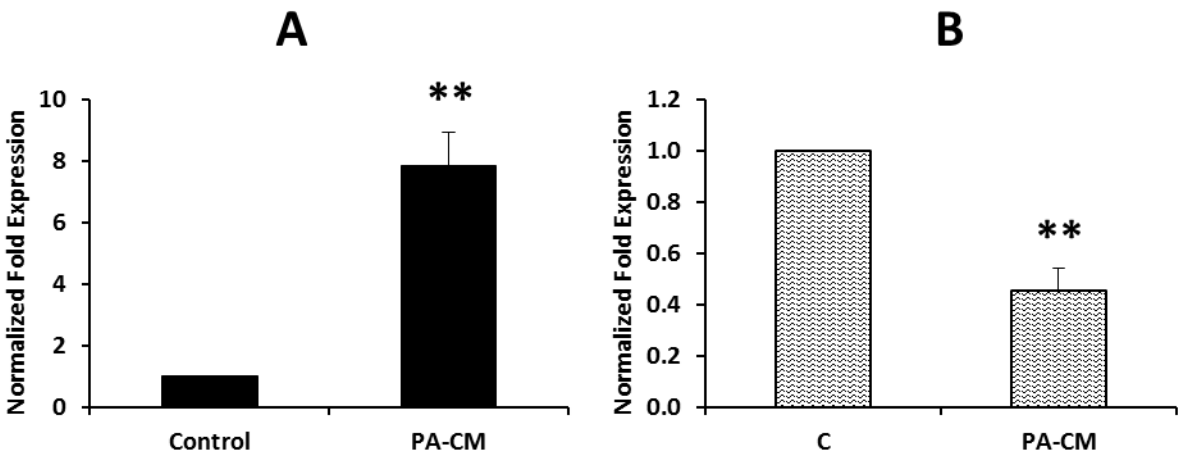

Figure 28: A) PRL and B) TNFa gene expression in MDMs after 24 hours

PRL protein production mirrors gene expression in monocytes and MDMs treated with PA-CM

To measure whether or not protein production follows the expression patterns observed above, we took medium from three PA-CM treated monocyte experiments and three PA-CM treated MDM experiments and compared them against their controls from the same experiment. We also tested PA-CM itself for PRL and although there have been reports of adipocytes producing ePRL de novo[113], we were unable to measure any PRL in the medium, allowing us to assume that all PRL detected from these experiments would be monocyte and MDM derived.

We see a clear increase in ePRL protein production from both monocytes and MDMs exposed to PA-CM. Shown together, it is also easier to appreciate that while treated MDMs do not show as drastic of a fold increase in PRL expression when compared to treated monocytes, their overall increase in ePRL protein output is quite substantial, resulting in almost 10x more protein being secreted in the same time period.

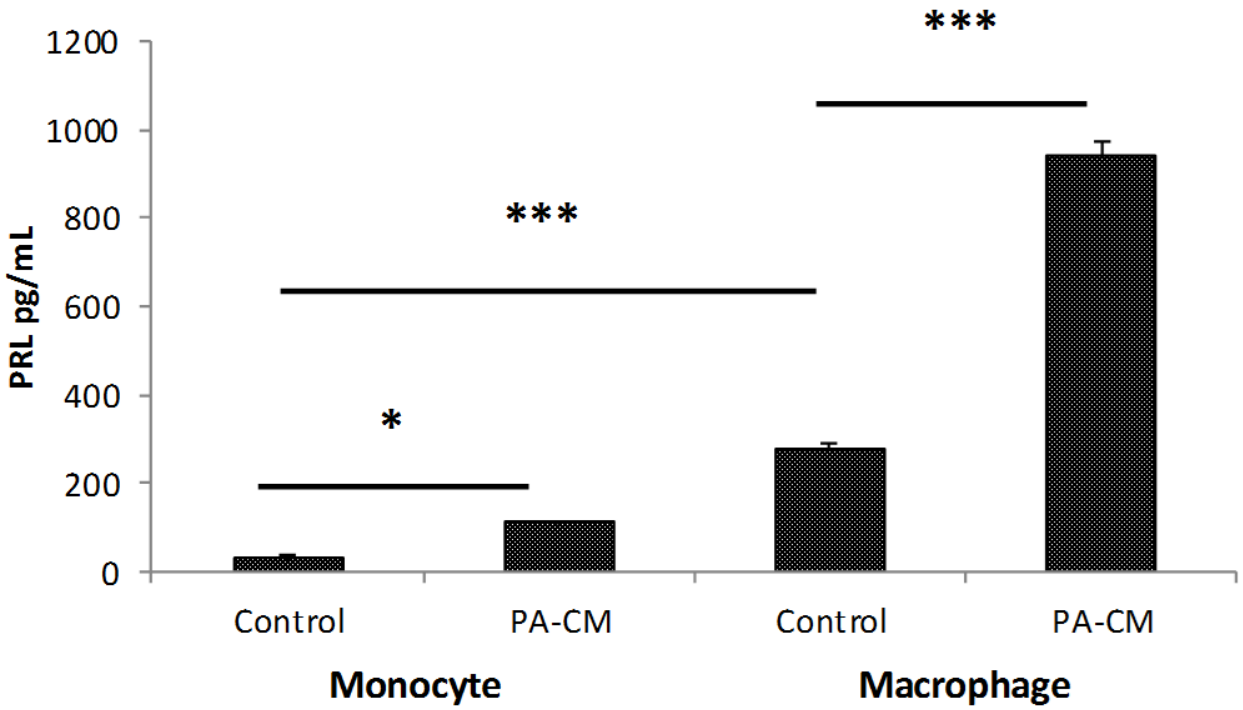

Figure 29: ELISA data for monocyte PA-CM treatment (left pair) and MDM PA-CM treatment (right pair).

SGBS preadipocytes lose their ability to stimulate PRL in monocyte/MDM when differentiated to mature adipocytes.

In order to study our initial aims of understanding how monocyte/macrophage derived PRL may influence adipocyte biology we differentiated the SGBS-PA into mature adipocytes (SGBS-MA) and repeated the transwell coculture and conditioned medium experiments. Differentiation percentages were consistently around 90% meaning that some undifferentiated preadipocytes remained in the culture system although at minor levels.

To our surprise, SGBS-MA no longer retained the ability to increase PRL expression in either monocytes or MDMs in either coculture or via conditioned medium. In fact, in some trials PRL gene expression was actually decreased compared to that of controls. This failure to increase PRL gene expression was reflected in the medium as no significant change in PRL protein concentration was measured for monocytes or macrophages treated with CM from mature adipocytes. (Figure 30)

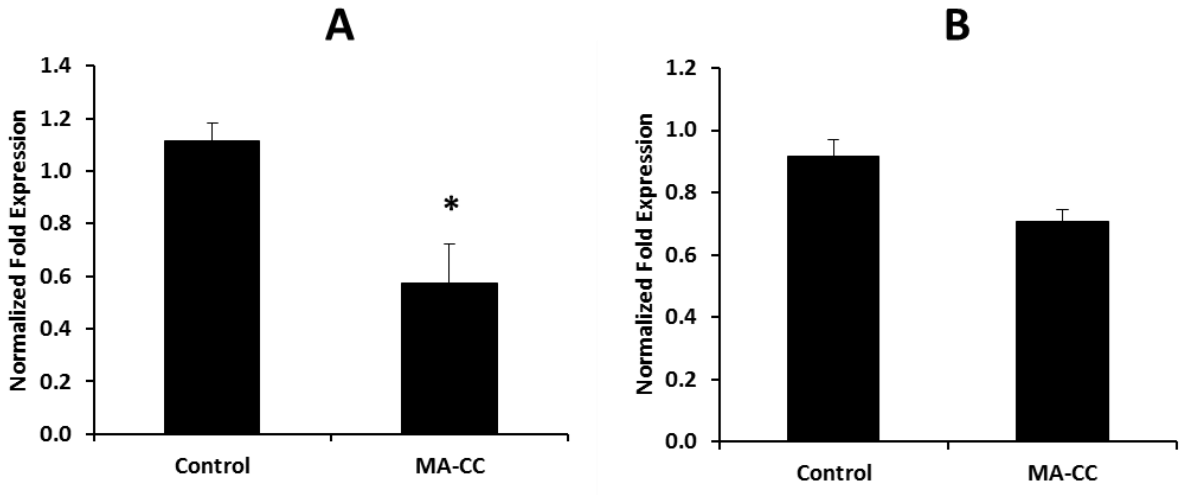

Figure 30: A) PRL gene expression in monocytes cocultured with MA. B) PRL gene expression in MDMs cocultured with MA.

