HISTONE DEACETYLASE REGULATES THE EXPRESSION OF PRO-INFLAMMATORY CYTOKINES IN PALMITATE TREATED ASTROCYTES

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ABSTRACT

Neuroinflammation is defined as the activation of the brain's innate immune system upon injury. Cytokine release is a significant hallmark of neuroinflammation, and leads to neuronal death and neurogenerative disease. Palmitate, release cytokines via the Toll-like receptor (TLR) based NF-kB inflammatory pathway. Additionally, histone deacetylases (HDAC) is associated with the upregulation of cytokine and NF-kB expressions. NF-kB is a transcription factor that upregulates the gene expression of proinflammatory cytokines; they are bound to the Ikβα complex in the inhibitory state and activated upon degradation of the Ikβα complex by the IKK complex. The major gap that this project aims to investigate is the role of HDACS, specifically in the palmitate treated astrocytes. I hypothesize that **HDACs lead to the activation of the IKK complex to translocate NF-kB components in the nucleus to upregulate cytokine expression in palmitate treated astrocytes.** This study demonstrates the impact of HDACs on cytokine expression upon palmitate treatment and molecular mechanisms involved. Pro-inflammatory cytokine IL-6, was significantly upregulated upon palmitate treatment, and treatment with palmitate and HDAC inhibitor valproic acid downregulated the expression of the cytokines. However, there was no significant change in cytokine expression when treated with valproic acid alone, suggesting that HDACs are involved in the palmitate induced cytokine pathway. Our results also suggest that HDACs impact cytokine expression through its catalytic activity, i.e., histone acetylation, or protein-protein interactions with the IKK complex. The latter is based on molecular docking analysis.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AP-1	Activator protein 1
ADP	Adenosine diphosphate
BACE-1	Beta-secretase1
BBB	Blood Brain Barrier
BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
COA	coenzyme A
DNA	Deoxyribonucleic acid
FFA	Free fatty acid
GCN5	General control non-derepressible 5
GNAT	GCN5-related N-acetyltransferases
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IKKβ	IkappaB kinase
IL-1β	Interleukin 1β
IL-6	Interleukin-6
LPS	Lipopolysaccharide
L-CS	Levo Cycloserine
MYD88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NLRP3	NLR family pyrin domain containing 3
PAGE	Polyacrylamide gel electrophoresis
PCAF	p300/CBP-associated factor
PKC-δ	Protein Kinase C-δ
PPARα	Peroxisome proliferator-activated receptor alpha
PPI	Protein-protein interaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SIRT	Sirtuin family

Smases Sphingomyelin phosphodiesterase

SPT Serine palmitoyl transferase

TAK-1 TGF-B-activated kinase 1

TLR Toll like receptor

TNF-α Tumor necrosis factor α

Ubc8 Ubiquitin-conjugating Enzyme

VPA Valproic Acid

WB Western blot

Chapter 1: Introduction

1.1 Saturated Free fatty acid palmitate and its association in neuroinflammation related neurodegenerative diseases pathology

Saturated fatty acids such as stearic acid (18 carbon), palmitic acid (16 carbon) and myristic acid (14 carbon) have an essential part in the production of hormones, cellular signaling, and stabilization process in the body[1]. On the contrary, high levels of saturated fatty acids have shown to impact insulin resistance and β cell dysfunction[2 3]. Free Fatty acids (FFA) have also been shown to cause lipotoxicity and its accumulation promotes inflammation via the accumulation of reactive microglia and macrophages in the brain[4].

1.2 Palmitic acid

Palmitic acid (16:0) is a fatty acid representing around 20-30% membrane phospholipids and adipose triacylglycerols is the most common saturated fatty acid found in the human body[5 6]. Diet which acts an environmental factor is a vital source of palmitic acid, especially in dairy products, cocoa butter, olive oil, and most importantly palm oil[7] associated with many irregularities, especially lipid accumulation and apoptosis in hepatocytes[8]. With palmitoylation, palmitate is covalently linked to proteins through thioester bond[9]. This is one-way palmitic acid regulates the functionalities of the proteins. Dysregulation of palmitoylation leads to disease development such as breast cancers[10], Alzheimer's Disease[11], and metabolic dysregulation[12]. Review by *Sarwat et al. (2019)*[9], states palmitate to be unique among the saturated fatty acids due to its pathogenetic role in metabolic syndrome, cardiovascular disease, cancer, neurodegenerative diseases, and inflammation. Additionally, palmitic acid is an important component of cell membranes, secretory and transport lipids, with crucial roles in protein palmitoylation and palmitoylated signal molecules.

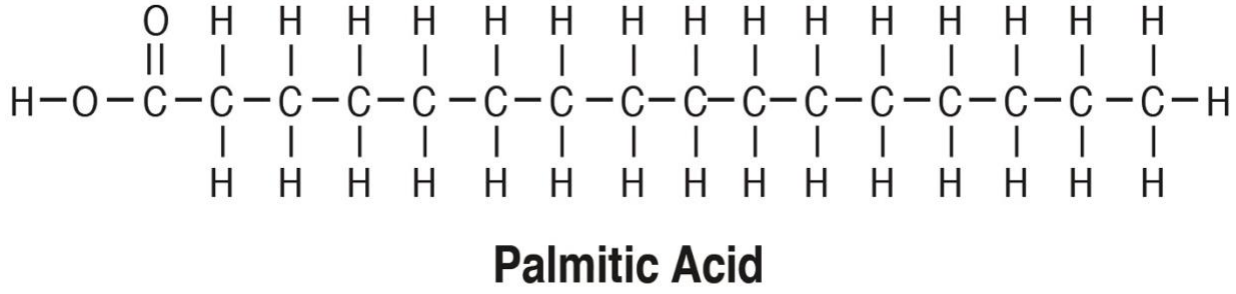


Figure 1: Structure of 16 carbon saturated fatty acid, palmitic acid. Image is reproduced from med.libretexts.org.[13]

1.3 Palmitate and its association in Alzheimer's Disease (AD) pathology

According to the Alzheimer's Association, more than 6 million Americans live with Alzheimer's Disease (AD) and by 2050, the numbers are projected to reach about 13 million. This progressive neurogenerative disease is usually associated with aging. The symptoms arise due to slow ongoing injuries to the brain especially in the neo-cortex and hippocampus of the brain. This is characterized by wilting of the cortex of the AD brain leading to damage in the areas of thinking, planning and memory. Neuroinflammation is defined as the activation of the brain's innate immune system in response to an inflammatory change. This is characterized by a host of cellular and molecular modifications involving release of cytokines, chemokines and the generation of reactive oxygen and nitrogen species within the brain[14].

Elevated levels of FFAs in AD patients' brain have been shown in many studies when compared with the healthy brain. Our lab has demonstrated that ceramide generated through the serine palmitoyl transferase (SPT) pathway in the primary rat astrocytes mediate AD-like changes in primary rat neurons[15] by decreasing level of Aβ expression upon SPT suppression[16].

We have already reported astrocytes release cytokines such as TNF-α and IL-1β upon treatment with palmitate[17]. Before adding conditioned media from palmitate-treated astrocytes to the cortical neurons, the cytokines TNF-α and IL-1β were neutralized via treatment with antibodies specific to these cytokines. The conditioned media treated cortical neurons showed downregulated expression of BACE1 protein levels upon neutralizing the cytokines. The presented evidence supports that neuroinflammation and AD pathology are connected (figure 2).

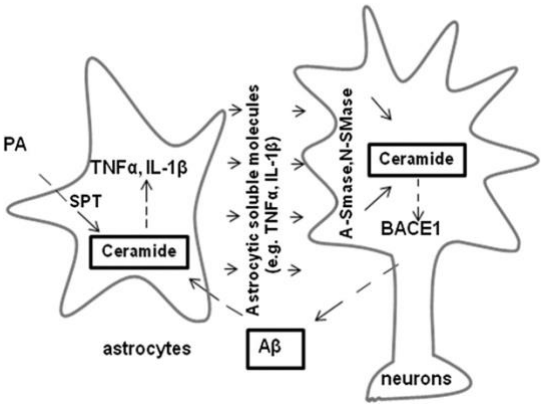


Figure 2: Palmitate treated astrocytes released cytokines via the de novo ceramide pathway. The cytokines enter the neurons and activate ceramide again that upregulates BACE1 factors in the neurons, causing release of Aβ pathological factors of AD. Reproduced with permission from ref 16 (2013 Neurobiology of Aging).

Not just Alzheimer's Disease, palmitate is also known to impact other neurodegenerative diseases such as Parkinson's Disease, a progressive neurogenerative disease affecting the elderly population with symptoms such as hyposmia, fatigue depression, behavioral disorders and constipation[18]. Palmitate treatment has been associated with the upregulation in the expression α-synuclein(ASN) an important pathology for Parkinson's Disease[19]. Similarly palmitate is also associated with pathologies including Multiple Sclerosis(MS) and Huntington Disease(HD)[18] in both cell line and primary cells.

1.4 Neuroinflammation

Neuroinflammation is connected to the release of an inflammatory response in the regions of the brain and spinal cord. In general, inflammation stands for the activation of the innate immune system that protects and defends the body due to triggers onto the body. Immune response can arise due to sterile surgery where there is involvement of a mechanical injury or factors where there is involvement of bacterial or viral invasion[20]. The inflammatory response is coordinated by a variety of cells especially the white blood cells (leucocytes) and endothelial cells, which are derived from the bone marrow, lymph nodes and spleen. According to the National Institute of Environmental Health Sciences[21], Inflammation is a normal part of the body's defense to injury or infection but if it last too long, it can be lead to chronic inflammation. At a stage of acute inflammation, the initial response confers to increased movements of plasma and leucocytes from the blood to the injured tissues and the chronic stage is characterized by the destruction as well healing. Based on varying pathogenic factors, in response to a tissue injury, the body can initiate a cascade of signaling events that aids in healing of injured tissues[22]. Overall, the inflammatory response is dependent upon the initial stimulus followed by recognition of stimuli by receptors. This is followed by activation of the inflammatory pathways, release of inflammatory markers and recruitment of inflammatory cells.

Our brain is protected from the direct impact of external and internal stimuli due to the presence of the blood brain barrier. These highly selective semi permeable border of endothelial cells that prevents solutes that are circulating in the blood from crossing over to the central nervous system where there is presence of the neurons[23]. They are overall helpful in preventing the entry of pathogens into the central nervous system. But there are extreme instances that lead to the entry of pathogens and other pathogenic factors that can breach the blood brain barrier and in turn affect the central nervous system. Lipopolysaccharide(LPS), the cell wall content of the gram negative

bacteria is able to damage functional components of the blood brain barrier and can cause sepsis-associated encephalopathy(ESE)[24]. High fat diet rich palmitic acid has been shown as a factor to influence the permeability of the blood brain barrier[25].It is in such scenarios, there is a breach in the permeability of the blood brain barrier and entry of external factors in the central nervous system, the immune cells in the brain gets activated. The immune cell in the central nervous system comprises majorly astrocytes and microglia, and evidence also showcase entry of external T-cells and macrophages at the edges of the CNS. Together these factors help the healthy brain to function and defend it from diseases[26]. Astrocytes and microglia are the important immune cells in the central nervous system. Other than the regular function of immunity, these glial cells are important in maintaining homeostasis and provide the structural integrity of the neurons. There are evidences that show the glial cells to function via cross talks within themselves[27].

Integral to the overall understanding of immunology, these glial cells consists of pattern recognition receptors (PRRs).These are proteins that are able to identify molecules associated in pathogens such as Pathogen Associated molecular Patterns(PAMPS) or molecules released from degraded cells such as Damage Associated molecular Patterns(DAMPS)[28]. PRRs specific for microglia and astrocytes are the Toll like receptors (TLRs). There are 11 known TLRs that are widely expressed by the microglial cells[29]while TLR1, TLR3, TLR4, TLR5 and TLR9 are mostly expressed in the astrocytes[30]. The activation of microglia and astrocytes is categorized as neurotoxic (M1 for microglia and A1 for astrocytes) and neuroprotective (M2 for microglia and A2 for astrocytes)[31]. For our study, we were primarily focused on neuroinflammation via the astrocytes. These astrocytes are primarily responsible for the formation and maintenance of the blood brain barrier and they are directly associated with its endothelial cells, making them the first line of defense in case of rupture in the BBB[32].

In general, neuroinflammation can be demarcated as a defense mechanism that tends to protect the CNS by beneficial effects such as tissue repair and removal of cellular debris, but persistent or chronic level of inflammatory response can be detrimental, and it tends to change the fate of the glial cells from neuroprotective to neurotoxic. Chronic levels of neuroinflammations can be accessed by the release of excessive levels of pro-inflammatory cytokines such as IL-1β,TNF-α and IL-6, chemokines such as CCL2, CCL3 and CCL5, CXCL1, CXCL2 and CXCL8, reactive oxygen species (ROS) and Nitric oxide(NO)[31]. Persistent inflammatory responses involving

microglia and astrocytes, one of the major causes for neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS)[33].

1.5 Epigenetics and Histone modifications

Chromatin is complex macromolecule consisting of RNA, DNA, and proteins. It is the state in which DNA is packaged within the cell. The nucleosome is the fundamental unit of chromosome and is composed of octamer of the histones (H3, H4, H2A, H2B) around which 147 base pairs of DNAs are wrapped. The core histones are globular except for the N-terminal tails which are unstructured. The striking feature of the tail is the large number and type of modified residues they possess[34]. These modifications are the basis for epigenetics and there are at least eight different types of modifications on histone residues listed in the table below (Figure 3).

Table 1. Different Classes of Modifications Identified on Histones

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Figure 3: Overview of different classes of modification identified on histones. Reproduced from ref 33 (Cell Reports,2007).

