Saikosaponin B2 Protects Against LPS Induced Inflammation In Neuronal Cell Lines

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Supervisor: Dr. Adeeb Shehzad

Department of Biomedical Sciences and Engineering

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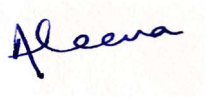
Student Name: Aleena Sajjad Signature: 

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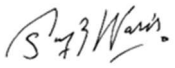
Name of Dean/HOD: Dr. Asim Waris Signature: 

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Dedicated to my beautiful daughters, Aina and Mariam for bringing light into my life. My loving husband, Asfandyar for his unwavering support throughout this journey. And my exceptional parents, Mohammad Sajjad Haider and Naila Sajjad for giving me the confidence and equipping me in all possible ways to meet all challenges, head on.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

SSB2	Saikosaponin B2
LPS	Lipopolysaccharide
A.D	Alzheimer's Disease
P.D	Parkinson's Disease
ROS	Reactive Oxygen Species
NO	Nitric Oxide
TNFα	Tumor Necrosis Factor-Alpha
BBB	Blood Brain Barrier

ABSTRACT

This study explores the protective effects of Saikosaponin B2, a triterpenoid extracted from the roots of *Radix bupleurum* against lipopolysaccharide-induced inflammation, a common model for studying neurodegenerative diseases. LPS induces strong immune reactions, activating pro-inflammatory cytokines, increases ROS generation, ultimately leading to neuronal damage and loss. We theorized that Saikosaponin B2 holds an anti-inflammatory and anti-oxidant profile capable of mitigating LPS-induced effects. An in-vitro model was used to determine the extract's effectiveness in reducing inflammatory gene expression, ROS generation and activation of injury mechanisms. Our results show that Saikosaponin B2 reduces LPS-induced inflammation, signifying its possible utilization as a therapeutic agent against neurodegenerative diseases.

Keywords: Neuroinflammation, Neurodegenerative Diseases, Alzheimer's Disease, Saikosaponin B2, Lipopolysaccharide (LPS)

CHAPTER 1 : INTRODUCTION

Neuroinflammation can be defined as the activation of immune system mediated by brain to protect Nervous System against diseases or chemical and mechanical insults and injuries. Research in the past years has established that neuroinflammation plays active roles in diseases characterized by degeneration of neurons like Alzheimer's Disease and Parkinson's Disease. It activates a number of components and mechanisms including; immune cell recruitment, changes at cellular and molecular levels, release of inflammatory chemical messengers, generation of Reactive Oxygen Species and cross-talk between signaling pathways. All this combined together results in a vicious cycle that results in chronic neuroinflammation, ultimately resulting in dysfunction of neurons and death. This information, supplemented by research in other aspects of A.D indicates that Neuroinflammation is a key player in the progression and initiation of neurodegenerative diseases. (Zhang, Jiang et al. 2015)

Neuroinflammation triggered as a consequence of peripheral inflammation involves the blood-brain barrier, microglial cells and astrocytes. It was previously thought that the Blood-brain barrier completely separated the peripheral system from the central one. On the contrary, the separation, mediated by a specialized endothelium, is only a partial one. In addition to being permeable to inflammation promoting cytokines of the peripheral inflammation, they allow the migration of leukocytes across the brain and themselves release these chemokines and cytokines. This neuroinflammation is responsible for progression and aggravation of several neuro-pathologies, impaired synapses and neuronal loss. (Lyman, Lloyd et al. 2014)

The major mediators of Neuroinflammation are microglia, astrocytes, EC's of the BBB and peripherally employed leukocytes. Otherwise, a protective mechanism, if activated for longer periods results in chronic activation of physiological, immunological and biochemical consequences. The degree and duration for which Neuroinflammation remains activated depends upon the type and duration of insult; therefor it is not an

equivalent concept and the context of Neuroinflammation determines its manifestation. (DiSabato, Quan et al. 2016)