Serum starved preadipocytes lose their ability to increase PRL expression in monocyte/macrophage

The differentiation process for the SGBS preadipocytes requires the cells be serum starved during the 12 day period of differentiation. In order to provide a more appropriate comparison between our SGBS-PA results and SGBS-MA studies, preadipocytes were also serum starved for 12 days and used in parallel with SGBS-MA. DMEM:F12 is designed to promote cellular survival even in the absence of serum and SGBS-PA showed no sign of stress or apoptosis even when serum starved for periods longer than 1 month. However, serum starved

preadipocytes did cease to proliferate and a slight decrease in size was evident that became more noticeable with longer starvation time periods.

Surprisingly, preadipocytes starved for 12 days no longer retained any of their previous ability to increase PRL expression in monocytes and macrophages in coculture or via CM. (Figure 1)

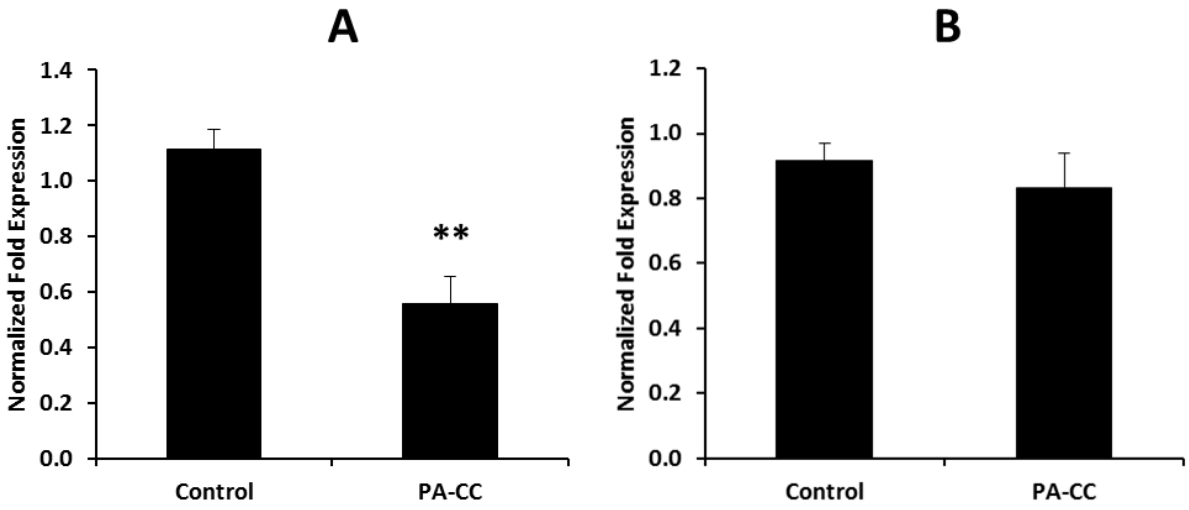

Figure 31: A) PRL gene expression in monocytes cocultured with starved PA. B) PRL gene expression in MDMs cocultured with starved PA.

Since our CM itself is produced with serum free medium and SGBS-PA are still releasing the unknown factor within day one of serum starvation, we wanted to measure how different durations of serum starving affect SGBS-PA ability to condition the medium. We measured the

effect of the CM from 0 day (0d), 1d, 4d, 8d, and 12d starved SGBS-PA and immediately noticed a significant drop in the cells ability to stimulate PRL in monocytes after just 1 day starvation. (Figure 32) Despite this immediate decrease, the PA-CM did maintain the ability to stimulate PRL expression in monocytes even after multiple days of serum starving, though in general, the greater the time of serum starvation, the lesser the ability of the CM to stimulate PRL in the monocytes.

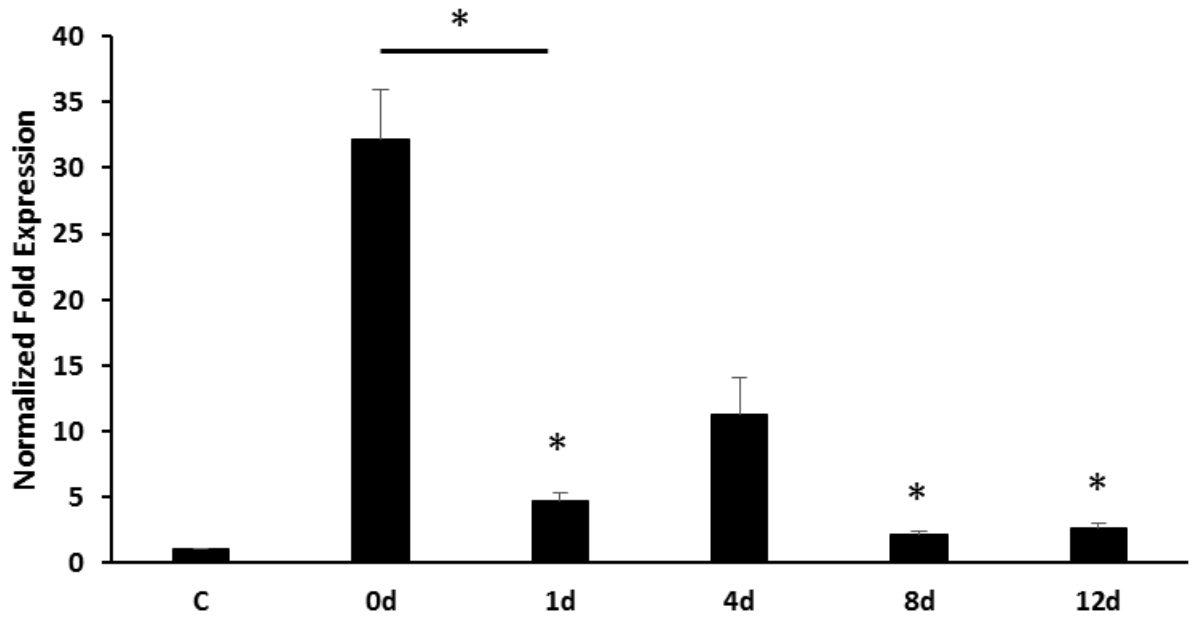

Figure 32: PRL gene expression in monocytes treated with CM from PAs serum starved for the indicated number of days

Interestingly, we again noticed TNFa gene expression indirectly correlating with PRL gene expression. Longer starvation of the SGBS-PA amounted to an attenuated ability to lower TNFa expression.