Epigenetics is a mechanism of gene transcription regulation that does not change the DNA sequence and is usually reversible. Histone modifications regulate transcription either by affecting the chromatin structure directly, i.e., by changing the interactions of histones with DNA, and/or by recruiting non histone proteins such as transcription factors. These interactions manipulate the affinity of histone binding and the topology of the DNA that winds around them. Therefore, the combination of different histone modifications, which has been called the histone code, adds an additional level of transcriptional regulation besides regulation by activators or repressors at the promoter level.

There are different enzymes which have been identified for histone acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, sumoylation etc. Most of these studied modifications are quite dynamic and enzymes have been identified that are known to reverse the process, except for methylation of arginine where the demethylating activity is yet to be known[35].

Of all the enzymes that modify histones, the methyltransferases and kinases are the most specific. This is perhaps the reason why methylation is the most characterized modification to date.

1.6 Histone acetylation and modifiers

The acetylation and deacetylation of the ε- amino groups of conserved lysine residues present in histone tails has long been linked to transcriptional activity and has been the most intensively studied histone modification. Histone acetylation is a modification that neutralizes the positive charge of the target lysine, and it occurs at specific lysines on the four core histones. Acetylated histones are generally linked with transcriptionally active chromatin and deacetylated histones are associated with inactive chromatin[36]. This modification is catalyzed by histone acetyltransferases (HATs) through the transfer of the acetyl moiety from acetyl-coenzyme A to the ε-amine of target lysine residues. Histone acetylation can be reversed by the enzymatic action of the histone deacetylases (HDACs). The interplay between HAT and HDAC activities thus regulates cellular histone acetylation levels.

1.7 Histone acetylase

Histone modification being regulated by histone acetyltransferases (HAT) and histone deacetylases (HDACS) lead to reduction in the positive charge of lysine residues that prevent the binding of the histone tails and the negatively charged DNA[37]. This way, histone acetylation acts as an active histone mark. The lysine acetylation consists of a writer and an eraser. Histone deacetylases are considered as erasers and Histone acetylases are considered as writers for the histone modification[38]. Histone acetyl transferases are of two types based on cellular localization. Type A HATs are localized in the nucleus, and they are further subdivided into five family. GNAT family consisting of PCAF, GNC5 and ELP, CBP family consisting of p300 and CREB, MYST family consisting of TIPO, MOZ, MORF, MOZHBOI and HMOF, and followed by TAFI and TIFIIIC90 family. Type B HATS are localized in the cytoplasm[39]. GCN5 and PCAF are homologous to yeast GCN5 and paralogous to each other, they are utilized by SAGA and ATAC complex to acetylate nucleosome histone H3[40]. CBP and P300 are also paralogs and capable of inducing lysine acetylation in histone residues[34]. Genome wide mapping have indicated correlation of these two HAT complexes with multiple gene expressions and its location at the transcription sites.

1.8 Histone deacetylase

Histone deacetylases or HDACS are enzymes that act as erasers to the acetylation of the histones by removing the acetyl group. They cause reduction in the positive charge of lysine residues that prevent the binding of the histone tails and the negatively charged DNA[37]. The HDACs are generally composed of 18 members and categorized into four classes of two different protein families. Zinc-dependent metalloprotein family consist of Class I HDACs composed of HDACs 1-3 and 8, class II HDACs composed of HDACS 4-7,9 and 10 and Class IV HDAC composed of HDAC11. The NAD+ dependent protein family consists of Class III HDACs composed of SIRT1-7[41]. Based on datasets and evidence so far, H3K9ac epigenetic modification is corelated to downregulation and upregulation of SIRT1 in the hypothalamus[42], HDAC1 and HDAC2 in primary microglia of alcohol fed mice[43]. The evidence mentioned above predicts downregulating levels of the HDACS being synonymous with the increasing expressions of H3K9ac and thus giving an idea of how the epigenetic mechanism is controlled by the HDACs. It also showcases the catalytic activity of HDACs.

Chapter 2: The role of Histone Deacetylases in the expression of pro-inflammatory cytokine expressions in palmitate treated astrocytes

2.1 Palmitate induced inflammatory pathways

Long chained free fatty acids are made up of 10 or more carbon atoms. Palmitic acid is a 16-carbon fatty acid molecule that are insoluble in water or any polar solvents. These fatty acids being non-polar, tends to repel polar solvents such as water and blood plasma. This is also reason why they are required to be transported to different parts of our body by specific transporters. The major bulk of transportation of fatty acid is performed by albumin[44] whereby these reach the cells and dissociates from albumin[45]. Palmitic acid are taken up by factors such as fatty acid translocase CD36, fatty acid transporter proteins such as FATP 1-6 and fatty acid binding proteins[46 47].

Palmitate can induce cytokine expression in multiple immune and non-immune cells. There are multiple pathways involved in the cytokine expression by palmitate. The inflammatory response via the Toll-like receptors (TLR) is a major pathway for cytokine expression by palmitate. Palmitate so far was thought to be a direct agonist for the TLR-4 receptors but recent shreds of evidence suggest otherwise, it is instead predicted to bind the receptors via different TLR-4 ligands[48] such as TLR4 adaptor protein MD2 to initiate cytokine expression based signal transduction[49]. There are also evidence of palmitate forming complex with CD36[12] through which TLR4 can be activated. TLR-4 receptor activation correlates with the expression of cytokines via NF-kB as well as the AP-1 transcription factors[50]. Association of TLR4 and MYD88 activates the kinases that degrade IKKβ leading to the activation of the NF-kB factors and their translocation into the nucleus and binding to the promoter region of cytokines IL-6 and IL-1β[51](**Figure 4B**). Palmitate is also involved in the de novo ceramide synthesis. In the ceramide synthesis pathway, the palmitate in the cells gets converted to Palmitoyl-CoA (coenzyme A derivative of palmitate formed by acyl-CoA synthase), which also acts as precursors to the ceramide synthesis. They are catalyzed by Serine palmitoyl transferase (SPT) to produce 3-ketosphinganine, which in is associated with further reduction in acetylation-based enzymatic activities to produce the final product ceramide[52]. Previous studies from our lab[53] have associated ceramide with upregulation in the levels of cytokines IL-1β and TNF-α. Palmitate treated astrocytes showed high expression of the cytokines when compared to BSA only treated control. Inhibitors specific to SPT, L-cycloserine (L-CS) attenuated the cytokine levels (**Figure 4A**). Ceramide is also related to NLRP3 inflammasome[54] and IPAF inflammasomes[55] dependent caspase 1 cleavage activity which in turn

aid in the maturation of IL-1β cytokine. Palmitate can also impact cytokine expression through metabolized product diacylglycerol (DAG). DAGs can activate protein kinase C PKC or accumulate in the endoplasmic reticulum (ER) to promote ER stress[56]. ER stress sensors IRE1α and PERK can modulate NF-kB activity and impact cytokine expression[57] [58]. Palmitate can generate reactive oxygen species (ROS) .These generated ROS can further impact the activation of NF-kB[59].

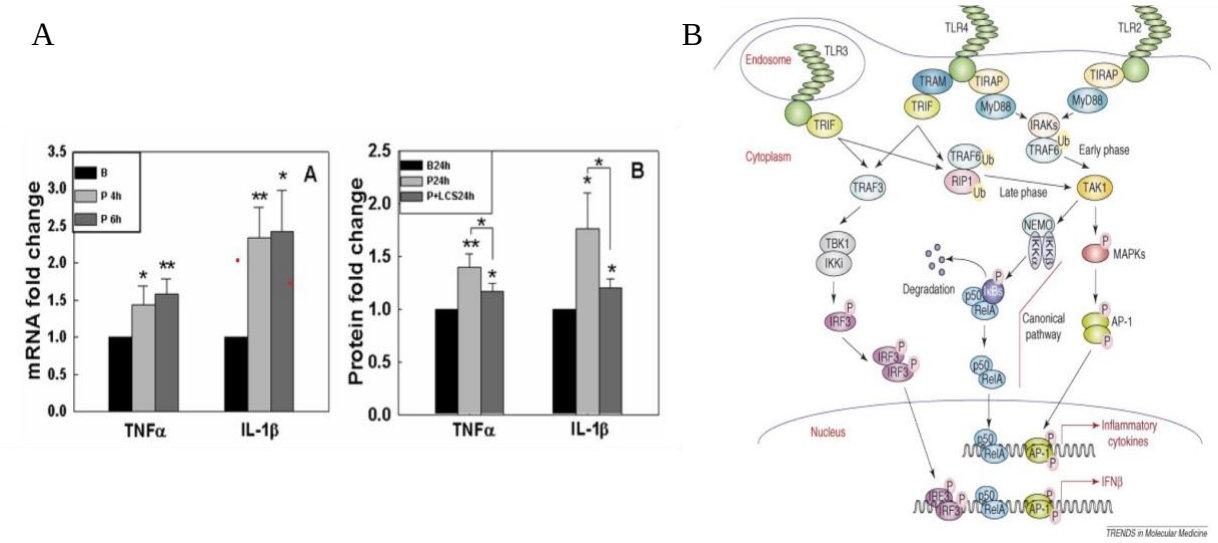


Figure 4: Palmitate and its associated inflammatory pathways. (A) Astrocytes treated with Palmitate(P), BSA(B) or P plus L-cycloserine(P+LCS) for indicated time. Palmitate treatment shows increase in TNF-α and IL-1β expressions and presence of inhibitor attenuates their expression. (B)Association of TLR-4 pathway in the expression of cytokines. Reproduced with permission from ref 16 (2013 Neurobiology of Aging) and ref 50 (2007, Trends in Molecular Medicine).

2.2 Effect of palmitate in the players of the NF-kB based inflammatory pathway

Based on previous studies in our lab[17], palmitate is involved in the expression of proinflammatory cytokines in the astrocytes and it also further shown that these cytokines when released from the astrocytes reach the neurons where they are able to regulate the pathologies associated with Alzheimer's Disease. The overall cytokine expression is dependent upon factors that ultimately cause the activation of NF-kB leading to the expression of cytokines. As discussed before palmitate can activate the TLR4 based inflammatory pathway, and experimentally when rat astrocytes were treated with TAK-242, an inhibitor of TLR-4 along with palmitate the expression of cytokines decreased. The expressions of both IL-6 and TNF-α remained unchanged in comparison to the palmitate only treated astrocytes[60](figure 5).This evidence supports TLR-4 based inflammatory pathway in astrocytes.

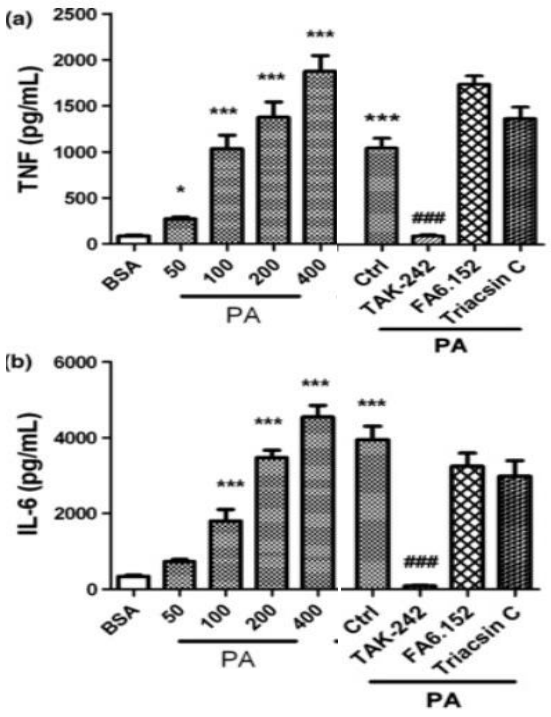


Figure 5: A) Primary rat astrocytes treated with palmitate at different concentrations ranging from 50-400μM and the protein concentrations of TNF-α and IL-6 were measured. B) Treatment of rat astrocytes with palmitate along with TAK-242 a pharmacological inhibitor of TLR-4, FA6.152 inhibitors of CD36 and Triacsin C inhibitor of acetyl-COA synthase. Reproduced and modified from ref 51(2012, John Wiley and Sons).

Palmitate binding of TLR-4 is correlated with the association of a cascade of proteins such as the TRAM and TIRAP domain that can bring in the MYD88 proteins towards the TLR-4 receptor. Binding of MYD88 with the TLR-4 receptors is followed by the involvement of multiple kinases that ultimately leads to the activation of the TAK-1 kinases. TAK-1 is related to the phosphorylation of IKK complexes and activation of MAP-kinases that are responsible for the activation of transcription factors that regulate the cytokine expressions. How the TAK-1 is able carry on these functions is a matter of current study worldwide.

IkB kinase(IKK) is an enzyme complex that is involved in the cellular response upstream to the NF-kB signaling cascade based inflammation[61]. They are composed of three subunits IKK1, IKK2 and NEMO, where the IKK 1 and IKK2 are homologous in structure and together composed of domains that allows the third subunit NEMO to bind. The overall IkB kinase activity is important for the activation of NF-kB family of transcription factors. There is a canonical and a non-canonical pathway that is involved in the NF-kB activation. IKK2 is an important factor for the canonical pathway whereas IKK1 is more utilized in the non-canonical pathway for NF-kB

activation[62]. IKK2 subunit can phosphorylate the IkB factors, by which it gets released from the NF-kB factors in the cytoplasm and the NF-kB factors are free from its bound state with IkB. These activated NF-kB get phosphorylated by external kinases and can enter the nucleus where they act as transcription factors for cytokine expression[63].

Overall palmitate has been shown to be responsible for cytokine expression following the NF-kB signaling cascade for cytokine expression. The palmitate based cytokine expression is TLR-4 dependent and previous works in our lab with palmitate treated astrocytes had shown upregulation of NF-kB factors p50 and p65[64]. It is these factors that form heterodimers and act as transcription factors of pro-inflammatory cytokines. This supports that palmitate-based cytokine expression is dependent upon the TLR-4 and NF-kB signaling cascade. Moreover, palmitate treated human mesenchymal stem cells have shown upregulation in the phosphorylated IKK1 and IKK2 levels[65](figure 6).