Neurodegenerative diseases have become more prevalent owing to ever-increasing average age of the elderly people, worldwide. The next 15-to-20-year estimate of WHO states that neurodegenerative diseases will take over cancer as the most common cause of death after cardiovascular disease as a result of the increasing percentage of senior citizens especially in developed countries. A number of risk factors have been enlisted as the causes of ND's but aging tops this list. Determining the incidence of Neuroinflammation has remained a problem as it is a pathological mechanism and not a single disease. As Neuroinflammation remains a major hallmark of neurodegenerative diseases, its incidence is reported by explaining the epidemiology of these diseases. WHO reports that at present in excess of 55 million people are suffering from A.D, more than 60% of these numbers are reported from second and third world countries. 10 million new cases of A.D are reported annually. Globally reports state it as the major cause of dependance and disability in older population. The global prevalence for P.D in the past 25 years has doubled. WHO reported over 8.5 million cases in the year 2019. Disability adjusted Life years (DALY's) in 2019 increased by 81 % since 2000; prevalence was 5.8 million and reported deaths were 329,000. (Zaib, Javed et al. 2023) Treatment for A.D is currently making use of 2 pharmaceuticals. The first belongs to the Cholinesterase inhibitors class of drugs including Donepezil, Rivastigmine and Galantamine. (Howard, McShane et al. 2012) Second drug prescribed to patients suffering from varying degrees of A.D in Memantine which works both as a dopamine agonist and a competitive inhibitor of NMDA receptor. This is prescribed to patients who suffer from a short attention span and cant remain alert.(Grossberg, Manes et al. 2013) A.D is also managed by supplementation of Vitamin D and through Nutraceutical therapy combined with Huperzine usage. (Littlejohns, Henley et al. 2014, Shi, Lin et al. 2018) A precursor of dopamine, Levodopa (l-dopa, 1-3,4-dihydroxyphenylalanine) is used to treat the deficiency of dopamine in P.D patients. Due to first-pass effect it doesn't stay stable and is prescribed in combination with carbidopa and benserazide. Dopamine (Goldenberg and Therapeutics 2008) Dopamine agonist such as Pergolide and Monoamine Oxidase B (MAO-B) inhibitors are also used which prevent dopamine metabolism. (Jankovic, Aguilar et al. 2008, Emamzadeh and Surguchov 2018)

Drugs that target the inhibition of α-synuclein oligomers are in trial phases. (Oertel 2017) In cases where dopamine therapy fails, deep brain stimulation and gene therapy is used. (Herrington, Cheng et al. 2016)

Natural extracts have been used since thousands of years for therapeutic purposes. Natural products and their bioactive compounds have been explored in the recent past owing to a multitude of benefits including nutritional value and biological profiles. A number of compounds have been termed safe to use and declared as neuroprotective for the treatment of diseases that involve neuronal loss and decrease in synaptic plasticity.(Mohd Sairazi, Sirajudeen et al. 2020) Honey, containing over 200 compounds is reported to have neuroprotective effects. In kainic-acid induced excitotoxicity it significantly reduced brain oxidative stress and improved the morphology of the hippocampus.(Mohd Sairazi, KNS et al. 2017) Propolis, a bee product contains terpenes, phenols, flavonoids and alcohol. It is reported to reduce NO levels, generation of ROS and TNF-α and glutamine synthase levels. It also reduced neuronal loss in kainic acid-induced excitotoxicity.(Kwon, Park et al. 2004) Indian ginseng, more commonly known as Ashwagandha (*Withania somnifera*) is a medicinal plant reported to have restored dopamine levels and reduced the expression of acetylcholinesterase enzyme.(Jayawanth Manjunath 2013) Traditional Chinese Medicine has made use of Radix bupleuri (Bupleureum chinense DC) for more than two thousand years for the treatment of a number of ailments. Known for its neuroprotective effects it is a commonly used treatment of depression and neurologic disorders. The underlying mechanisms of its protective effects are however scarce.(Wang, Li et al. 2023)