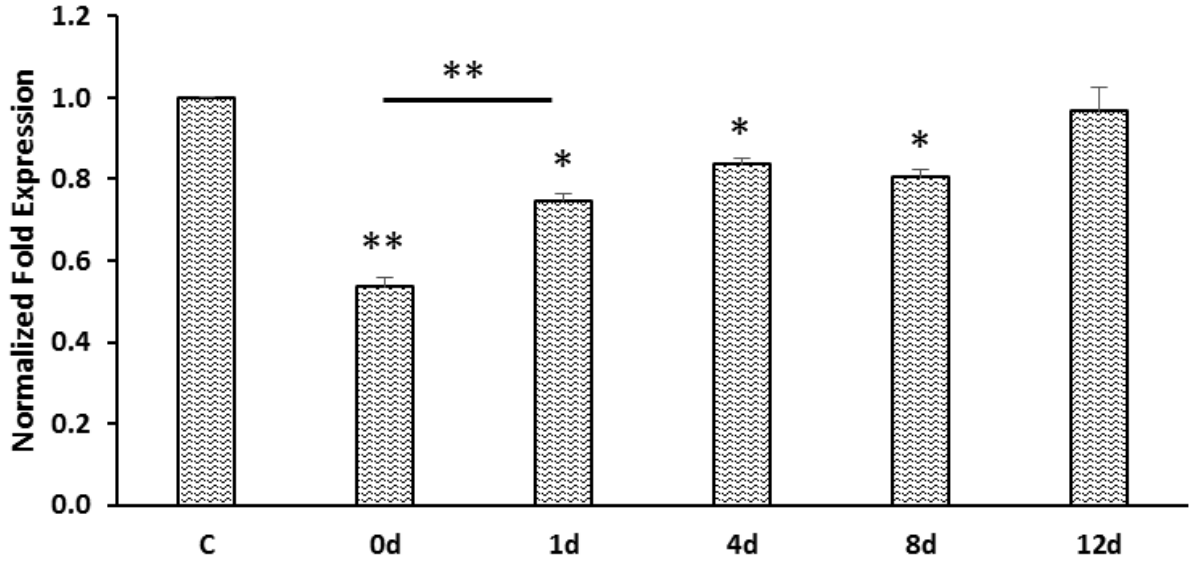

Figure 33: TNFa gene expression in monocytes treated with CM from PAs serum starved for the indicated number of days

<u>Unknown factor stimulating PRL in monocytes and macrophages is under 3kDa</u>

Our original intent was to use mass spectrometry to help identify the possible factor transferring the PRL stimulation signal from preadipocytes to monocyte/macrophage. Our mass spectrometry setup allowed analysis of molecules under 3kDa in mass. In order to explore the

feasibility of using this method for analysis we ran PA-CM from proliferating preadipocytes through 3kDa centrifuge filters. After centrifugation, a low molecular weight (LMW) fraction was isolated as wash-through. The high molecular weight (HMW) fraction consisted of the portioned retained by the filter, resuspended in serum free medium. This fraction was obtained by running the medium in the opposite direction through the filters as to collect and resuspend the larger molecular weight components, such as most proteins, filtered out. These two fractions were then compared in their ability to stimulate PRL.

The LMW fraction maintained much of its ability to stimulate PRL in monocytes although slightly reduced. The HMW fraction maintained very little if any of its ability to stimulate PRL (Figure 34). The slight stimulation seen from the HMW fraction is likely due to LMW factors left over after filtration, as complete filtration was not feasible using these filters. From 2mL of starting CM, about 200uL was consistently left in the upper chamber after filtration and trials with a more complete filtration showed lower induction from the HMW fraction. However, we cannot rule out that whatever factor being released could be around 3kDa, a common size for many peptides, thus resulting in incomplete filtration.

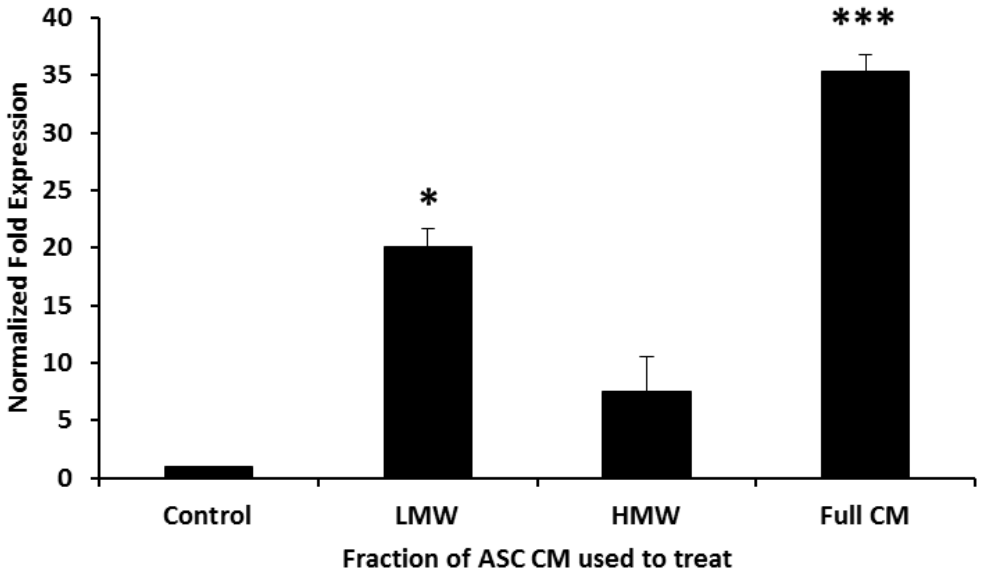

Figure 34: Effect of different PA-CM fractions on monocyte PRL expression

Complete medium is required for transferring PRL stimulating signal

In order to gain a better understanding of what type of factors could be influencing signaling from SGBS-PA to monocytes and MDMs, we repeated our experiments using HBSS. Small molecule biosynthesis frequently relies on a steady supply of precursors from the medium so by minimizing medium contents to only salts and buffers, we would expect to see a reduction in the ability of SGBS-PA to condition the medium.

Using HBSS as the culture medium resulted in a drastic decrease in the ability of SGBS-PA to condition the medium; however, some stimulation ability was retained and just over a 2

fold increase in ePRL expression was observed in monocytes upon exposure to HBSS PA-CM (HCM). This was in comparison to about a 25-fold increase in expression for monocytes and a 10-fold increase in MDMs treated with DMEM:F12 conditioned medium (DCM). Another interesting effect of HBSS in these experiments is that it resulted in 2 and 3 fold decrease in basal ePRL expression in monocytes and MDMs respectively. (Figure 35)

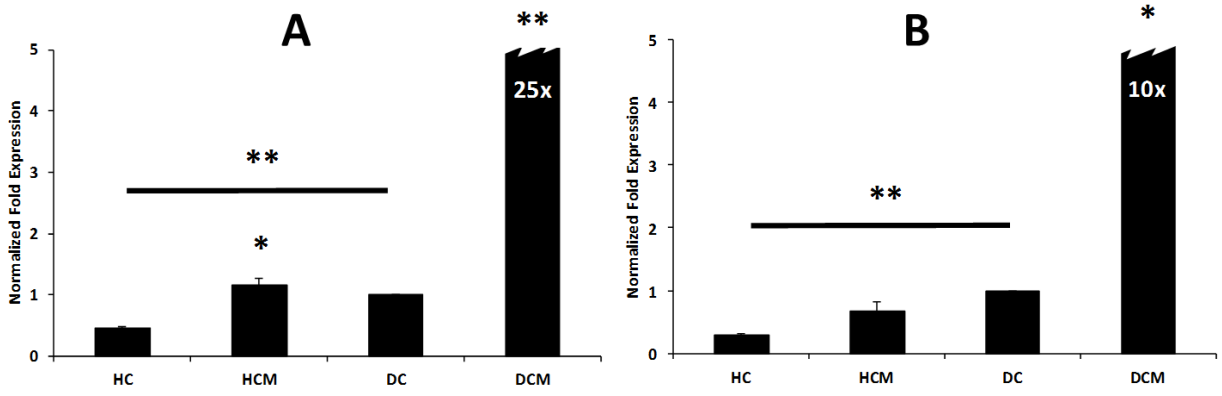

Figure 35: PRL gene expression in A) monocytes treated with PA-CM B) MDMs treated with PA-CM

Next we wanted to narrow down possible pathways involved in SGBS-PA signaling by returning selected amino acids to the medium and seeing if it rescued the SGBS-PA ability to condition HBSS. Our first goal was to investigate whether ePRL regulation from SGBS-PA could be through adrenergic hormones, a mechanism we showed previously, or by some other

catecholamine pathway. For this, we made a media formulation with aromatic amino acids (Aro) that contained tryptophan, phenylalanine, tyrosine, histidine, and also cysteine. Tyrosine and phenylalanine are precursors to most all catecholamines and tryptophan is a precursor to serotonin, another more distant member of the catecholamine group. If the SGBS-PA were signaling via these molecules or their byproducts, we would expect to see an increase in the ePRL stimulating ability of the CM by providing these precursors. We also tested amphipathic amino acids (Amp) containing lysine, arginine, proline, and also cysteine. These amino acids are important mediators of a variety of metabolic pathways.