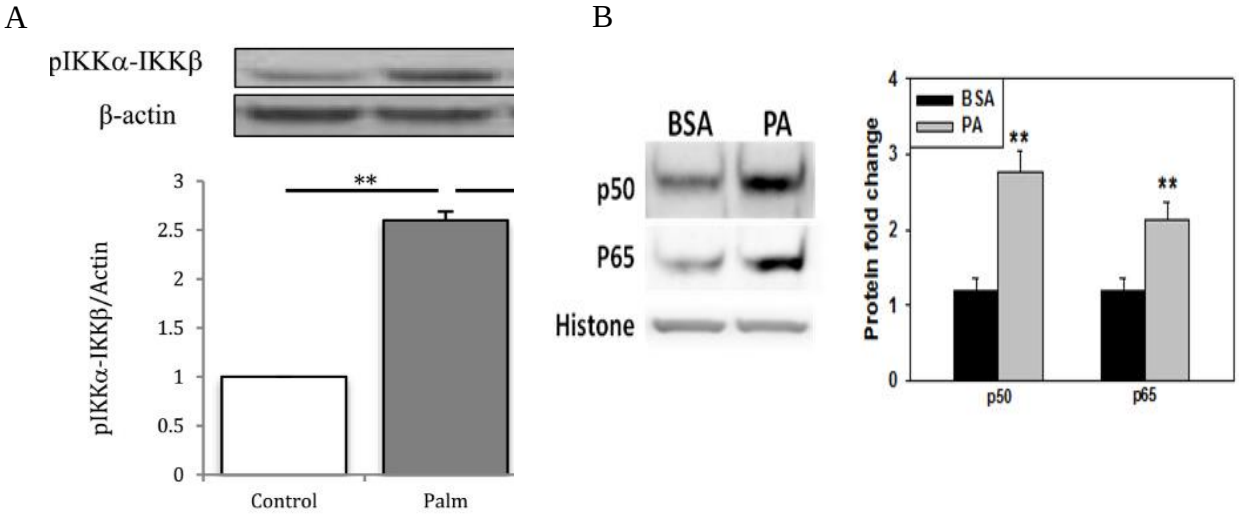


Figure 6: A) Treatment of human mesenchymal stem cells with palmitate at a molar ratio of 3:1 led to the increase in the expression of the IKK complex (IKK1-IKK2). B) Treatment of palmitate at a molar ratio of 3:1 with rat astrocytes increased the protein expressions of p50 and p65 subunits of NF-kB. Reproduced and modified from ref 55 (2014, Neurobiology of Aging) and ref 56 (2019, International Immunopharmacology).

2.3 Histone Deacetylases are involved in the expression of cytokines

DNA is negatively charged and is held on by the histone proteins via its positively charged amino acid residues. During addition of acetyl group to the to the N-terminal amino groups such as lysine, there is not just an overall change of charge but also in the hydrophobicity[66]. Histone acetylation occurs mostly at the lysine residues and causes an open state of the chromatin thereby allowing easy access for the gene regulatory factors causing an overall activation of gene expression and in

certain cases, progression in cell cycle and replication[67]. On the other hand, histone deacetylases are catalysts that remove the acetyl group from the amino acid residues that leads to the original state of the histone and DNA molecules and causing decrease in gene expression. So overall via its catalytic activities the HDACs can cause a reduction in gene expressions. HDACs class I, II and IV share sequence similarity and are dependent on Zn2+ for enzymatic activity while Class III HDACs are NAD+ dependent[68].

It was originally that acetylated histone and non-histone proteins to be an important regulator for cytokine expression given their function to actively express genes. Evidence showcased in the previous sections abide by the similar thought process. Contradictory to the present scenario, multiples evidence shows otherwise. Class I HDACs, known to be repressor complexes have shown to promote targets genes in type 1 interferon (IFN) signaling pathway[69].Class II HDACs that can act both in cytoplasmic as well as nuclear environments, can function in a deacetylase dependent manner and deacetylase independent manner to promote gene expression[70]. Current studies have shown that inhibition of specific HDACs downregulates the expression of cytokines and the factors that activate the expression of the cytokines.

Among the class I HDACs, experimental evidence has shown that silencing HDAC2 on the MG-63 and the HOS human osteoblast cell line led to the downregulation of proinflammatory cytokine expression IL-6 and overexpression of HDAC2 upregulated the expression of IL-6 cytokines (figure 7A). Moreover, overexpression of HDAC2 in the same cell lines have shown an increase in expression of the phosphorylated p65 and IKK2 levels[71](figure 7B). Murine microglial cells, when treated with Lipopolysaccharide(LPS), showed an upregulation of the pro inflammatory IL-6 and TNF-α cytokine expression whereas there was downregulation of the same LPS treated cells when there was a knockdown of HDAC1 and HDAC2 suggesting an essential role of HDAC1 and HDAC2 in the LPS based cytokine expression in microglia[72]. Specific knockdown of HDAC3 showed downregulation of proinflammatory cytokine expression in LPS treated monocyte[73] and HDAC8 suppression was associated with the decreasing IL-6 and IL-8 cytokine expression in human (acute myeloid leukemia)AML cells[74].

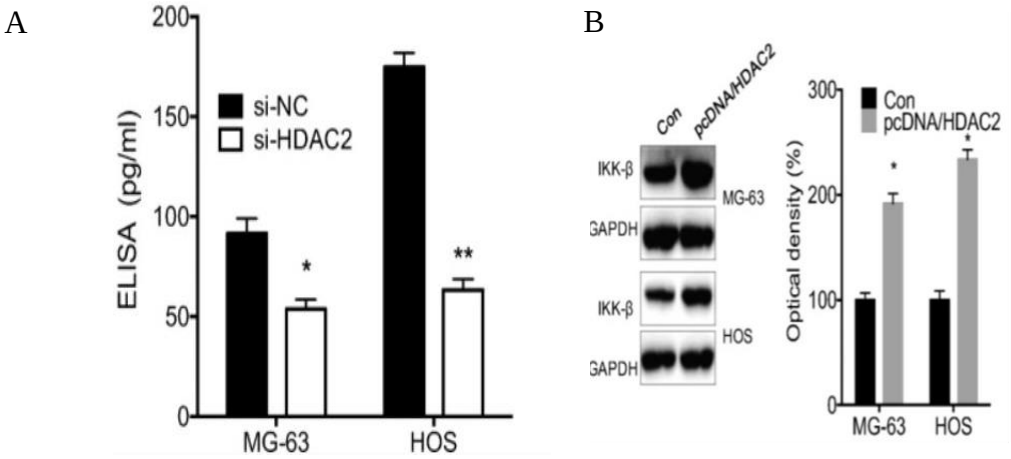


Figure 7: Impact of class I HDACs on the expression of pro-inflammatory cytokines. Silencing of HDAC2 on MG-63 and HOS cell lines caused downregulation of IL-6 cytokine expression and overexpressed HDAC2 increased the expression of IKK-2 in the same cells. Reproduced and modified from ref 62(2019, Springer Link).

Among the class II HDACs, knockdown of HDAC5 in murine macrophages led to the downregulation of TNF-α cytokine expression[75], overexpression of HDAC7 upregulated the expressions of IL-1β and IL-6 proinflammatory cytokines in mice astrocytes[76](figure 8A), and knockdown of HDAC9 in ischemia and reperfusion(I/R) induced mice brain cortex led to the downregulation of cytokine expressions[77](figure 8B).

Based on the evidence showcased and the discussions made so far, it can be understood that HDACs play an essential role in the expression of cytokines in different immune cells, such as microglia and macrophage. But it remains unknown how the HDACs moderate this process. In the following sections, we will discuss in detail, based on literature evidence, how HDACs can moderate cytokine expression.

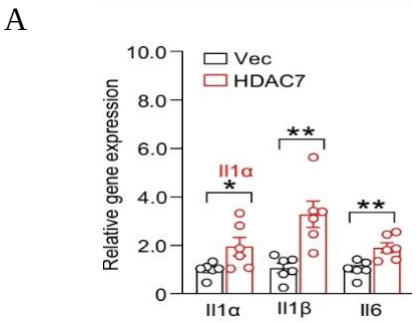
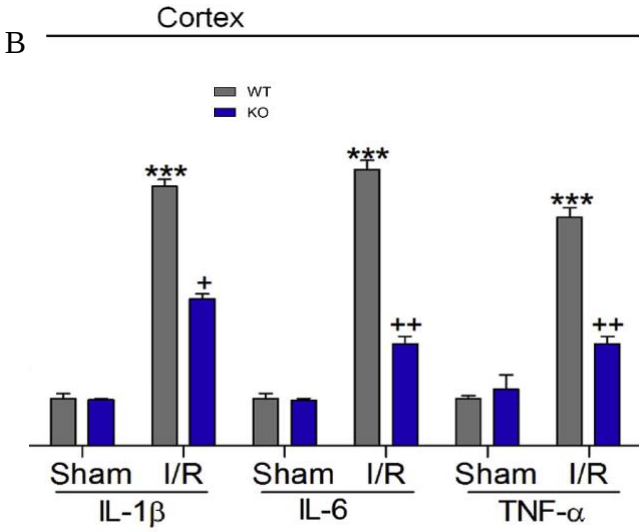


Figure 8: A) Impact of class II HDACs on the expression of pro-inflammatory cytokines: Overexpression of HDAC7 upregulated the expression of cytokines in mice astrocytes.

Figure 8 (cont'd)

B) Impact of class II HDACs on the expression of pro-inflammatory cytokines: Knockout of HDAC9 in ischemia and reperfusion(I/R) induced mice brain cortex leads to downregulation of cytokine expression. Reproduced and modified from ref 67 (8A) (2022, Springer Nature) and ref 68 (8B) (2018, biochemical and Biophysical Research Communications).



2.4 Objective and Hypothesis

Previous research in our lab has shown palmitate induced the release of pro-inflammatory cytokines from rat cortical astrocytes. The cytokines have a detrimental effect when produced at a chronic level. In addition, they can reach the neurons, impacting the pathologies of different neurodegenerative diseases. This gives us an overall understanding that palmitate is involved in neuroinflammation. Literature evidence showcases palmitate-treated rat astrocytes depend on the TLR-4 pathway for cytokine expression. Evidence also showcases the activation of IKK factors and p65 factor of NF-kB upon palmitate treatment, confirming that palmitate is an essential factor responsible for proinflammatory cytokine expression through the TLR-4 expression pathway in astrocytes.

The canonical pathway for TLR based cytokine pathway is known. With new evidence suggesting histone deacetylases can play an essential role in cytokine expression and the factors involved in TLR4 based cytokine pathway, it leads me to the **hypothesis that HDACs are involved in the activation of the IKK complex to translocate NF-kB components to the nucleus and upregulate cytokine expression in palmitate treated astrocytes.** To test this hypothesis, we examined the role of histone deacetylases in palmitate-induced cytokine production in astrocytes.

2.5 Experimental Procedure

2.5.1 Cell Culture and treatment

CTX-TNA2(CRL-206), a rat astrocyte cell line, was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM medium (catalog number-11995065, Thermo Scientific, Asheville, NC, USA) and 10% fetal bovine serum (FBS). Aseptic conditions for the growth of cells were maintained by filtering the media in 0.22 μM filters and cultured in Class II Type A2 Biological Safety Cabinet. The cells must be grown at 70-80% confluency in T-75 flasks and then seeded into six-well plates before treatment. The seeding density of cells in the six well plate should be maintained around 7.5*10^5 cells/well, and after the cells achieve confluency of 70-80%, they are treated with the treatment media. Continuous passaging was maintained by using 0.25% trypsin and then seeded onto 6 well plate for treatment. The cells are ready for treatment only after they reach a confluency of 70-80%.

Long-chain fatty acids like palmitate are poorly soluble in an aqueous medium and thus become a hindrance for any in vitro or in vivo studies. Moreover, neurons have a low uptake of palmitic acid directly and requires to be effective upon cells by conjugating with albumin in the mammalian body [78] [79]. Thus, palmitate needs to be conjugated with free fatty acid Bovine Serum Albumin (BSA) to prepare treatment media. The effectiveness of palmitate on the cells depends upon the accessibility of unbound palmitate via the ratio of the total free fatty acid to BSA. Albumin-bound free fatty acids cannot enter cells, bind to proteins, or serve as substrates for free fatty acid utilizing proteins. Instead, it is the unbound free fatty acids that can be transported across membranes, bind to specific proteins sites, and cause enzymatic reactions. Keeping the concentration of BSA constant (37.5 μM), a gradient concentration of palmitate of 0.2 mM was maintained so that the molar ratio of palmitate and BSA remains at 6:1. With CTX- TNA2 at 70-80%, palmitate-BSA treatment was done and incubated for 24 hours. BSA-only treated astrocytes will serve as control. To the determine the impact of histone deacetylases on the palmitate treated astrocytes, treatment of the cells was made with 10mM valproic acid, a class I and IIa specific HDAC inhibitor both with palmitate and without palmitate conditions. As positive control treatment onto the astrocyte cells were made with 5mM L-cycloserine(L-CS) with or without palmitate. Treatments with saturated fatty acid stearic acid and non-saturated fatty acid oleic acid at molar ratio 6:1 with BSA was also being made as positive control.