1.1 Problem Statement:

The part played by neuroinflammation in degenerative diseases like Alzheimer's Disease gained attention in the past years. Ground-breaking research has led to a significant increase in understanding of Alzheimer's Disease, but the mechanisms by which neuroinflammation progresses this disease remains unraveled. This gap has retarded the development of personalized therapeutic regimens, hindering the ability to prevent, manage and treat Alzheimer's Disease. The expected burden of Alzheimer's combined

with an ever-increasing aging population is going to overwhelm the healthcare systems and cause socioeconomic problems. This study aims to explore the underlying pathways of inflammation of neurons in the context of A.D and determine the role of Saikosaponin B2 as a potential therapy against it.

1.2 Null Hypothesis:

Saikosaponin B2 does not protect against LPS induced Inflammation in Neuronal Cell Lines.

1.3 Alternative Hypothesis:

Saikosaponin B2 protects against LPS induced Inflammation in Neuronal Cell lines.

1.4 Aim of Study:

I. Scientific Validation of the traditional knowledge of Saikosaponin B2.
II. Determination of underlying molecular mechanism of LPS-induced inflammation.
III. Cost effective therapy for mitochondrial dysfunction.

CHAPTER 2 : LITERATURE REVIEW

2.1 Inflammation:

A defense mechanism employed by the immune system is inflammation. Septic injuries and non-septic invasions can trigger the innate immune system which activates inflammatory responses. This response ranging from being systemic and local is mediated by the activation and interaction of different cells and molecules that transmit signals between different cell compartments. The main players of inflammation include the leukocytes (WBC's) and the Endothelial cells (EC). Derivatives of phagocytes, which include leukocytes and monocytes have the ability to cross the tissues and they are mainly involved in antigen presentation and phagocytosis.

2.2 Innate and Adaptive Immunity:

The chemical messengers are either locally acting molecules in the form of Nitric Oxide (NO), lipid molecules, prostaglandin and its derivatives and a huge number of proteins that are complex in nature; the cytokines. Pathogen-Associated-Molecular Patterns, otherwise known as PAMP's drive the responses of innate immunity which are generic; these are short lived and prompt. In contrast the adaptive immunity which includes Tand B type lymphocytes takes much longer to come in action and produces very specific response to insults.

2.3 Cellular Adhesion Molecules:

In the beginning, resident or inactive leukocytes provokes the EC's to present the Cellular Adhesion Molecules or CAM's that result in the induction of more leukocytes to the place where the tissues were damaged. These CAM's bind and attract the leukocytes from blood circulation, and allow them to attach to the EC after retarding their speed. As a result, the endothelium undergoes a change in its morphology and becomes penetrable to the newly attracted leukocytes at the site of injury. At the tissue site, macrophages arise from the monocytes which are capable of phagocytosis and secretion of chemokines that

activate and induct more responsive elements from the periphery. In this manner, the response is regulated; from a local one to a full-blown systemic response. (Quan, Stern et al. 1999)

Initially aimed at clearing and controlling the primary insult by using mechanisms of phagocytosis, enabling tissue regeneration, formation of scars and triggering apoptosis; inflammation is mainly a protective mechanism. An excess of the same can be a causative factor and main pathological mechanism of permanent tissue damage and disease. In such a scenario, the periphery involved and the main site of damage are the targets of the activated inflammatory system. (Fernandes-Alnemri, Yu et al. 2009)

2.4 Peripheral Inflammation:

Inflammation at the periphery activates a response from the Nervous System; the Neuroinflammatory response. This encompasses inflammation of the microglia, the blood brain barrier and the astrocytes. It is different from the peripheral inflammatory response in that the main cells employed are essentially different i.e. neurons. It was earlier thought that the nervous system enjoyed the status of 'immunity' however recent studies have concluded that the endothelial cells of the blood brain barrier do not 'separate' the central nervous system from the peripheral nervous and immune systems. (De Vries, Blom-Roosemalen et al. 1996) In addition, it is permeable to pro-inflammatory cytokines allowing activation following release, releases these cytokines itself and allows the migration of leukocytes in to the brain. This permeation and cytokine response manifests the impaired synapses, loss of neuron and progression of neurodegenerative diseases such as Alzheimer's and Parkinson's.(Kitazawa, Oddo et al. 2005)