Addition of our selected amino acids yielded negative results as neither aromatic or amphipathic amino acids were able to rescue PA-CM conditioning ability. This line of testing was continued using a variety of other amino acid concoctions (data not shown), none of which were able to return more than a 4 fold response to PA's ability to condition HBSS. (Figure 36)

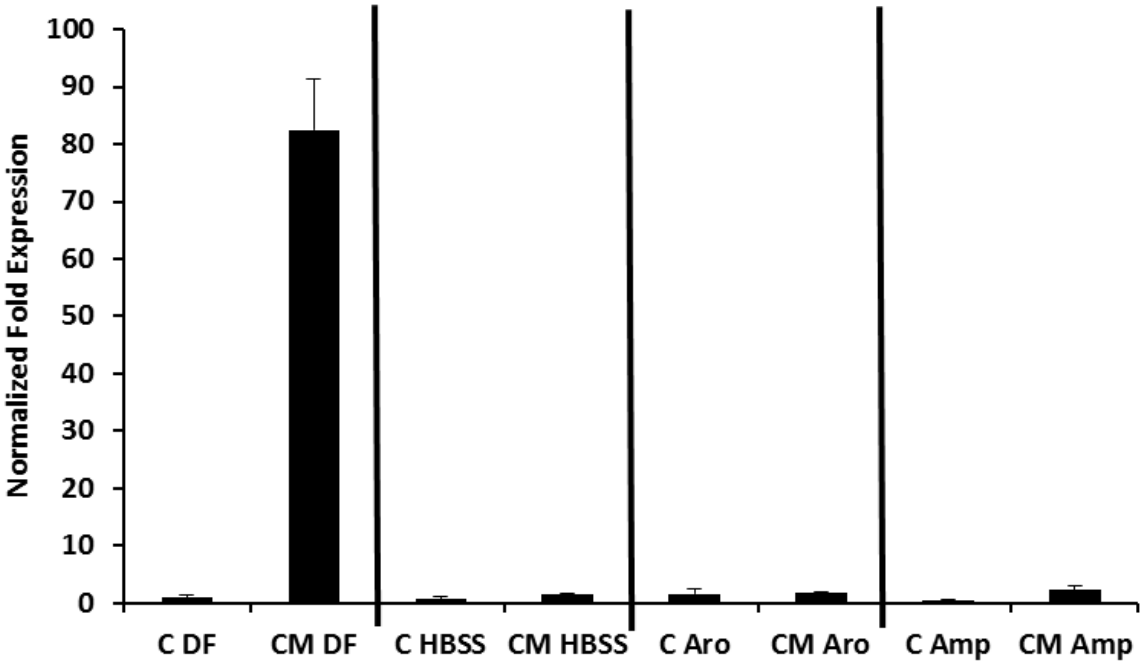

Figure 36: PRL gene expression in monocytes treated with PA-CM of different basal medium formulations

To make sure that HBSS as culture medium did in fact cause the ablation of SGBS-PA ability to stimulate ePRL in monocyte/MDM, we also ran ELISA to determine PRL protein content in the medium of the trials. Protein levels reflected the genetic expression changes with monocytes having no detectable protein in either control or treated and MDM's having markedly reduced levels compared to those in DMEM:F12. MDM protein data however, does reflect the finding that some stimulative ability is maintained in HBSS after 24 hours, although this is also reduced.

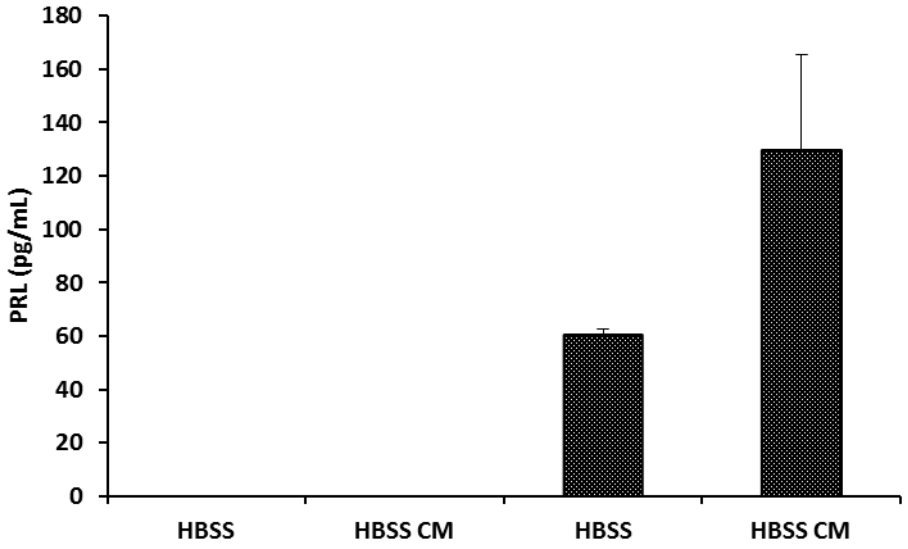

Figure 37: PRL protein content in medium of monocytes and MDMs treated with HBSS PA-CM

CM treated monocyte gene array

For the monocyte vs. PA-CM treated monocyte gene array we used the same cells from the original ELISA experiments that showed the increase in secreted PRL into the medium after 24 hour PA-CM treatment. The RNA was extracted in house and sent to Qiagen to perform the service. Seeing as few studies have been done in respect to immune production of ePRL, we

selected a wide reaching array covering genes from the following signaling pathways: TGFβ, WNT, NFκB, JAK/STAT, p53, Notch, Hedgehog, PPAR, oxidative stress, and hypoxia.

Upon initial review we noticed a variety of genes significantly up regulated (Table 1) and downregulated (Table 2) in CM treated monocytes. Some of the most heavily upregulated (over 2 fold increase) genes include: PPARD, ICAM1, LRG1, SOCS3, BTG2, FAS, LFNG, and FTH1. While genes from many of the pathways are both upregulated and downregulated, it appears that overall genes from STAT-induced, p53, Notch, and Oxidative pathways seemed generally upregulated.