2.5.2 Western Blot analysis for protein expression

Whole cell extracts were lysed with Radio- Immunoprecipitation Assay (RIPA) lysis buffer and the extracted proteins were quantified using BCA assay. 30 μg of lysate protein was run through 10-14% SDS-PAGE and transferred to PVDF membranes (catalog number-1620177, Bio-Rad laboratories, Hercules, CA, USA). Membranes was blocked by 5%milk in 0.05% Tween 20-TBS (Tris buffered saline) for 1 hour and incubated with specific primary antibodies overnight at 4^{O}C. The membrane was kept in contact with anti-rabbit HRP-conjugated secondary antibody and incubated for 1 hr. The blots were washed three times with 0.05% Tween 20-TBS, and then visualized by SuperSignal West Femto maximum sensitivity substrate (catalog number- 34095, Thermo Scientific, Asheville, NC, USA). Each condition was performed at least in triplicates and student t-test was used to test for analyzing statistical significance. Primary antibodies used for aim1 were HDAC1(catalog number-34589), HDAC2(catalog number-2540), HDAC3(catalog number-85057), HDAC8(catalog number-66042) and β-actin (catalog number-8457). All antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA.

2.5.3 Total messenger RNA (mRNA) extraction and quantitative real-time polymerase chain reaction

Total mRNA from the cells was extracted using the miRNAeasy plus kit (catalog number-217004, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total mRNA was extracted from the astrocyte cell line using TRIzol and the miRNAeasy plus kit. The total mRNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (catalog number-4368814, Thermo Scientific, Asheville, NC, USA) using the protocol as stated by the manufacturer. The following primer sets (Eurofin Genomic, Louisville, KY, USA) were used for polymerase chain reaction (PCR) for aim 1 are shown in table 1. The primers were designed and analyzed using primer3 and benchling software. Quantitative real-time PCR was performed using PowerTrack SYBR Green Master Mix ((Thermo Scientific, Asheville, NC, USA). The reaction program was cycle 1- Enzyme Activation -95 C for 2 minutes (1X), Denature-95 C for 15 seconds (1X) and Anneal- 60C for 1 minute (40 X) and cycle 2-dissociation step at 0.075 C/second for 15 seconds.

Gene Name	Primer Sequence
B-actin	5’- CCTGTGGCATCCATGAAACTAC-3’ (F) 5’- CCAGGGCAGTAATCTCCTTCTG-3’ (R)
TNF-a	5’-TCTCAAAACTCGAGTGACAAGC-3’ (F) 5’GGTTGTCTTTGAGATCCATGC-3’ (R)
IL-1B	5’- TGTGATGTTCCCATTAGAC-3’ (F) 5’- AATACCACTTGTTGGCTTA-3’ (R)
IL-6	5’- ACCACCCACAACAGACCAGT-3’ (F) 5’-ACAGTGCATCATCGCTGTTC-3’ (R)
HDAC1	5’-CATGCCAAGTGTGTGGAGTTCGT-3’ (F) 5’- GTCCAGCACCGAGCGACATT-3’ (R)
HDAC2	5’- GGCCTCAGGATTCTGCTACG-3’ (F) 5’- GACGGTCATCACGCGATCT-3’ (R)
IL-10	5’- ACCTGGTAGAAGTGATGCCCC-3’ (F) 5’- GGTCTTCAGCTTCTCTCCCAGG-3’ (R)

Table 1: Forward and reverse primers of genes used in RT-PCR experiments in aim 1.

2.6 Results

2.6.1 Palmitate treated astrocytes showed an increase in the HDAC2 protein expression

First and most important aspect is to check the expression of histone deacetylases in rat astrocytes upon the treatment of palmitate. As referenced in the method section, astrocytes cultured in palmitate at a molar ratio of 6:1. Based on evidence from previous literature studies, we have noted multiple treatments as positive controls for our treatment. Datasets from previous works in our lab has shown downregulation of cytokine expression upon treatment with palmitate and L-cycloserine. Thus, we were also intrigued to know how HDACs behave for the same. Treatment was also made with valproic acid (VPA), a class I and class IIa HDAC inhibitor, stearic acid, an 18 saturated fatty acid, and oleic acid, a 9-carbon unsaturated fatty acid. Overall, we checked the protein expression levels of all the class I HDACS.

Upon comparison with the expression of HDAC2 on astrocytes with no treatment, the expression of HDAC2 when treated with palmitate showed an upregulation in its protein expression. Conversely, upon treatment with L-CS and VPA, there was downregulation in the protein expression of HDAC2. There was no significant change in expression upon treatment with stearic acid and oleic acid (figure 9).

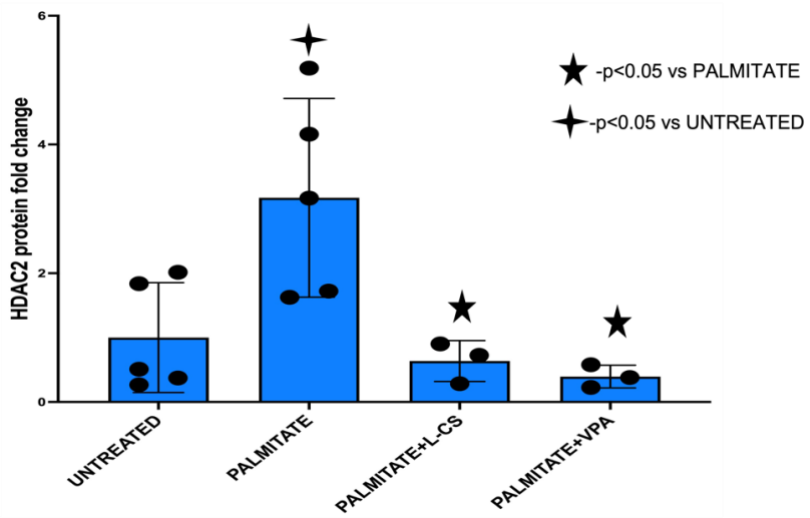


Figure 9: Quantitative Western blot dataset of HDAC2 proteins in untreated, Palmitate, Palmitate +LCS, Palmitate+VPA, Stearate, and Oleate treated CTX-TNA2 astrocyte cell line. Statistical analysis is done using student t-test. Number of biological replicates are greater than or equal to 3.

2.6.2 Palmitate treatment of astrocytes showed no significant change in the HDAC1 protein expressions

Saturated fatty acid palmitate treated astrocytes showed no change in HDAC1 expression compared to the untreated. In comparison, inhibition of HDACs with VPA decreased the expression of HDAC1(figure 10).

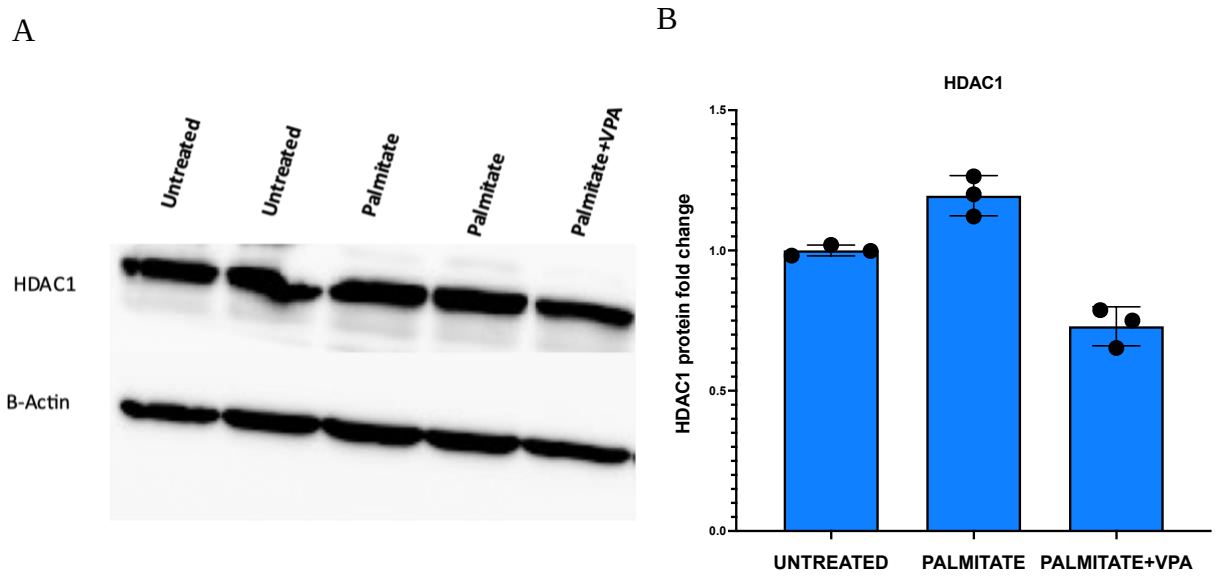


Figure 10: A) Western blot dataset of HDAC1 proteins(n=3) in untreated, Palmitate, Palmitate+VPA, treated CTX-TNA2 astrocyte cell line. B) Quantitative analysis of the blots. Statistical analysis is done using student t-test. Results are based on 3 biological replicates**.**

2.6.3 Palmitate treatment of astrocytes showed no significant change in the HDAC3 and HDAC8 protein expressions

Palmitate treatment and treatment with VPA did not show any significant change in protein expressions of the remaining class I HDACs HDAC3 and HDAC8(figure 11).

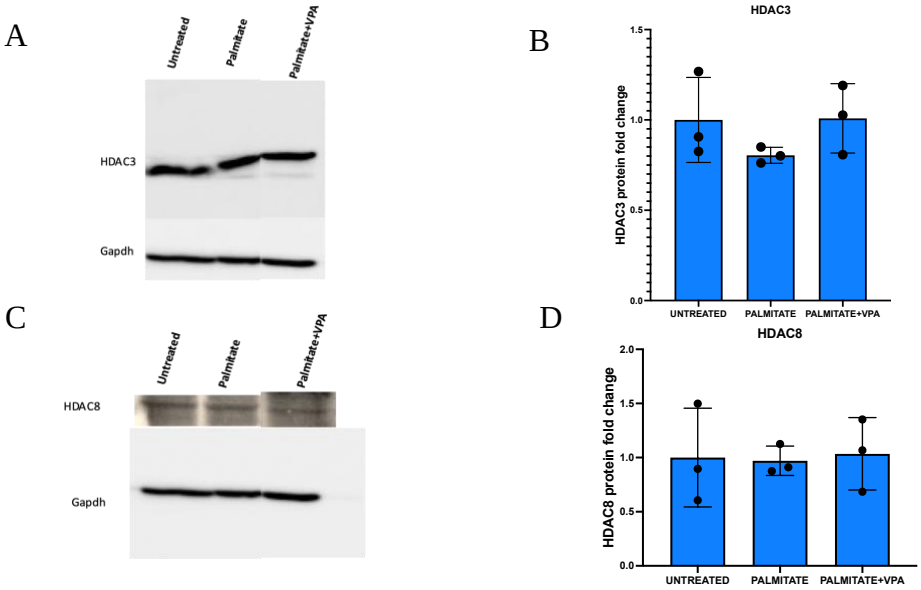


Figure 11: A) Western blot dataset of HDAC3(n=3) proteins in untreated, Palmitate, Palmitate+VPA, treated CTX-TNA2 astrocyte cell line. B) Quantitative analysis of the blots. Statistical analysis is done using student t-test. C) Western blot dataset of HDAC8 proteins (n=3) in untreated, Palmitate, Palmitate+VPA, treated CTX-TNA2 astrocyte cell line. D) Quantitative analysis of the blots. Statistical analysis is done using student t-test. Results are based on 3 biological replicates.

2.6.4 Expression of cytokine IL-6 upon treatment with palmitate and inhibitors specific to ceramide pathway and class I and II HDACs

To determine the connection between HDACs and the expression of cytokines, we studied the gene expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1β. The treatment conditions were the same as discussed in the previous results. The IL-6 cytokines expression was determined at a 6-hour interval. Unfortunately, the other cytokines, TNF-α and IL-1β cytokines) showed high CT values beyond 35(data not shown) for the RT-PCR results.

Palmitate treated astrocytes increases the gene expression of IL-6 cytokine compared to untreated cells. As a positive control and based on a previous study in our lab, palmitate plus L-CS treatment of the astrocytes decreased the expression of the cytokines. Interestingly, in palmitate plus treatment with VPA, a class I and class II a HDAC inhibitor, there was downregulation of the cytokine expression compared to the palmitate only treated astrocytes. To further study the

connection of the histone deacetylases and the expression of cytokines, L-CS and VPA were treated without the presence of palmitate. The VPA and the L-CS only treatment did not show any significant change in the cytokine expression, suggesting that HDAC activity depends on the palmitate dependent cytokine expression(figure 12).

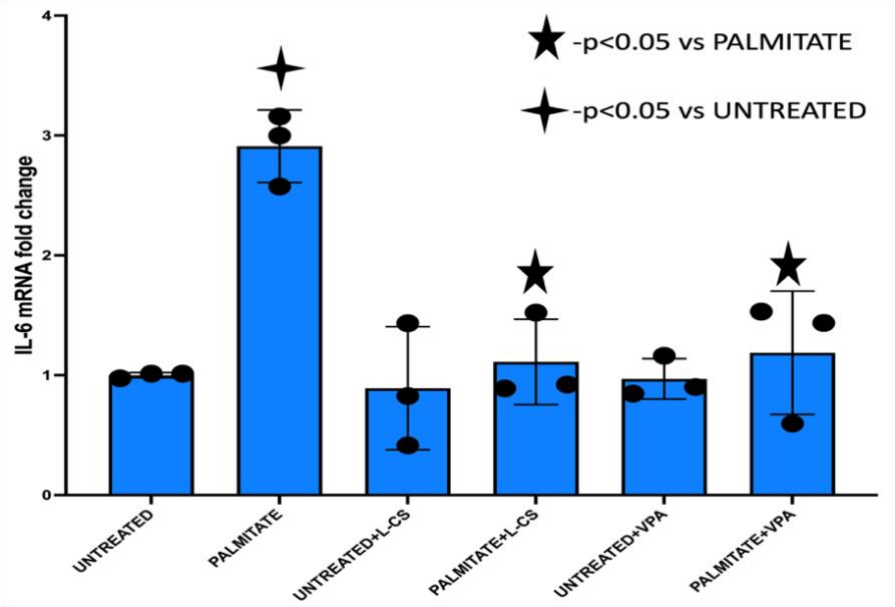


Figure 12: RT-PCR datasets of IL-6 pro-inflammatory cytokines. Statistical analysis is made using student t-test**.** Results are based on 3 biological replicates.