2.5 Neuroinflammation Cellular Components:

To understand what role peripheral inflammation plays in neuroinflammation, the endothelial cells of the Blood Brain Barrier and transport mechanism needs to be understood. Chemical messengers and other proteins were considered to be large; the concept that these substances could not reach brain from blood was rejected recently when research about transport mechanisms evolved. It was observed that active transport

mechanisms were employed to transport TNF and Inter-leukin molecules from and to the brain.(Gutierrez, Banks et al. 1993)

2.5.1 Cytokines:

Organs that have incomplete blood-brain barriers such as the circum-ventricular organs are particularly involved in bulk transport of cytokines. These molecules such as IL-1β and IL-6 are involved in a reduction of permeability and BBB integrity; thereby facilitating the entry of leukocytes in to the brain. (Laflamme, Lacroix et al. 1999)

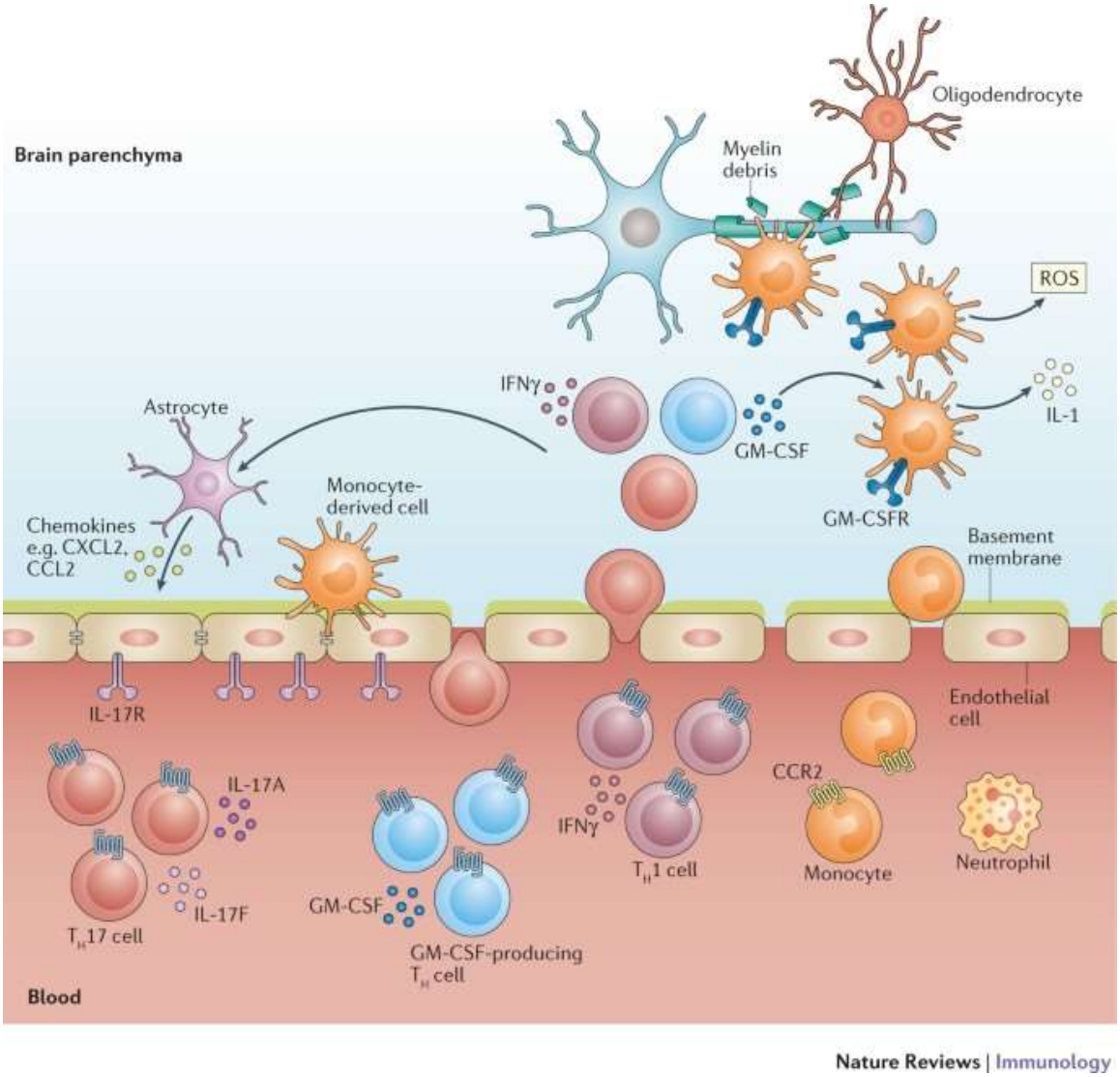

Figure 2.1: Delivery of Cytokines in inflamed CNS(Becher, Spath et al. 2017)

2.5.2 Tight Junctions:

Tight Junctions are important component of the blood-brain barrier. They are present between the Endothelial Cells and an alteration in the resistance between them modulates changes in permeability of the BBB. (Wong, Dorovini-Zis et al. 2004) This alteration in the resistance of tight junctions may result from damage to proteins such as "Occludin". When it's interaction with the cytoskeleton is affected, the BBB permeability increases. Another translation of the peripheral inflammation into the neuroinflammation results from the vagal stimulation of peripheral cytokines. This directly affects the CNS and mediates behaviours related to neurologic disease .(Fung, Vizcaychipi et al. 2012).

2.5.3 Chemokines:

Some humoral factors regulate the movement of leukocytes across the endothelial cells of BBB. Chemokines such as CCL19 and CCL 21 mediate the adhesion of T cells to the Blood Brain Barrier. On the other hand, CXCL 12 plays a negative role in T cell adhesion and infiltration. Astrocytes are the major producers of these factors. These along with the glial cell activation and the subsequent release of chemokines regulate the reduction of integrity of the BBB. (Engelhardt and Neuroimmunology 2010)

2.5.4 Microglial Cells:

Research has determined the pivotal role played by microglia in neuroinflammation. These are resident cells in the brain, having very fine processes for picking up pathogens or damage. As resident cells, their morphology is such that they have ramified cell bodies in the brain. In case of acute inflammation, upon activation, they become phagocytic cells and secrete pro-inflammatory cytokines. In case of chronic inflammation they have the potential of remaining activated for longer durations and remain a source of cytokines and other harmful molecules that progress mechanisms such as neurodegenerative diseases. (Liu, Hong et al. 2003)

Activation of macrophages occurs in different manners. For ease, this activation is classified into two major forms; M1 and M2. In M1 activation mechanism, a robust immune mechanism is displayed by the effector macrophages, activated by TNF-αand IFN-γ. This is the classical mechanism of macrophage activation. In an alternatively activated mechanism, all the macrophages other than the effector macrophages are involved. Their stimulation depends upon IL-4 and they mediate mechanisms of healing of wounds. The development of peripheral inflammation is to some extent dependent upon the shift from M2 to M1 mode of macrophage activation. This is also linked to the activated microglia in the CNS although the underlying mechanism of this relevance is still not understood.