Table 2: Genes significantly upregulated in monocytes treated with ASC CM

Gene	Fold Change	P-value	Pathway
HERPUD1	1.54	0.00131	**TGFβ**
TNFSF10	1.48	0.00026	**TGFβ**
DAB2	1.82	8.9E-05	**WNT**
PPARD	2.41	4.3E-05	**WNT**
ICAM1	2.18	0.00406	**NFκB**
STAT1	1.31	0.00505	**NFκB**

Gene	Fold Change	P-value	Pathway
IRF1	1.14	0.02828	**JAK1 & JAK2 / STAT1**
CEBPD	1.44	0.0194	**STAT3-Induced**
MCL1	1.35	0.00327	**STAT3-Induced**
LRG1	2.55	1.1E-05	**STAT3-Induced**
SOCS3	4.40	0.00088	**STAT3-Induced**
BBC3	1.45	0.04398	**p53**
BTG2	2.85	0.00089	**p53**
CDKN1A	1.35	0.00122	**p53**
FAS	2.04	0.01478	**p53**
ID1	1.39	0.02793	**Notch**
JAG1	1.42	0.00441	**Notch**
LFNG	2.92	4.7E-05	**Notch**
ACSL4	1.37	0.00238	**PPAR**
FTH1	2.14	2.2E-05	**Oxidative**
SLC2A1	1.40	0.01056	**Oxidative**
SQSTM1	1.19	0.00173	**Oxidative**
VEGFA	1.5948	0.00834	**Oxidative**
ARNT	1.37	0.00263	**Hypoxia**

The most heavily downregulated genes (0.50 cutoff) include: EMP1, FCER2, and BCL2. Of particular note though, is the downregulation of TNFa by 0.56, a downregulation similar to what we frequently observed in the lab. Downregulated genes include many inflammatory type genes from the TGFβ and NFκB pathways as well as oxidative stress pathway.

As mentioned before, many genes from these pathways were both up and downregulated. CM is constituted of many different factors and it is likely that some of these factors have counteracting roles causing genes within the same pathway to react differentially. Also, many genes respond in as little as 4 hours and having taken readings from a 24 hour experiment, we cannot rule out that some of gene responses could be secondary or tertiary responses as many genes stimulate negative feedback loops.

Table 3: Genes significantly downregulated in monocytes treated with ASC CM

Gene	Fold Change	P-value	Pathway
ATF4	0.7684	0.00223	**TGFβ**
EMP1	0.1539	0.00897	**TGFβ**
MYC	0.6721	0.00037	**TGFβ/WNT**
CCL5	0.7756	0.00688	**NFκB**
TNF	0.5586	0.00429	**NFκB**
FCER2	0.2892	0.03693	**JAK1 & JAK3 / STAT6-Induced**
PCNA	0.8665	0.01508	**p53**
HEYL	0.6071	0.03488	**Notch**
BCL2	0.4796	0.00638	**Hedgehog**
SLC27A4	0.6029	0.00932	**PPAR**
GCLM	0.7457	0.00401	**Oxidative**
NQO1	0.8726	0.00433	**Oxidative**
TXN	0.7738	0.02536	**Oxidative**

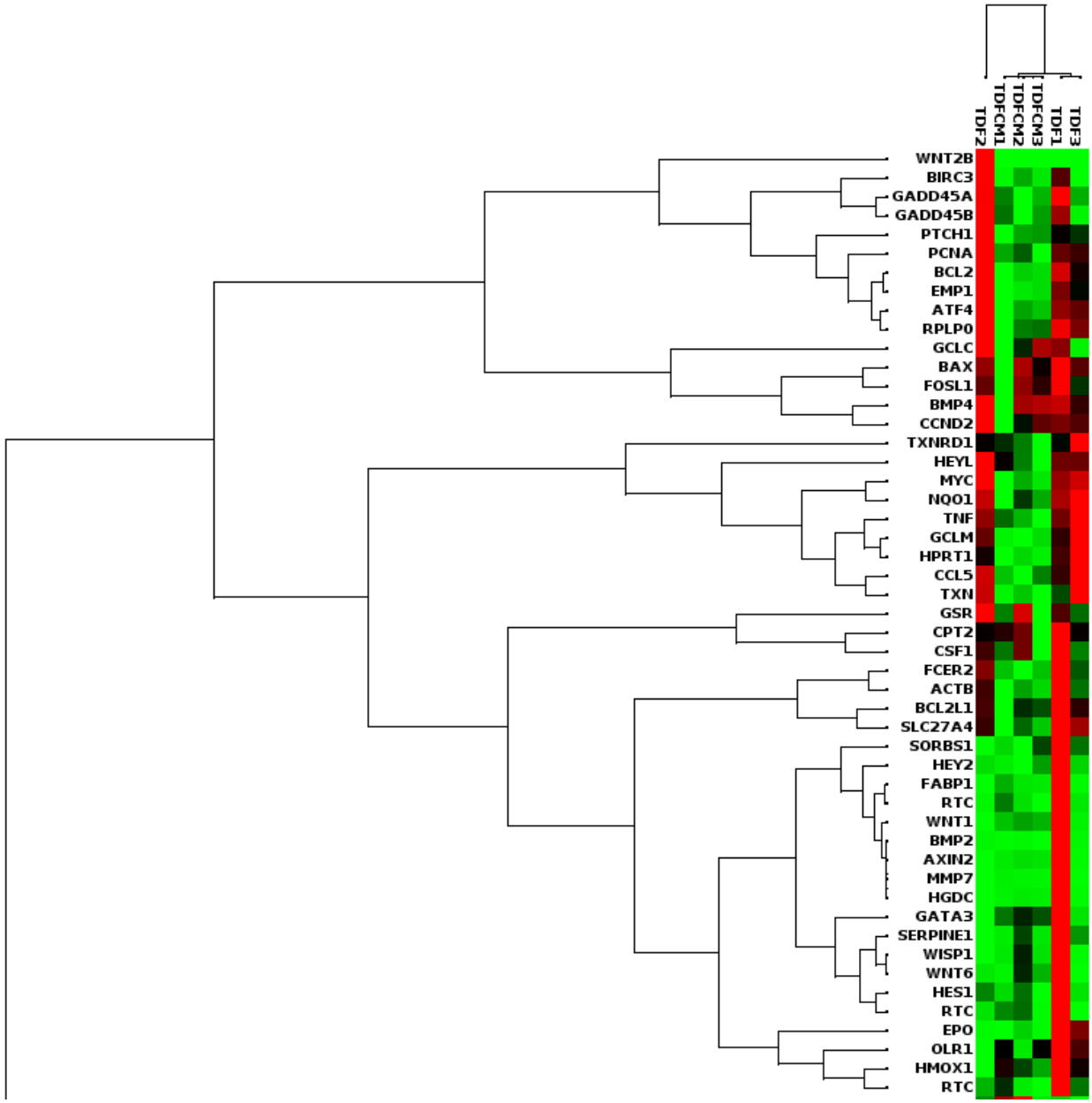

Figure 38: First gene grouping from gene array heat map for individual monocyte samples