2.6.5 Effect of palmitate and valproic acid on anti-inflammatory IL-10 cytokine gene expression.

We can determine the state of astrocytes by the presence or expression of anti-inflammatory cytokines. The reactive astrocytes referred to as A1 are known to produce large amounts of pro-inflammatory cytokines and chemokines and A2 type astrocytes are known to regulate the expressions of anti-inflammatory factors[63 80].

Palmitate treatment of cortical astrocytes did not show any significant change compared to control. However, treatment of palmitate plus valproic acid caused a significant increase in the expression of IL-10 cytokines. Treatment with VPA only did not show a change in the expression of the cytokines. Though there was no significant change in the expression of IL-10 cytokines upon palmitate treatment, but the pro-inflammatory IL-6 expressions increased with palmitate treatment. This implies a neuroinflammatory state of the cytokines. Furthermore, the mRNA fold change of IL-10 cytokines was higher than IL-6 cytokines upon treatment with valproic acid, suggesting a neuroprotective state of the astrocytes.

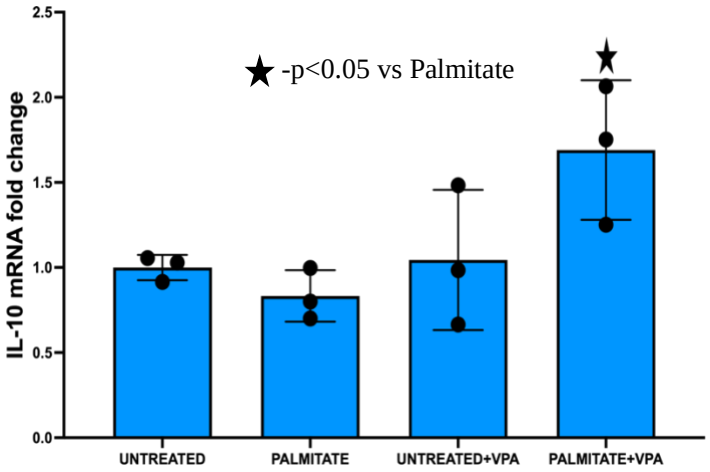


Figure 13: RT-PCR datasets of IL-10 anti-inflammatory cytokines. Statistical analysis is made using student t-test. Results are based on 3 biological replicates.

2.7 Discussion

The literature data suggests that histone deacetylases are involved in the expression of cytokines in most of the cells involved in immune signaling, such as microglia and macrophage. Previous studies in our lab and other literature evidence suggests palmitate upregulates the expression of pro-inflammatory cytokines in the astrocytes, and the release of high levels of cytokines is associated with neurodegenerative diseases. Literature also suggests palmitic acid to be dependent on the TLR-4 based pathway for cytokine expression. This pathway is dependent on the activation of NF-kB factors which is an important transcription factor for the cytokine gene expression. The major gap in this scenario is the role of HDACs in the palmitate based inflammatory pathway. This led to our hypothesis that HDACs impact the expression of pro-inflammatory cytokines in palmitate treated astrocytes. Interestingly, based on the datasets there was an increase in the expression of HDAC2 in palmitate treated rat astrocyte cell lines and no change in the protein expression of the other class I HDACs like HDAC1, HDAC3, and HDAC8. So far, we have not shown specific HDAC2 knockout experiments relative to the cytokine expressions to suggest our point, and it is also to be noted that increased protein expression is not indicative of the HDACs catalytic activity or its ability to perform protein-protein interactions.

Valproic acid (VPA) are short chain fatty acids considered pan inhibitors of class I and II HDACs. HDAC inhibitors (HDACi) have been used for a long time as treatment for multiple diseases; for example, VPAs that have been utilized long-term for the treatment of epilepsy as well as bipolar or mood disorders. So far, 5 HDACi have been approved by the US FDA and Chinese FDA.

Among them, SAHA, Belinostat, Panobinostat, and Romideposin is approved by the US FDA, while the Chinese FDA approves Chidamide. Valproic acid was known to inhibit the catalytic activity of HDACs, but until recently, the mechanism and nature of inhibition was relatively unknown. Literature evidence, VPA is bound to the amino acid chain via hydrophobic tunnels of class I and class IIa HDACs, performing a competitive inhibition that prevents the substrates from binding at the catalytic site and, thereby a decrease in the HDAC activity[81]. Interestingly, in addition to inhibiting catalytic activity, VPA can degrade HDAC2 via proteasomal interactions. VPA can increase the expression of Ubc8, an E2 ubiquitin conjugase that is part of the ubiquitination process and whereby the RLIM E3 ubiquitin ligase is activated. HDAC2 are specific substrates of RLIM and thus undergo proteasomal degradation[82]. We did notice a decrease in the expression of HDAC2 proteins compared to other class I HDAC proteins when treated with palmitic acid.

We wanted to understand the role of histone deacetylases in the expression of cytokines and thus treated the palmitate treated astrocytes with valproic acid. We observed that when there is inhibition of histone deacetylases via VPA, there is downregulation in the expression of IL-6 cytokines, thus supporting our hypothesis that histone deacetylases are involved in the expression of cytokines in palmitate treated astrocytes. We also studied the gene expression of pro-inflammatory cytokines such as TNF-α and IL-1β but unfortunately, the CT values were above 35 at all time periods of treatments (1hrs, 3hrs, 6hrs, 12 hrs, and 24 hrs.). The readings or data analysis for these cytokines could not be made. Based on the available results for IL-6 cytokines, the treatment of astrocytes with palmitate upregulated the expression of IL-6. When the same palmitate treated astrocytes were treated with VPA, there was a decrease in their expression. Based on the previously shown dataset in our lab, ceramide inhibitor L-CS was known to inhibit the expression of pro-inflammatory cytokines. Thus, we used this treatment as a positive control for our experiment. Where there is L-CS only and VPA only treatment without palmitate, there was no significant change in the expression of cytokines supporting that the impact of HDAC on the cytokine expression is specific to palmitate treatment.

Cytokines are small proteins released by cells that affect cell interactions and communication. There is presence of both pro-inflammatory and anti-inflammatory cytokines in the immune cells. The anti-inflammatory cytokines are the immunoregulatory molecules that control the pro-inflammatory cytokine response[83] and IL-10, a major anti-inflammatory cytokine that have the

principal function to repress inflammatory cytokine expression like IL-6[84] and TNF-α[85]. As mentioned before, the astrocytes can be regulated in multiple phases, A0 or resting phase, A1 or neurotoxic phase, and A2 or neuroprotective phase. In most scenarios, activation of A1 markers is accompanied by increasing pro-inflammatory cytokine expression, while A2 markers for astrocytes are denoted by increasing anti-inflammatory cytokine expressions. To get an idea of the preliminary state of the astrocytes upon palmitate treatment, we analyzed the mRNA expressions of IL-10 cytokines. Based on our data, the IL-10 cytokines remained unchanged with palmitate treatment but showed increased expression upon treatment with valproic acid. This suggests the presence of neurotoxic expression of astrocytes upon palmitate treatment and suggests the neuroprotective nature of astrocytes upon treatment with VPA. It is to be noted that the neuroprotective nature of VPA in palmitate treated astrocytes is a preliminary viewpoint and an in-depth study is required, especially the impact of markers for A1 and A2 astrocytes upon treatment with palmitate and VPA.

Chapter 3: Molecular mechanisms of Histone Deacetylases in NF-kB activation

3.1 Cytokine expression is dependent on the catalytic activity of HDACs

With a preliminary understanding that HDACs are an essential factor in the expression of pro-inflammatory cytokines, a significant gap remains about the mechanism of action as to how it assists in cytokine expression. Scientists have developed multiple scenarios based on theoretical and experimental setups to denote a possible pathway, but concrete explanations remain unresolved.

Some scientists have speculated the overall catalytic activity of the histone deacetylases could be an essential factor in the expression pathway. Scientists who have showcased the evidence of HDACs as a regulator of cytokine expression concluded that the catalytic activity of HDACs could cause downregulation of factors known to regulate the NF-kB negatively. There is a possibility for a potential pathway, especially when there is evidence of negative regulators playing a part in the cytokine expression pathway.

An example of a negative regulator of NF-kB factors is the Peroxisome proliferator activated receptor alpha (PPAR-α). It belongs to the nuclear hormone receptor family and play an essential role in modulating energy homeostasis by modulating glucose, lipid metabolism, and transport. Upon getting activated by agonists, PPAR-α has a high chance of suppressing inflammation via the downregulation of IL-6, TNF-α and NF-kB in the liver. In PPAR-α knockout mice, there is a decrease in the expression of IKβα proteins (factors that prevent the activation of NF-kB) compared to the wild type. In the same scenario, the same knockout mice showed upregulation of NF-kB factors and IL-6 cytokines. Transfection of PPAR-α onto HEPG2 cells showed decreased NF-kB luciferase activity. Using computational and wet lab analysis, PPAR-α was indeed found to bind the promoter regions of the IKβα genes, thereby providing an overall understanding of how PPAR-α negatively regulates pro-inflammatory cytokine expression[86].Now, the question arises about how HDACs or even histone acetylation can play a role in this. The answer lies in the evidence itself. Mouse liver lysates, when treated with grape seed procyanidin extracts, show a decrease in the HDAC activities of both class I and II HDACs. It is in co-relation with the upregulation of PPAR-α gene and protein expression[87]. This evidence showcases that receding HDAC catalytic activity is related to highly expressed PPAR-α and thereby causing the inhibition of NF-kB factors and overall cytokine expression and increasing HDAC activity is related to activation of NF-kB factors via decreased expression of PPAR-α.To be noted, PPAR-α is not the

only negative regulator of NF-kB; there are also factors such as NLRC3 and other NLR proteins such as Nod1[88] and Nod2[89] that are known to attenuate TLR receptor signaling by proteasomal degradation of the TRAF6 factors that ultimately inhibits NF-kB[90].

3.2 HDAC as a recruiter of transcription factor

Not much has been known about the other functions of the HDACs, but recently there has been evidence of HDAC2 being a recruiter of transcription factor such as paired box 5(PAX5) to the promoters of target genes.

A recent research article[91] showed HDAC2 to recruit a transcription factor PAX5 to the promoters of target genes in an HDAC activity-independent manner. The authors studied the differentiation of HL-60 cells, a human acute myeloid leukemia cell line, by treatment with 12-0-tetradecanoyl phorbol-13 acetate (TPA). They performed ChIP-seq on the HL-60 cells with antibodies specific to HDAC2, RNA pol II, acetylated H3, and H3K27me3. Heat map analysis revealed increased occupancy of acH3, RNA pol II, and HDAC2 upon TPA treatment, and they also showed HDAC2 to be associated with atypically active genes. They further showed the associated role of HDAC2 in the expression of these atypically active genes by siRNA knockdown of HDAC2. Interestingly, the knockdown of HDAC2 in differentiated HL-60 cells showed a decrease in the expression of the genes suggesting HDAC2 positively regulates gene expression despite having high acH3 levels. Overall, the evidence supports the idea that HDAC2 regulates gene expression independent of deacetylase activity. Furthermore, immunoprecipitation studies of overexpressed PAX5, a transcription factor, and TPA treated HL-60 cells showed an interaction of PAX5 with HDAC2. Moreover, PAX5 showed downregulated promoter activities of the atypically active genes, suggesting that HDAC2 recruitment of PAX5 is an essential factor in the expression of the atypically active genes involved in HL-60 cell differentiation.

The evidence discussed above gives a detailed account of the function of HDACs. Here the HDAC2 acts as a scaffold to bring in transcription factor without its canonical catalytic activity to express target genes. This could be a viewpoint ahead, especially in studying the role of HDACs in the expression of proinflammatory cytokines.

3.3 HDACs can undergo protein-protein interactions with the factors of IKK complex

Protein-protein interaction (PPI) is the high specificity contacts established between two or more protein molecules. These interactions can be electrostatic force-based interactions centered on polar and charged residues of proteins, hydrogen bonding, and hydrophobic effects, which are

primarily noncovalent. They are essential for metabolic pathways and signal transduction. In some instances, covalent bonding exists in post-translational modifications. Protein-Protein interaction is vital for the functional and structural aspects of the cells or their different components. PPIs help carry out many molecular machines built from numerous organized protein components. Overall, from the cellular point of view, PPIs are essential for muscle contraction, cell signaling, cellular transport, and biochemical pathways within the cellular system.

Protein-protein interaction based on electrostatic force is vital to multiple cell-signaling processes and is currently being studied by many scientists worldwide. Electrostatic force is related to the polar and charged residues of the proteins. There can also be short-range interactions such as salt bridge and hydrogen bond. Charges in proteins can be modulated by pH, phosphorylation, and dephosphorylation. Amino acids with charged ionizable chains, such as aspartate, glutamate, lysine, and arginine, are modulated in their charges when there is a change in pH. Phosphorylation and dephosphorylation of serine, threonine, and Tyrosine modulates the charges of the amino acid and, in turn, affect the signal transduction network. In many cases, acetylation of lysine that changes the charges of the lysine amino acid residues of histones leads to the protein-DNA interaction. The binding of Ca2+ or ATP at specific sites of proteins also impacts protein-protein interactions due to change in charge. Interestingly nonpolar amino acids undergo folding stability through hydrophobic interactions. Basic understanding showcases that like charges of amino acids repel each other, unlike charges attract each other. This charged interaction determines the binding specificity for ligands with receptors and substrates onto enzymes.