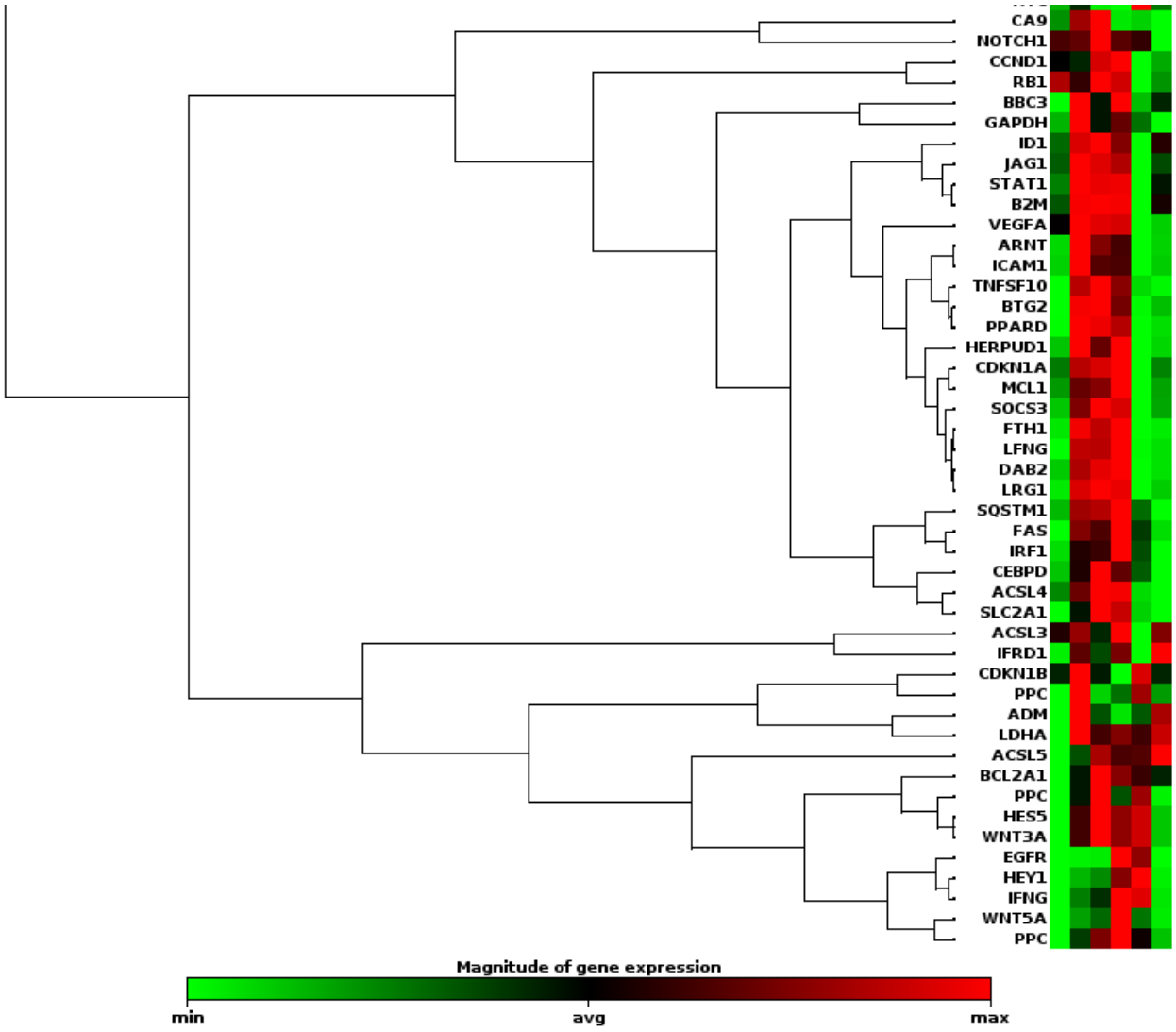

Figure 39: Second gene grouping from gene array heat map for individual monocyte samples

CM treated macrophage gene array

Compared to monocytes, MDM's showed fewer genes responding to PA-CM. The genes WISP1, and OLR1 show the highest fold induction of the upregulated genes. Also of note, LRG1 showed a large fold-increase although fell just short of the statistical significance cutoff of P-value 0.05. However, this was a gene also upregulated in monocytes so is worth considering.

Table 4: Genes significantly upregulated in MDMs treated with PA-CM

Gene	Fold Change	P-value	Pathway
PPARD	1.4439	0.00466	**WNT**
WISP1	5.5919	0.03771	**WNT**
BCL2A1	1.2142	0.03933	**NFκB**
CCND1	1.6396	0.0081	**WNT**
LRG1	3.3404	0.07589*	**STAT3-Induced**
MCL1	1.2454	0.04986	**STAT3-Induced**
HES1	1.7818	0.02295	**Notch**
JAG1	1.4709	0.01025	**Notch**
NOTCH1	1.4743	0.00254	**Notch**
WNT6	1.2805	0.02048	**Hedgehog**
ACSL4	1.154	0.02365	**PPAR**
OLR1	4.801	0.00087	**PPAR**
FTH1	1.3287	0.0285	**Oxidative**
LDHA	1.1975	0.04153	**Oxidative**
SLC2A1	1.4675	0.00943	**Oxidative**
ARNT	1.3044	0.02746	**Hypoxia**
EPO	1.8067	0.04864	**Hypoxia**

*large “fold change” but not significant

The most heavily downregulated gene (0.50 cutoff) was BIRC3 although CSF1, TNFa, and FAS were close to the cutoff. Looking at the significantly downregulated genes as a whole though, there is a clearer trend of inflammatory genes from the TGFβ and NFκB pathways being downregulated.

Table 5: Genes significantly downregulated in MDMs treated with PA-CM

Gene	**Fold Change**	**P-value**	**Pathway**
ATF4	0.7792	0.01722	**TGFβ**
BIRC3	0.3431	0.0011	**NFκB**
CSF1	0.6271	0.03346	**NFκB**
TNF	0.6314	0.01963	**NFκB**
FAS	0.5164	0.01574	**p53**
BCL2	0.7544	0.00274	**Hedgehog**
ACSL5	0.9096	0.03983	**PPAR**
GCLM	0.8293	0.01622	**Oxidative**

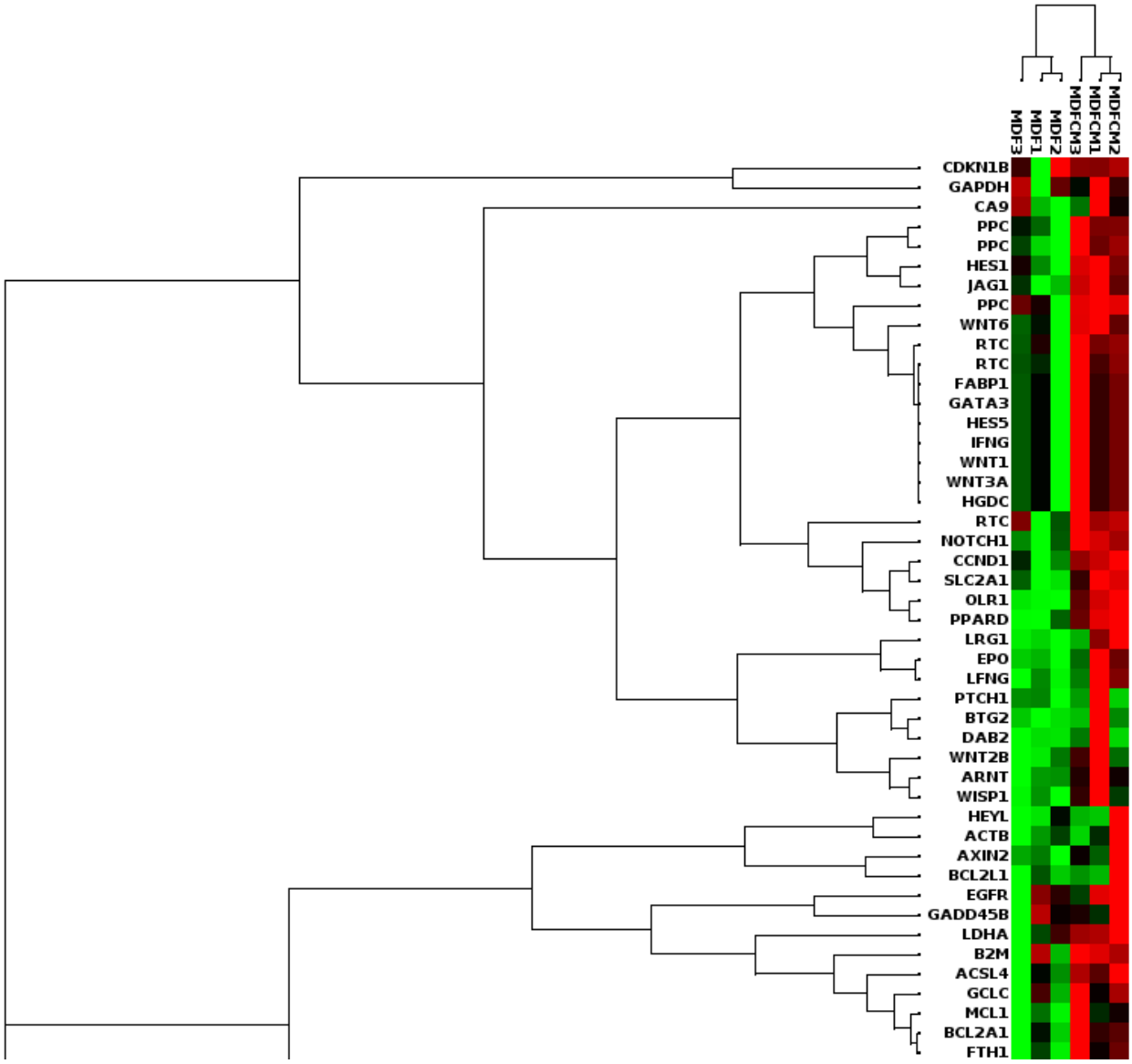

Figure 40: First gene grouping from gene array heat map for individual MDM samples