Interestingly, new evidence suggests the possibility of a protein-protein interaction between HDACs, especially of class I and II, with the activators of NF-kB factors such as IKK-2. An important point to notice from this is that the interactions were leading to an increase in the expression of pro-inflammatory cytokines. Among the class IIa HDACs, HDAC4[92], HDAC5[75], HDAC7[76] and HDAC9[77] are specifically involved in the positive regulation of proinflammatory cytokines. Class I HDACs HDAC1, HDAC2, and HDAC3 are involved in similar functions to class II HDACs for cytokine expression. But as mentioned beforehand, most of these observations have been showcased recently, so the overall mechanism of how the HDACs can positively regulate cytokine expressions is unknown. But the scientists who specifically worked on the HDAC7 and HDAC9 based inflammatory pathway did point out by experimental evidence that

there is a possibility of protein-protein interaction of the HDACs with the activators of NF-kB, especially the IKK-2 subunit of the IKK complex.

In the hippocampal and cortical mice brain, LPS treatment led to the expression of pro-inflammatory cytokine, which is dependent upon the upregulation of HDAC7 protein levels. Similar observations were observed in the cultured mice astrocytes. Overexpressed HDAC7 was relative to the increased expression of IKK-1 and IKK-2 subunits of the IKK complex and their activities. In the HEK293 cells, co-transfection of HDAC7 with the IKK complex subunits or Ikβα factors were being made. Now, coimmunoprecipitation experiments further corroborated the evidence of HDAC7 binding to the IKK-2 factor and other factors such as IKK-1 and NEMO factors of the IKK complex. Similar co-immunoprecipitation studies in HEK293 cells with co-transfection of HDAC9 with the IKK complex protein showed binding of HDAC9 with IKK-1 and IKK-2. So far it has been HDAC7, and HDAC9 are predicted as protein-protein interactors. However, how the other known HDACs regulate cytokine expression activity is still unknown. Some, like the evidence mentioned here, did show deacetylation of lysine residues of the IKK complex being involved in its activation upon binding with HDACs. Thus, giving us a broader scope of study for the association of HDACs in the expression of cytokines.

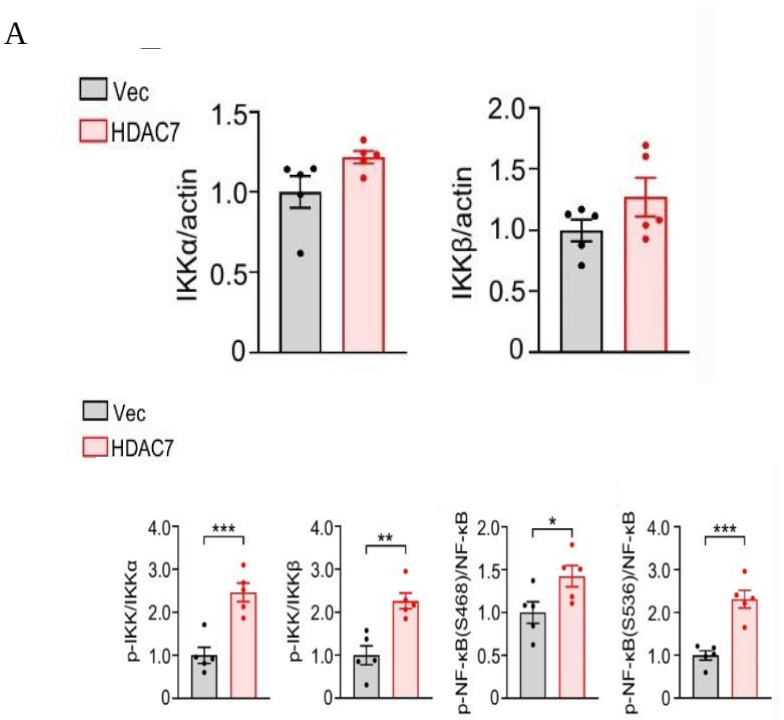
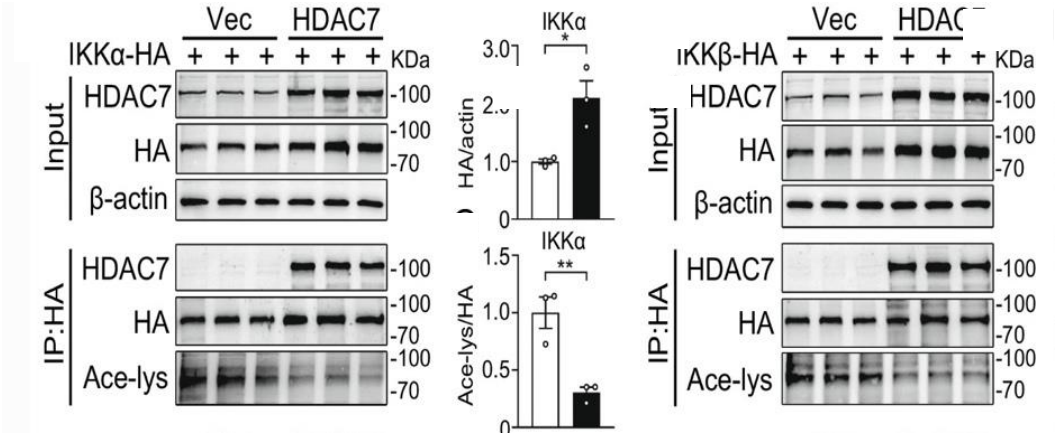


Figure 14: A) HDAC7 overexpression leads to increased phosphorylated IKK complex and NF-kB expression.

Figure 14 (cont'd)

B) HDAC7 binds to IKK complexes causing deacetylation. Reproduced from ref 67 (2022, Springer Nature) and ref 68 (2018, biochemical and Biophysical Research Communications).

B



3.4 Objective and hypothesis

Until now, there remains a major gap that is required to be studied: the possible molecular mechanism of HDACs in the palmitate treated astrocytes. Based on the evidence showcased, we notice multiple possibilities of molecular mechanisms being involved by which histone deacetylases can impact the cytokine expressions. They can impact the negative regulators of the TLR-4 cytokine pathway via its catalytic activity i.e., deacetylation of histone protein. They can recruit transcription factors that can impact the genes involved in the cytokine expression pathway or they can impact the cytokine expression by interacting with the protein factors involved in the cytokine expression pathway. These observations lead to the hypothesis to our second aim which is **Histone Deacetylases lead to the activation of factors associated with NF-kB by its catalytic activity or recruitment of transcription factor or protein-protein interactions.** To analyze molecular mechanism of HDAC activity, our major objective is to Identify the type of mechanism involved in the HDAC activity for cytokine expression.

3.5 Experimental Procedure

The cell culture and treatment protocols are same as the ones mentioned in 2nd chapter of this thesis report.

3.5.1 Western Blot analysis for protein expression

The protocol was same as the western blot analysis protocol mentioned in the 2nd chapter of this thesis report. The H3K9ac monoclonal primary antibody (catalog number-9649) and β-actin

monoclonal primary antibody (catalog number-8457) was used for this study. The antibodies were ordered from Cell Signaling Technology, Danvers, MA, USA.

3.5.2 Total messenger RNA (mRNA) extraction and quantitative real-time polymerase chain reaction

The protocol was same as what was mentioned in the 2nd chapter of this thesis report. The primer sets (Eurofin Genomic, Louisville, KY, USA) used for polymerase chain reaction (PCR) shown in table 2.

Gene Name	Primer Sequence
B-actin	5'- CCTGTGGCATCCATGAAACTAC-3' (F) 5'- CCAGGGCAGTAATCTCCTTCTG-3' (R)
IKK2	5'- GAGAGCGTCAGCTGTGTCC-3' (F) 5'- CCCCACACTTTCCTCATCTG-3' (R)
PPAR alpha	5'- TCAATGCCCTCGAACTGGAT-3' (F) 5'- TGCTCTGCAGGTGGAGCTT-3' (R)
p65	5'-GGGATGGCTTCTATGAGGCTGAAC-3' (F) 5'- GGGATGGCTTCTATGAGGCTGAAC-3' (R)

Table 2: Forward and reverse primers of target genes used in aim 2.

3.5.3 Theoretical Analysis for PAX-5 as a relative transcription factor at IKK2 genes using JASPAR

JASPAR (https://jaspar.genereg.net/) an open-access database was used to determine the binding of transcription factor onto specific promoter regions of IKK-2 genes. We tried to investigate possible binding of PAX-5 transcription factor onto the IKK-2 genes.

3.5.4 Theoretical analysis of protein-protein interaction using Molecular Docking

To obtain an understanding of the biochemical functions and the interactions of any protein on a molecular level, it is pertinent to have knowledge of the three-dimensional structure of the protein. In total about 52,500 solved crystal structures are reported in the protein data bank and UniProt database reports more than 6 million protein sequences. This creates a significant gap between the number of proteins in nature and the number of proteins that have their structures experimentally solved. The protein-protein interaction studies required in the project are the association of class I and II HDACS with the IKK complex proteins. The human HDACs crystal structure is available for all except HDAC5 and HDAC6. While the human IKK2, IKK-1, and NEMO factors of IKK

complex crystal structures are also available, the rat models of both HDACs and IKK complex still don't have an intact crystal structure. Homology remodeling is an alternative for the rat models that construct 3-D models of a protein from their amino acid sequences based on alignment in a similar protein of known structure. The critical steps of homology remodeling are: 1) Identification of relative proteins as templates having solved crystal structures that can serve as template to model protein of interest. 2) Sequence alignment to map residues of target sequence and template structure, 3) Generating 3-D structures based on the resultant alignment and 4) evaluation of the models.

The overall protein-protein interaction was studies using two types molecular docking software. First is a low-resolution H-DOCK software, this software was used to analyze the initial possibility of a protein-protein interaction. The data generates a model of possible binding whereby we can analyze it based on Docking score and Confidence score. Once the initial interaction was confirmed we moved on to the next step on high resolution docking using ROSETTA. The docking results in ROSETTA is generated via the HPCC provided by MSU and the structure were viewed using PyMol software

3.6 Results

3.6.1 Palmitate treated astrocytes showed downregulation in the levels of H3K9ac epigenetic modification

We not only checked the expression of histone deacetylases, but we also checked the expression of the H3K9ac histone modification to determine the activity of HDACs and provide us evidence for our theory of a possible histone modification activity that can impact the expression of cytokine expression. Palmitate treated cortical astrocytes showed a reduced expression of H3K9ac when compared to the untreated. Treatment with palmitate and VPA increased the levels of H3K9ac at a very high level (figure 14).

A

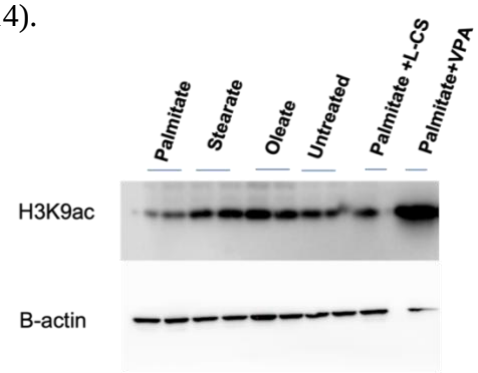
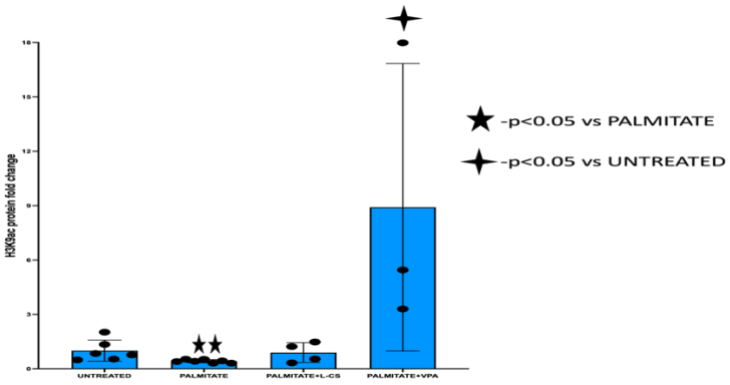


Figure 15: A) Western blot dataset of H3K9ac in untreated, Palmitate, Palmitate+VPA, treated CTX-TNA2 astrocyte cell line.

Figure 15 (cont'd)

B) Quantitative analysis of the blots. Statistical analysis is done using student t-test. Results are based on 3 or more biological replicates.

B



3.6.2 Histone Deacetylases as a recruiter of transcription factor

In certain HDACs as discussed in the background and review for literature, they have catalytic activity. Beyond that, they can also recruit transcription factors to the promoters of target genes and, help in gene expression. In the literature evidence discussed before, HDAC2 recruits' transcription factor PAX5 to the promoters of atypical genes. So, for our initial understanding, we wanted to notice the probability of transcription factor such as PAX-5 at the promoter sites of the activators of NF-kB such as IKK-2 genes. Theoretical analysis using JASPAR showcases the binding of Predominant motif binding sites in the 19-nucleotide motif sequence of IKK2 genes by PAX-5(figure 16 A). Before moving on to the stage where we want to show the binding of specific HDACs with PAX-5, it is necessary to see the gene expression of IKK-2 and subunit of NF-kB p65 when there is inhibition of HDACs.

The IKK-2 and p65 genes upon treatment with palmitate showed no significant change in the expression. This same non-significant change in gene expression is also noticed when treated with palmitate and valproic acid. Inhibition of HDAC has no impact on the gene expression of factors related to cytokine expression, suggesting HDAC has no part to play in the gene expression of factors such as IKK-2 or NF-kB and nullifies the possibility of recruitment of transcription factor (figure 16 B and C).

A

Matrix ID	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted sequences
MA0014.2	MA0014.2.PAX5	13.405265	0.884175476231003	seq1	1083	1101	-	ATAGTCAATAAAGCATGGA
MA0014.2	MA0014.2.PAX5	12.241392	0.870577580426497	seq1	1611	1629	+	ctgggtaacagagtgagac
MA0014.2	MA0014.2.PAX5	11.324827	0.859869060182066	seq1	1300	1318	+	ctgggcaactagcaagac
MA0014.2	MA0014.2.PAX5	10.375332	0.848775803884287	seq1	30	48	-	GCGGGCAGCGCGGCGCGAC
MA0014.2	MA0014.2.PAX5	8.395706	0.825647206064873	seq1	821	839	+	CAACGCAGACATGTGGGGC
MA0014.2	MA0014.2.PAX5	8.33477	0.824935271663927	seq1	1778	1796	+	ctaggtgacagagtgagac
MA0014.2	MA0014.2.PAX5	7.563901	0.815928960544278	seq1	147	165	-	GTcggcaggacgcgggggc
MA0014.2	MA0014.2.PAX5	7.379486	0.813774382935128	seq1	1484	1502	+	agaattagccaggcttggt
MA0014.2	MA0014.2.PAX5	7.3406515	0.813320666258952	seq1	1491	1509	-	acgtgccaccaagcctggc

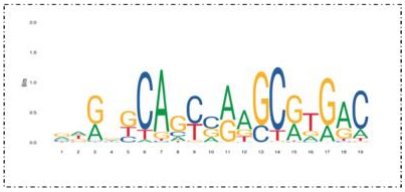
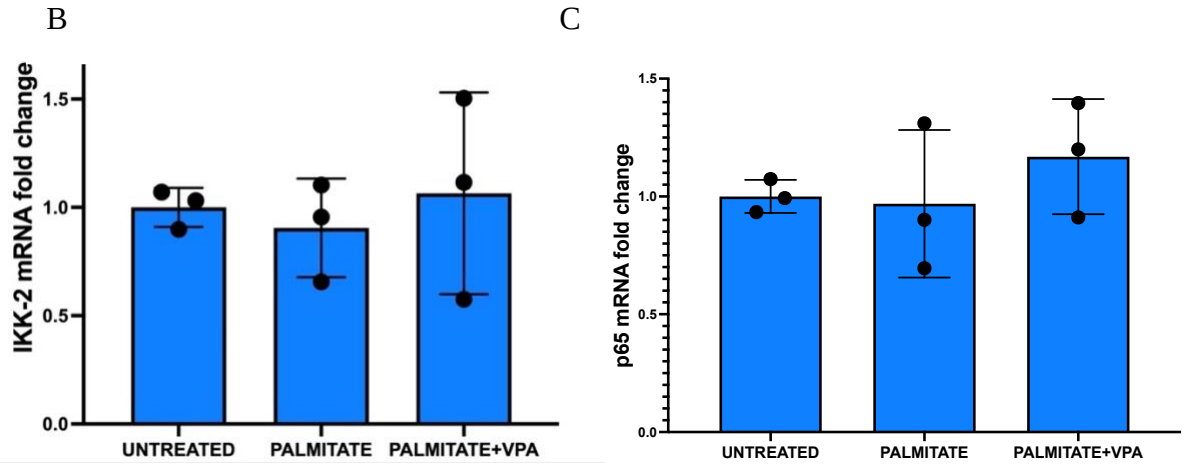


Figure 16: A) Jaspar dataset showcasing the binding sites of PAX5 on IKK-2 genes.

Figure 16 (cont'd)

B) RT-PCR datasets of IKK-2 and C) p65 factor of NF-kB. Results are based on 3 biological replicates.



3.6.3 Molecular Docking analysis to determine structural protein-protein interaction between IKK2 subunits of IKK complex and class I and class II HDACs

As mentioned in the experimental procedures, molecular docking studies will enhance the microscopic understanding of the HDACs-IKK interactions from a crystal structure perspective. We obtained six crystal structures of individual HDACs from the Protein Data Bank, depicted in Fig. 17A. The receptor crystal structure is also represented in Fig 17B. These structures were visualized in PyMol software to explore the possible molecular interactions stated in the following section.

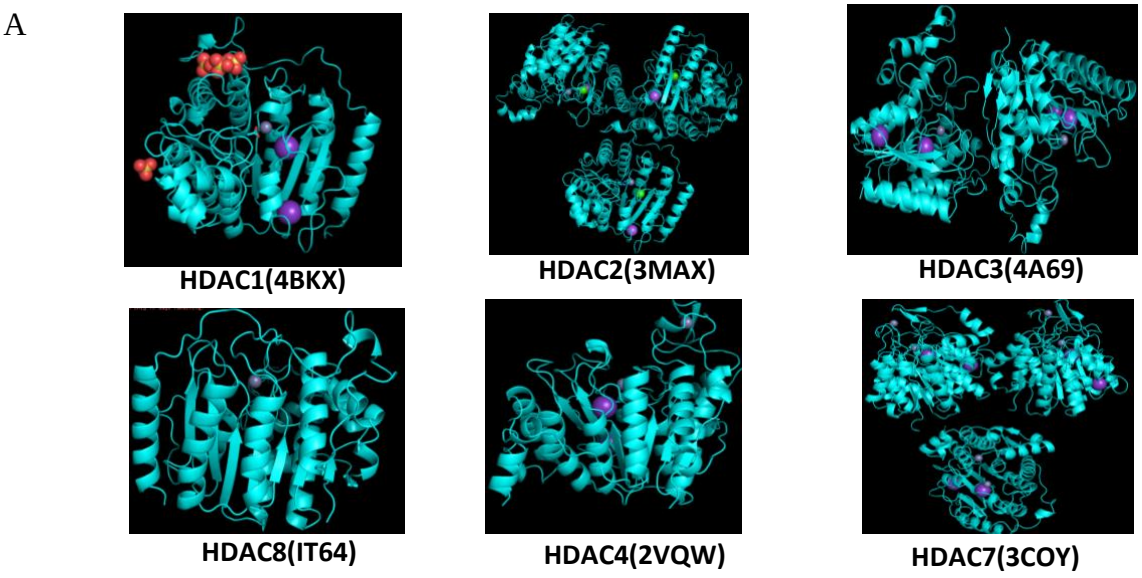
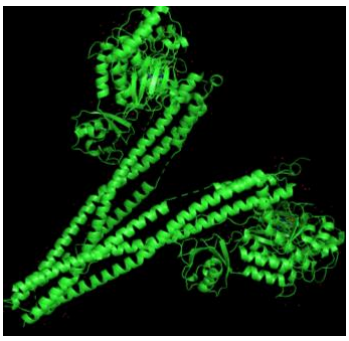


Figure 17: A) Crystal structures of Class I and Class II a HDACs with their PDB name.

Figure 17 (cont'd)

B) Crystal structures of IKK2 with PDB name.

B



IKK2 heterooligomer complex(4KIK)

After selecting proteins based on their crystal structure, we analyzed their interaction using molecular docking software. The proteins interacted with each other via noncovalent bonds among the amino acid residues of the individual proteins. In Figure 4, we have shown interactions of individual HDACs and IKK2. In Table 1, we noted all the interacting residues obtained from molecular docking. The bond distance for all the interacting residues was within 1-3 A, except HDAC8-IKK2 interaction in the range of 3-4 A. Molecular Docking was also performed between the HDACs and IKK1 subunits of the IKK complex (data not shown).

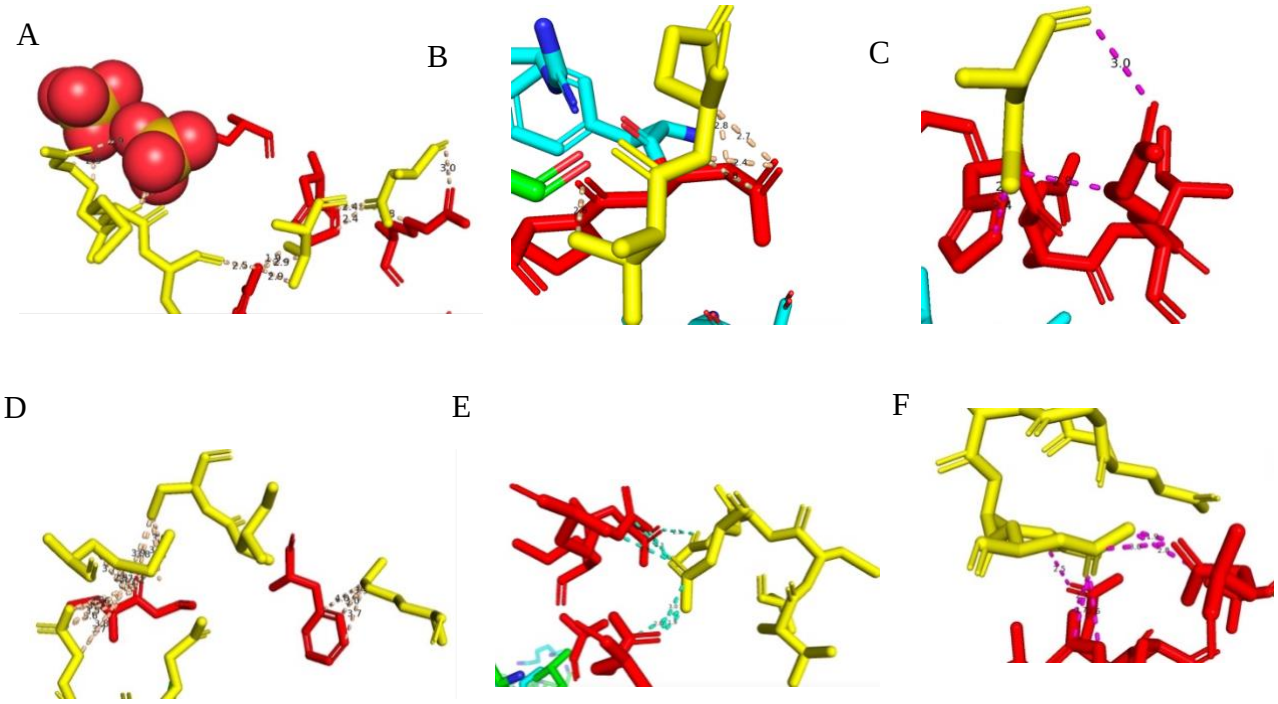


Figure 18: Amino acid interactions between specific HDACs and IKK-2. A) HDAC1, B) HDAC2, C) HDAC3, D) HDAC8, E) HDAC5 and F) HDAC7.Red residues- HDACs and yellow residues-IKK2.

Protein-ligand	HDAC(n) residue	IKK-2 residue
HDAC1-IKK2	Pro-324,Arg-36,His-33,Y-335,Arg-585, Phe-333	Arg-459,Asp-545,Thr-541,Met-536 Val-544, Lys-466
HDAC2-IKK2	Asp-296,F-295,Glu-257, Met-88,Glu-30	Phe-181,Val-288,Glu-229,Lys-300,Phe-295, Asp-296
HDAC3-IKK2	Phe-47,Thr-4,Tyr-324,Phe-328,Pro-333, Lys-366,Asp-369	Glu-137,Lys-300,Ser-130,Tyr-134,Asp-138,Glu-191,His-231,Tyr-293
HDAC8-IKK2	Phe-190,Arg-222,Tyr-223,Gly-211, Arg-355, Pro-272	Lys-364,Met-383,Glu-355, Ser-377,Arg-459, Asp-545, Arg-535,Leu-531
HDAC4-IKK2	Glu-1047,Asp-1048,Glu-1000,Lys-663, Leu-1000	Glu-515,Gln-646, Arg-650, Leu-634, Glu-229,Arg-525
HDAC7-IKK2	Leu-877,Glu-873,Lys-874,Arg-862,Thr-514, Glu-787	Asp-138,Arg-139,Tyr-135,Leu-68,Glu-137,Arg-139,His-283

Table 3: Representation of the interacting amino acid residues between HDACs and IKK-2. All the interactions with the interchain amino acids are taking place within the range of 1-3 A.

3.7 Discussion

The datasets obtained from palmitate treated astrocytes showcase the expression of class I HDACs as well the gene expressions of cytokines. The understanding we get so far is that there is an involvement of HDACs in the expression of cytokines. The requisite next step to understand is how HDACs impact cytokine expression. HDACs, in general, can catalyze the removal of acetyl functional groups from the lysine residues of histone and nonhistone proteins. In addition, they can act as recruiters of transcription factors at the promoter of genes and interact with NF-kB activators, thereby impacting cytokine expression. Therefore, the next set of experiments was performed to examine the functions of HDAC that affect cytokine expression. First, we wanted to determine the catalytic activity of HDACs, and for this, we wanted to investigate the expression of H3K9ac histone acetylation, the final product of HDAC catalytic activity. The product of the catalytic activity of HDACs is deacetylation of histones at its lysine residues. This determines the rate of histone modification, such as H3K9ac or acetylation of histone 3 at the 9^{th} lysine residue. For the preliminary determination of HDAC catalytic activity, we checked the protein expressions of H3K9ac. Interestingly, treatment with palmitate decreased the H3K9ac protein expressions, and treatment with valproic acid HDAC inhibitor increased H3K9ac protein expressions at exceptionally high levels. Based on this evidence, we can confirm the catalytic activity of HDACs. Still, we cannot confirm the impact of the catalytic activity in the expression of cytokines as an essential missing link in this aspect is the measurement of negative regulators of NF-kB and their

direct implications via HDACs and histone modification. It is also to be noted that palmitate plus L-CS treatment (positive control based on previous evidence from our lab) did not show any significant change in the expression of H3K9ac. Thus, further studies in the future are required regarding the catalytic activity of HDAC.

HDACs such as HDAC2 are known to recruit transcription factors such as PAX-5 that are known to enhance the expression of atypically active genes [91]. Based on this rationale, we tested the possibility of PAX-5 being recruited to the promoters of IKK-2 genes using the JASPAR database. Then to confirm the impact of HDACs via transcription factor recruitment at the promoters of activators of NF-kB, we measured the gene expression of IKK-2 and p65 factors upon treatment with palmitate and valproic acid. Finally, to test the protein-protein interactions of HDACs directly with the activators of HDAC, we performed molecular dock using H-DOCK software. Then we analyzed the amino acid residues that interact with each other.