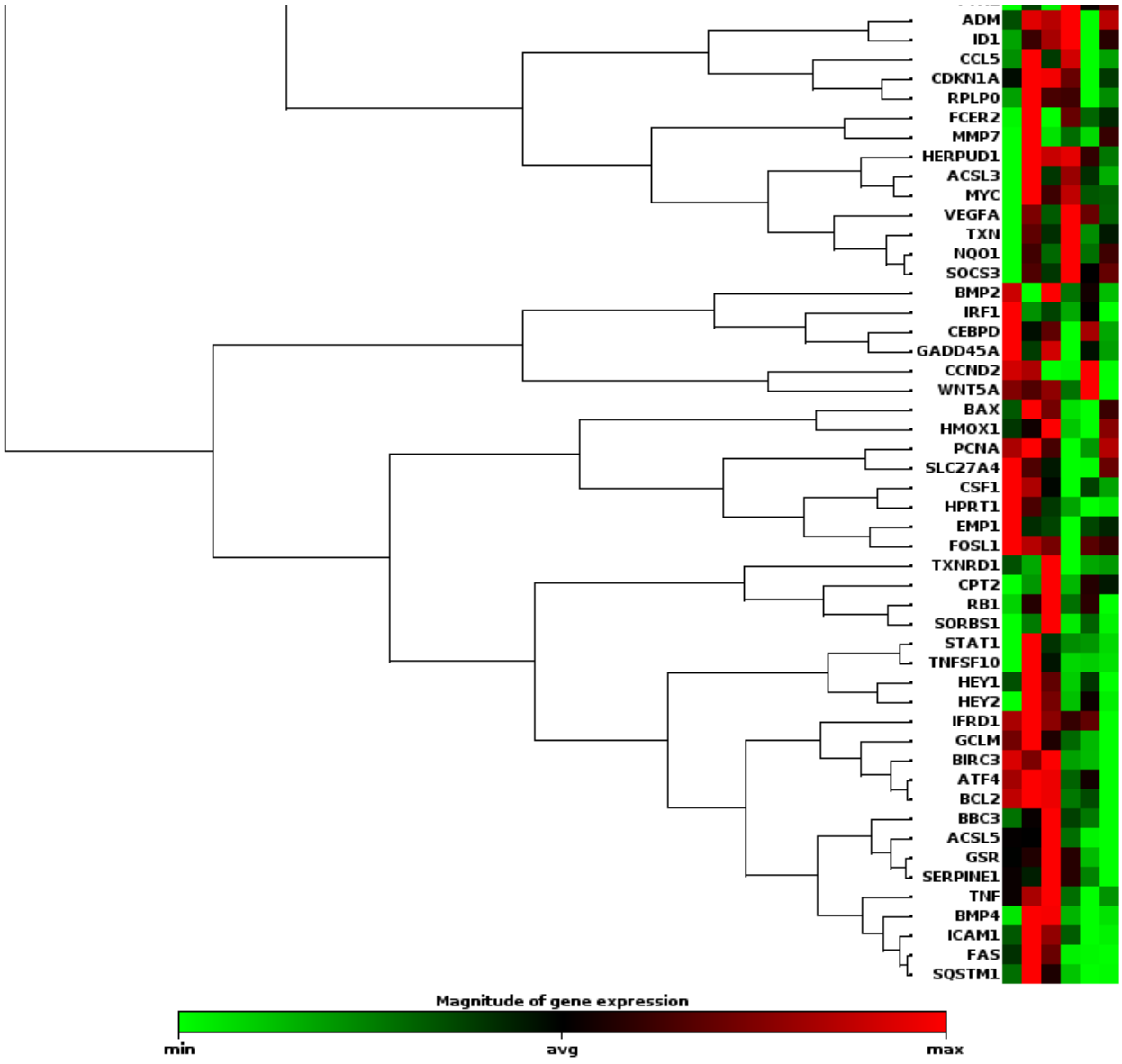

Figure 41: Second gene grouping from gene array heat map for individual MDM samples

Discussion

The original goal in this line of experimentation was to investigate some of the hypotheses developed during our adrenergic and macrophage differentiation work. We had proposed that monocyte/macrophage ePRL expression would be altered in the presence of mature adipocytes and in turn, ePRL could cause changes in how lipids were stored and metabolized in adipocytes. While our data does not disprove such a relationship, it does lead us in a different direction and suggests a more likely role for ePRL in adipose tissue homeostasis may lie in tissue remodeling.

Our first set of surprising results came with our initial coculture experiments where PA were able to stimulate ePRL in monocytes and MDMs but MA and serum starved PA had essentially no effect on ePRL expression. It is difficult to pinpoint exactly what is causing such a change in signaling as multiple variables are being altered at once during serum starvation and differentiation. Clearly, with the removal of serum from the experimental conditions we are removing a large number of signaling molecules that could be important precursors or cofactors in the process of stimulating PRL in the immune cells. Also, removing these components likely alters a variety of metabolic or other normal housekeeping pathways within the cells of which a PRL stimulating factor could be a byproduct.

In line with serum removals effects on baseline pathways within a cell, we must also take into account another similarity between MA and serum starved PA in that both groups of

cells cease to proliferate. In the case of MA, this is a terminal differentiation after which no more cell division can take place. For serum starved PA, it appears a component of the serum is necessary for proliferation although serum free medium is sufficient for survival. Nonetheless, we must consider that a PRL stimulating factor could be a byproduct of normal cell proliferation or the pathways activated during the process. Experiments that could separate these two variables, such as using an mTOR inhibitor to halt cell proliferation even in the presence of serum, would prove useful in gaining a better understanding of which variables have the most influence over this response and provide more insight into possible reasons for such a response.

Another interesting phenomenon regarding medium requirements by the cells is the observation that HBSS is unable to transfer PRL stimulation from the SGBS-PA to the immune cells. While it's clear that serum starvation alters the PA so that they are no longer able to stimulate PRL, it is also interesting to note that even in PA that should be able to do so, full medium is required.

We tried to take advantage of this finding by returning select amino acids to HBSS to see if we could rescue the response. For instance, we theorized that the factor from SGBS-PA involved in stimulating ePRL could be derived from catecholamine pathways in concert with our initial findings on this project. To test this we supplemented aromatic amino acids to the medium that are necessary precursors for all catecholamines. However, results from those

experiments and similar amino acid supplementation were unable to return full competency to the PA-CM. It is possible that another component of DMEM:F12 besides the amino acids we tested is necessary for the response or that certain components together are necessary and isolation of individual amino acids or amino acid groups is insufficient to return a full response.