The JASPAR datasets, with a relative confidence score beyond 8, confirmed that there are predicted binding regions of PAX-5 at the promoter region of the IKK-2 gene. However, gene expression of both IKK-2 and p65 factors of NF-kB remained unchanged upon treatment with palmitate and treatment with palmitate plus valproic acid. This implies that HDACs do not impact the gene expression of the cytokines or the factors dependent on its expression pathway. Instead, it puts up two possibilities. Firstly, HDAC catalytic activity-based histone modifications impact the expression of the inhibitors of NF-kB. Secondly, HDACs can directly impact the activators of NF-kB via protein-protein expressions.

Direct association of HDACs with the IKK complex is possible; there is multiple evidence to showcase that. Specifically, class II HDACs such as HDAC7 and HDAC9 are being shown to bind with the IKK2 factor via Co-IP experiment datasets in mice astrocytes and macrophages. But until now, we have no idea how this binding impacts the cytokine expression or have seen visual aspects of HDAC and IKK complex protein-protein binding. To base our theory that HDACs can directly impact the activators of the cytokine expressions, we performed molecular docking studies using H-Dock software and visually analyzed the protein-protein interactions between class I and class II HDACs with IKK-2(for canonical pathway) and IKK-1(for non-canonical pathway) of the IKK complex. Protein-protein interactions are high specificity contacts established between two or more proteins that are modulated by interactions such as electrostatic force, hydrogen bonding, or non-polar attractions such as hydrophobic interactions or Van der Waal's forces. The binding of

Ca2+ or ATP or SO4- at specific sites of proteins can also impact protein-protein interactions. Protein-protein interaction is essential for the proteins' stability and structural integrity. Functionally, PPIs can affect the signal transduction and expression of multiple pathways and factors in the biological system. The results show that class I and II HDACs can interact with the IKK-1 and the IKK-2 complex. The confidence score for the interaction was beyond 8 for all, and this suggested a high probability of the factors interacting with each other. We closely analyzed the interactions and noted down the interacting amino acid residue in the table shown. For analysis, we studied the interaction of only those interactions that are less than 3A. Most interactions occurred between positively charged amino acid residues such as lysine and arginine with negatively charged acidic amino acids such as aspartic and glutamic acids. This indicates a strong possibility of electrostatic interactions such as H-bonding. Interaction of non-polar amino acids with another non-polar amino acids or essential amino acids suggests a strong Van der Waal's interaction. Overall, the analysis of the interacting residues obtained from the docked structures illustrates a strong protein-protein interaction of HDACs with the IKK complexes. It will be interesting to examine the impact of these PPIs on cytokine expression in the future.

The datasets discussed here conclude that HDACs can impact the expression of cytokines in palmitate-treated astrocytes. The catalytic activity of HDACs is impacted upon palmitate treatment of astrocytes, though the role of histone modification upon the negative regulators of HDACs remains unknown. There is a high possibility of HDACs to impact the expressions of cytokines via PPI with the IKK complexes. Further study will be required to determine the theoretical datasets experimentally and the impact of cytokine expressions upon the HDAC-IKK complex formation.

Chapter 4: Future directions and experimental setup

We understand, so far, that histone deacetylases are involved in the expression of cytokines in palmitate-treated astrocytes. We also showcase the various ways by which HDACs can impact the expression of cytokines via catalytic activity or through protein-protein interactions. But many critical analyses are required to prove our preliminary observations further. Even though we understand the polarization state of astrocytes (A1 or A2) by the expression of pro-inflammatory and anti-inflammatory cytokines, it is requisite to confirm it by examining the expression of markers specific to them. Cascade component C3 is a marker specific for the A1 astrocytes and is generally expressed at a high rate in A1 astrocytes. While EMP1 is an important marker that is upregulated in the A2 state of astrocytes[93]. Thus, studying the expression of these markers is required to know the state and morphology of the astrocytes upon palmitate treatment. In addition to C3 other factors such as H2-T23, Serping1, H2-D1, Ggta1, Iigp1, Gbp2, Fbln5, Ugt1a1,Fkbp5, Psmb8, Srgn, Amigo2 are also well characterized markers for A1 astrocyte[94].Similarly, Clcf1,Tgm1, Ptx3, S100a10, Sphk1, Cd109, Ptgs2, Slc10a6, Tm4sf1, B3gnt5 and Cd14 are important markers of the A2 astrocytes[95].To confirm the state of astrocytes upon palmitate and valproic acid treatment, it would be important to note the expression of these markers using flow cytometry analysis. The flow cytometry-specific antibodies specific to these markers will be required for staining and analysis of cells, then will be made using flow cytometry.

Valproic acid (VPA) is a histone deacetylase inhibitor that can be used to identify the effect of HDACs on the biological system. However, it is not a very good indicator for identifying a specific HDAC's impact on the expression of cytokines. For example, based on our datasets, it can be concluded that class I and class II HDACs can inhibit the expression of cytokines. Still, we need further experiments to identify the specific HDAC responsible. It is also confirmed based on the datasets that VPA activity is specific to palmitate-based cytokine pathway itself, as treatment solely with VPA did not show any impact on the cytokine expression. VPA can bind the catalytic site of HDACs to prevent its catalytic activity and removal of acetyl group from histone and non-histone proteins. They can also increase the expressions of E2 conjugase enzyme Ubv8 that can associate with E3 ligases which have substrates specifically to HDAC2. The increasing expression of Ubv8 enzymes is an essential aspect of ubiquitination that leads to decreased HDAC2 protein expression. Thus, specifically for HDAC2 and based on our analyses, treatment with valproic acid, lead to the downregulation of HDAC2. We also confirmed this via the protein expression of other

class I HDACs such as HDAC1, HDAC3 and HDAC8, and showed no change in their protein expressions upon treatment with palmitate. This puts a bias towards our conclusion that HDAC2 is the specific HDAC that can probably impact the expression of cytokines. To prove this further, we would require a siRNA knockdown of HDAC2 by transfecting the astrocytes with siRNAs specific to HDAC2 using lipofectamine RNAiMAX in serum free Opti-MEM media. Since treatment with VPA itself did not show any change in cytokine expression, it is probable that siRNA knockdown of HDAC2 by itself will not show any change. Hence treatment with palmitate should also be made along with the transfection. We expect the HDAC2 silencing in palmitate treated astrocytes to impact the expression of both pro and anti-inflammatory cytokines as shown in palmitate plus valproic acid treated astrocytes. Given that we don't get the results as we expect, there is a possibility that the HDACs other than HDAC2 can also impact the cytokines. We did not check the activity of the HDACs so far except their expression. Expressions of HDACs, especially the ones we discussed so far did not show any significant change with both palmitate and VPA, will not determine two major factors. Firstly, the catalytic activity of the HDACs and the secondly the binding of HDACs to the IKK complex. If siRNA HDAC2 knockdown do not show the intended result, we need to identify the other HDACs part of the class I and class II HDAC in the cytokine expression pathway. HDAC activity assay could be another answer to test the catalytic activity of HDACs upon treatment with palmitate and VPA. It is a continuous colorimetric assay that can utilize cellular extracts or purified proteins. There is addition of HDAC specific substrates and then the products are recognized by specific antibodies provided by manufacturers. These antibodies contain fluorescence and when bound on the lysates or purified proteins, allows absorbance to read on a microplate spectrometer. The OD intensity measured will be proportional to the activity of the HDAC enzyme.

HDAC2 expression decreases to very low levels upon VPA treatment and that implies both the possibility of catalytic activity or binding activity. But to determine a particular method by which HDACs could impact cytokine expression, we would require a much in-depth study. Firstly, we would identify the specific as mentioned above. Once we identify the HDAC, we would mutate the genes specific to its catalytic residues using site specific mutagenesis. Aspartic acid, Histidine and Tyrosine residues can impact the Zinc co factor to form bonds with acetylated lysine residues of histone and non-histone proteins for most class I and II HDACs[96 97].Change in expression upon insertion or deletion of catalytic residue genes would make us proceed to the next step, which is

to identify the probable factors that can be impacted by the deacetylation activity. This analysis can be corroborated with our previous analysis where we show decrease in H3K9ac levels upon palmitate treatment and the reverse when there is VPA treatment. Finally, we would require identifying the factors that can directly or indirectly inhibition NF-kB transcription factors and showcase the involvement of Histone acetylation via ChIP sequencing. The ChIP-sequencing should be performed by antibodies specific to acetylated histones both in palmitate treated and palmitate plus VPA treated astrocytes. Based on datasets available we need to screen the factors that are downregulated upon palmitate treatment and upregulated upon treatment with VPA. The screened factors are required to be identified as inhibitors or related factors to NF-kB via literature study initially and then confirmed of its association via siRNA knockdown studies.

If the knockdown of catalytic residues does not show a change in the cytokine expression, we can move ahead with our observation showing protein-protein interaction individually with the IKK complexes. Theoretically, using molecular docking software, we show the possibility of a protein-protein interaction between the complexes and the class I and class II HDACs. Still, we need to prove it further using wet lab studies and analyze the domains of binding using high end stimulation software such as Molecular Dynamics. For co-immunoprecipitation studies, there are already prepared IKK2 and IKK1 plasmids as shown in the particular papers and then constructed onto pcDNA3.1 vector[76] can be utilized for transfection onto HEK-293 cells. HEK-293 provides the favorable scenarios for the expression large size proteins like IKK2. CTX-TNA2 has also shown favorable expression results with pcDNA3.1 vectors[98].In our future experiments it would be evident to transfect IKK1 or IKK2 onto the astrocytes followed by overexpression of specific HDACs and then perform co-immunoprecipitation assays to cross-validate our protein-protein interaction results. Molecular dynamic studies can give us the specific binding, the domain-based location of PPIs and structural changes in the interacting domains. It can also provide a specific interaction of the amino acid residue at different biological conditions such as temperature and PI.

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APPENDIX

Supplementary datasets

A.1 Gene expression study of PPARα

To check the impact on the expression of inhibitors of NF-kB upon palmitate and VPA treatment, we had measured the gene expression of PPAR alpha. Treatment with palmitate did not show a significant change in the PPAR alpha expression but showed a high increase in expression when treated with valproic acid. Overall, this dataset does not clearly indicate the impact of acetylated histone on the gene expression, so we require future experiments such as ChIP seq to confirm the association.

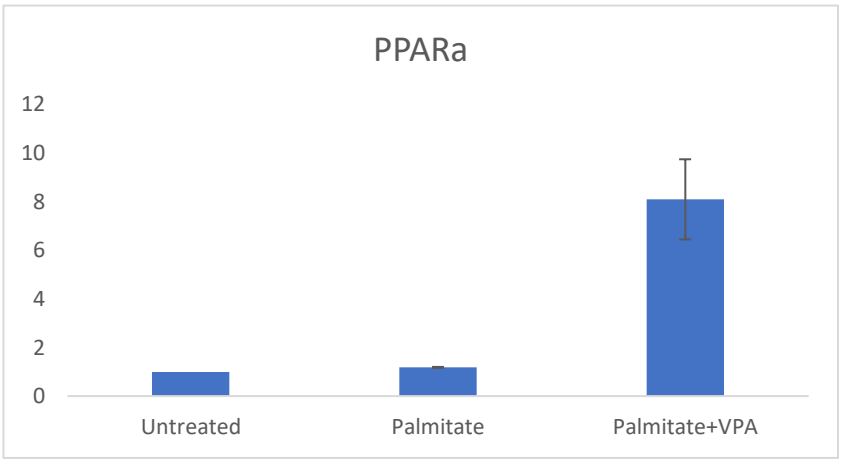
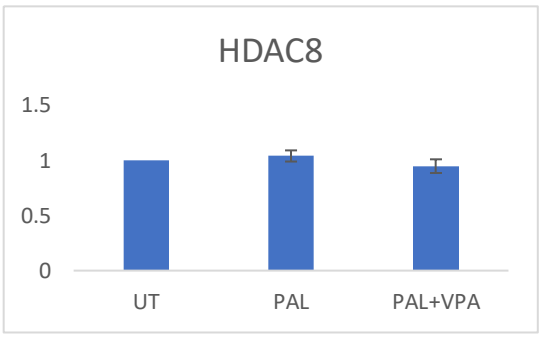


Figure A1: RT-PCR datasets of PPARα. Statistical analysis is made using student t test.

A.2 Gene expressions of class I HDACs

Not just the protein expressions, we had also measured the gene expressions of the class I HDACs. There was no significant change in the gene expressions of class I HDAC upon palmitate and VPA treatment.

A



B

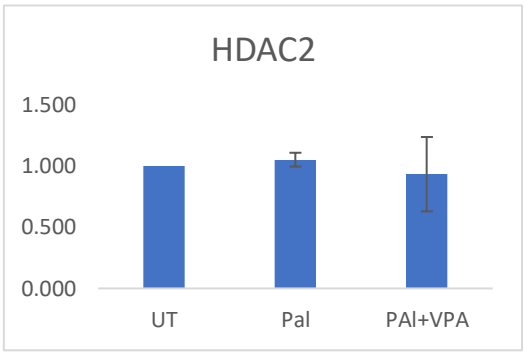
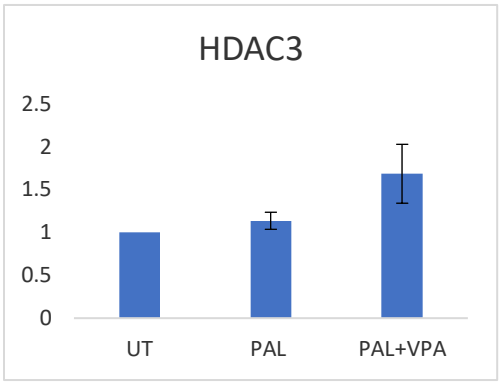


Figure A2: RT-PCR datasets of A) HDAC8 and B) HDAC2.

Figure A2 (cont'd)

RT-PCR datasets of C) HDAC3 and D) HDAC1.

C



D