Fractioning of the PA-CM also provided important insight into what is responsible for PRL stimulation. The fact that the LMW fraction retains the majority of the ability to stimulate ePRL allows us to rule out a large number of other possible components. For instance, 3kDa is a cutoff for which all proteins should be removed which allows us to exclude a large number of signaling pathways regulated by large proteins from consideration. However, this experiment does less in the way of identifying exactly what is causing the response as the LMW fraction could contain any number of small molecules, peptides, or nucleic acids.

Lastly, the most telling data collected throughout this project is analysis of the genes that are regulated alongside ePRL as these give us the best sense as to what conditions cells upregulate ePRL expression. In particular, we paid close attention to TNFa regulation, which seemed to closely negatively correlate with ePRL expression although our line of experimentation cannot delineate whether or not this is done by the same factor or a separate factor that is released under similar conditions.

This anti-inflammatory correlation of ePRL was further supported upon analysis of our gene arrays. We observed a variety of genes from different pathways that are known to be

important markers and regulators for general anti-inflammatory polarization in macrophages. For instance, we saw significant upregulation in PPARD, which acts as an important regulator of lipid metabolism. Metabolic reprogramming to lipid metabolism and beta-oxidation is a necessary step in M2 polarization and M2 macrophages cannot persist without PPARD activation.[114,115] Upregulation in other genes, such as ACLS4, that play vital roles in regulating lipid metabolism[116] also lead us to believe that PA-CM is capable of activating genes necessary for an M2 metabolic reprogramming in the immune cells.

More directly, we see specific markers of inflammation regulated in ways that correlate with an M2 phenotype when treated with PA-CM. In particular, the gene array shows downregulation of TNFa consistent with our own gene expression studies throughout our work. As mentioned before, TNFa expression closely negatively correlates with ePRL expression in our studies. Other inflammatory markers such as CSF1 and ATF4 were also shown to be downregulated. Also, after observing a variety of gene regulation hinting at M2 polarization, we also ran follow-up analysis for IL-10 and MRC1 expression, both key markers of M2 polarization. Interestingly, we saw a significant increase in IL-10 expression but did not see a significant increase in MRC1 expression.

Also of note, we observed upregulated genes involved in both angiogenesis and growth and development. WISP1 for example plays a role in mesenchymal stem cell proliferation and is a pro-mitogenic and pro-survival factor.[117,118] Other genes like LRG1[119], OLR1[120], and ARNT[121] all

play a role in growth and angiogenesis. OLR1 is of interest to the laboratory as this protein is suggested to recognize and internalize oxidized low density lipoprotein (Ox-LDL). Ox-LDL levels are increased in atherosclerosis, for which obesity is a known risk factor.

It is also interesting to note that many JAK/STAT pathways are upregulated in our PA-CM treated groups. In particular, we see a strong upregulation of SOCS3. These changes are of note because multiple STAT proteins are downstream targets of PRL signaling and genes such as SOCS3 have been shown to be directly regulated by PRL signaling.[122] Such regulation is likely the result of our time point chosen. We ran the gene array on our 24 hour treated samples from our ELISA experiment because the protein analysis gave us a high level of confidence that the experiment ran well and consistently. However, clearly being able to measure PRL in the medium means that cells were subjected to this signal at the time points tested and were likely already responding to their own PRL secretion. The effects of secondary and tertiary signaling could be more clearly defined by performing gene array at earlier time points but this was cost prohibitive for our laboratory. Nonetheless, it does lend credence to previously published work that ePRL functions in an autocrine fashion for immune cells.[66,123]

Our line of experimentation yields new insights on ePRL regulation in monocytes and macrophages. While our initial hypothesis was that ePRL played a primary role in modulating lipid metabolism and would thus more strongly respond to lipid loaded mature adipocytes, our observations throughout this course of study have yielded a surprising outcome that ePRL is far

more responsive to factors released by PA. In particular, it is important to remember that PA are in fact ASC and more broadly, mesenchymal stem cells. PRL as a growth and differentiation promoting hormone meshes well with these concepts. And the fact that much of the genetic regulation taking place alongside PRL induction also follows that general theme bolsters the idea that PRL may play an important role in stem cell development, growth, and tissue remodeling.

CHAPTER FOUR: CONCLUSION

The role of the immune system, and more specifically ATM, in non-classical functions such as tissue homeostasis is only beginning to be unraveled despite a flurry of recent research on the topic. We are beginning to understand that pathogen elimination is only a single arrow in the quiver available to these complex and plastic cells and that they in fact have a hand in almost every major process in the human body. On top of this complexity, we are uncovering more and more ways in which these cells differ between humans and many of the most common animal models such as mice and rats. Recent work on ePRL production shows how even in cases where the protein and signaling pathways vary little between species, millions of years of evolution can result in drastic differences in how the protein is expressed and distributed. It is of upmost importance to consider such differences when translating findings from animal models into the clinic and a better understanding of such nuances can hopefully increase the success of drugs and therapeutics as they progress into humans.

Originally we had set out to study how monocyte and macrophage derived PRL is regulated in the presence of mature adipocytes and whether or not such PRL responded to, or could interfere with general cellular metabolism in mature adipocytes. Our initial results showing that adrenergic hormones significantly increased PRL expression fit in well with this hypothesis and suggested that the two signals could work in opposition, helping to regulate each other through their opposing actions. Adrenergic hormones are well characterized as

activators of lipolysis and PRL has been shown to inhibit lipolysis and is correlated with weight gain across a number of studies.

The increase in PRL secretion observed as monocytes differentiate into macrophages also suggested a physiological significance as a drastic influx of macrophages into adipose tissue is observed in obesity. Even in the absence of stimuli, this increase in macrophage populations alone would increase adipose tissue exposure to the PRL proteins. And seeing as PRL tends to "stick" to proteoglycans on the surface of adipocytes[113], much of this PRL burden would likely be isolated to paracrine signaling within the adipose tissue and not be well reflected in total serum levels.

However, the data from our experiments points us in a different direction. Both our monocytes and macrophages show a significant and robust ePRL response when cultured with adipose stem cells, from which the mature adipocytes are seeded, and have generally no response to the mature adipose cells themselves.

While our line of experimentation cannot rule out that monocyte/macrophage-derived PRL influences adipocyte metabolism, our results suggest that PRL may be more relevant in a different context: when taken as a whole, it is clear that ePRL is being upregulated only in the setting of a high growth environment. Whether monocytes/macrophages are responding to the proliferating PA themselves, or factors in the serum used to stimulate growth in cell culture, we cannot yet say. Also, looking at the gene regulation in monocytes/macrophages surrounding

the increase in ePRL, we see a general shift towards an M2 phenotype. While our previous work does not show an increase in PRL expression upon M2 polarization, M2 polarization is far more complex than a binary event and M2 subsets with very different functions such as M2a, M2b, and M2c have been characterized along with tumor associated macrophages (TAM).[124,125] Perhaps PA-CM influences into an M2 state more complex than simple IL-4 signaling and more conducive to tissue remodeling.

Such a model makes sense when considering the massive changes in size that adipose tissue is required to undergo within very short time frames. During times of high nutrition, adipocytes must be able to undergo drastic increases in size as their lipid droplets swell to store energy, and during times of low nutrition, there can be just as drastic a decrease in adipocyte size in a matter of just weeks. All this must take place in a defined tissue space and must be accompanied by restructuring of the extracellular matrix (ECM) and vasculature.[126]

Given that PRL is shown to play a role in a variety of processes relevant to such actions, and that ePRL in monocytes and MDMs is upregulated along with other genes shown to play a role in tissue growth and remodeling, our work points to ePRL as likely being a human specific protein involved in this process. Further experiments will need to be performed in order to show a more direct role for ePRL from immune cells in this role, but as mentioned earlier, the goal of our research involving ePRLs regulation was to elucidate more about its possible

function within the human body and I believe our results point in a clear direction for future areas of experimentation.

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