Another class of neurons are the astrocytes which also release inflammation promoting chemical messengers. An example is the release of TNF-α. In comparison to microglia, they release less cytokines but play significant roles in the maintenance of synapses in terms of regulation and function.(Liu, Hong et al. 2003) In neurodegenerative diseases it is known that microglia, astrocytes and endothelial cells of the BBB are all in communication with each other, the neuroinflammatory responses of one kind of neurons impacts the response of others as well. (Abbott, Rönnbäck et al. 2006)

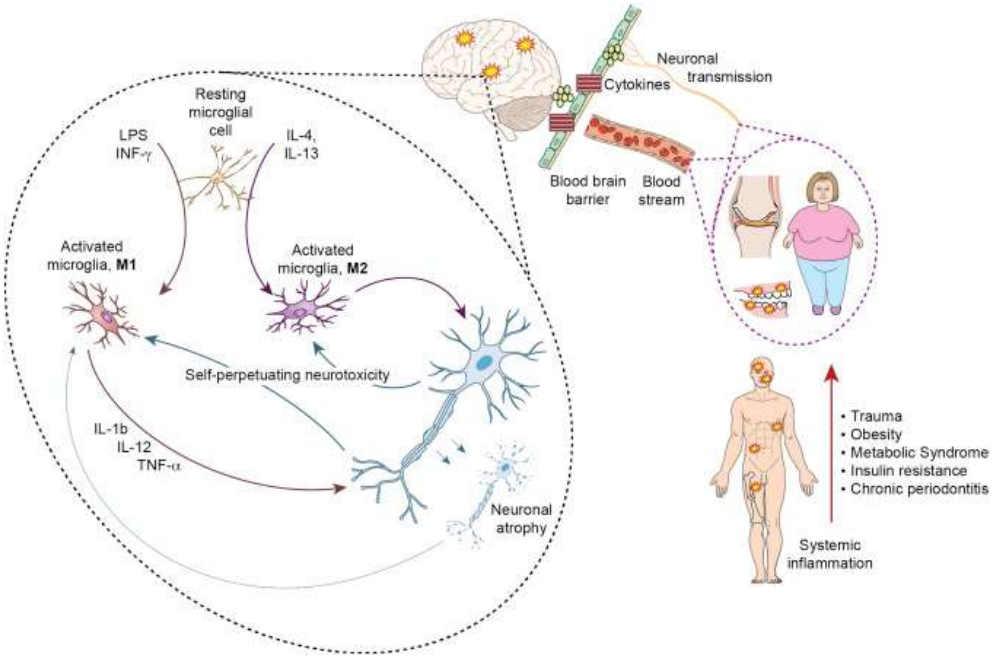

Figure 2.2: Mechanisms of Microglial activation and Neuronal Death (Calsolaro, Edison et al. 2016)

2.6 Neuroinflammation Molecular Components:

Cytokines are proteins in nature and they regulate the process of neuroinflammation. Signaling of Il-1 and TNF-α are reported to play important roles in progression of diseases and the inflammatory response. In contrast cytokines such as IL-4 play the opposite role; it reduces inflammation. Roles of cytokines, in disease and normal physiological states very significantly and as such one single label of pro-inflammatory or anti-inflammatory does not suffice the dynamic roles played by these signaling molecules.

2.6.1 Interleukin-6:

Release of pro-inflammatory cytokines fuels the release of more such molecules. As in the case of IL-6, the antigen presenting T cells release fibrinogen and C reactive protein (CRP). (Jung, Fan et al. 2002)

2.6.2 Tumor necrosis Factor – Alpha:

TNF-α binds its receptor and ultimately aids in the transcription of genes which promote degeneration and damage. TNF type-1 associated Death domain protein (TRADD) and TNF receptor-associated factor 2 protein (TRAF 2) proteins, which activate NF-κB, JNK and other transcription factors which promote cell death and damage. The culmination of the TNF signaling cascade culminates in apoptosis mediated by the Fas-associated protein with death domain (FADD). This pathway activates Caspase 8 and ultimately results in cell death. (Kischkel, Lawrence et al. 2000) .

2.6.3 Interleukin-1 Beta:

MAPK (Mitogen Activated Protein Kinase) pathways are activated by the binding of IL-1β to its receptor. Like JNK, p38 MAPK is involved in responses against stress mechanisms and it secretes cytokines like IL-8 and 6. (Sokolova, Hill et al. 2009)

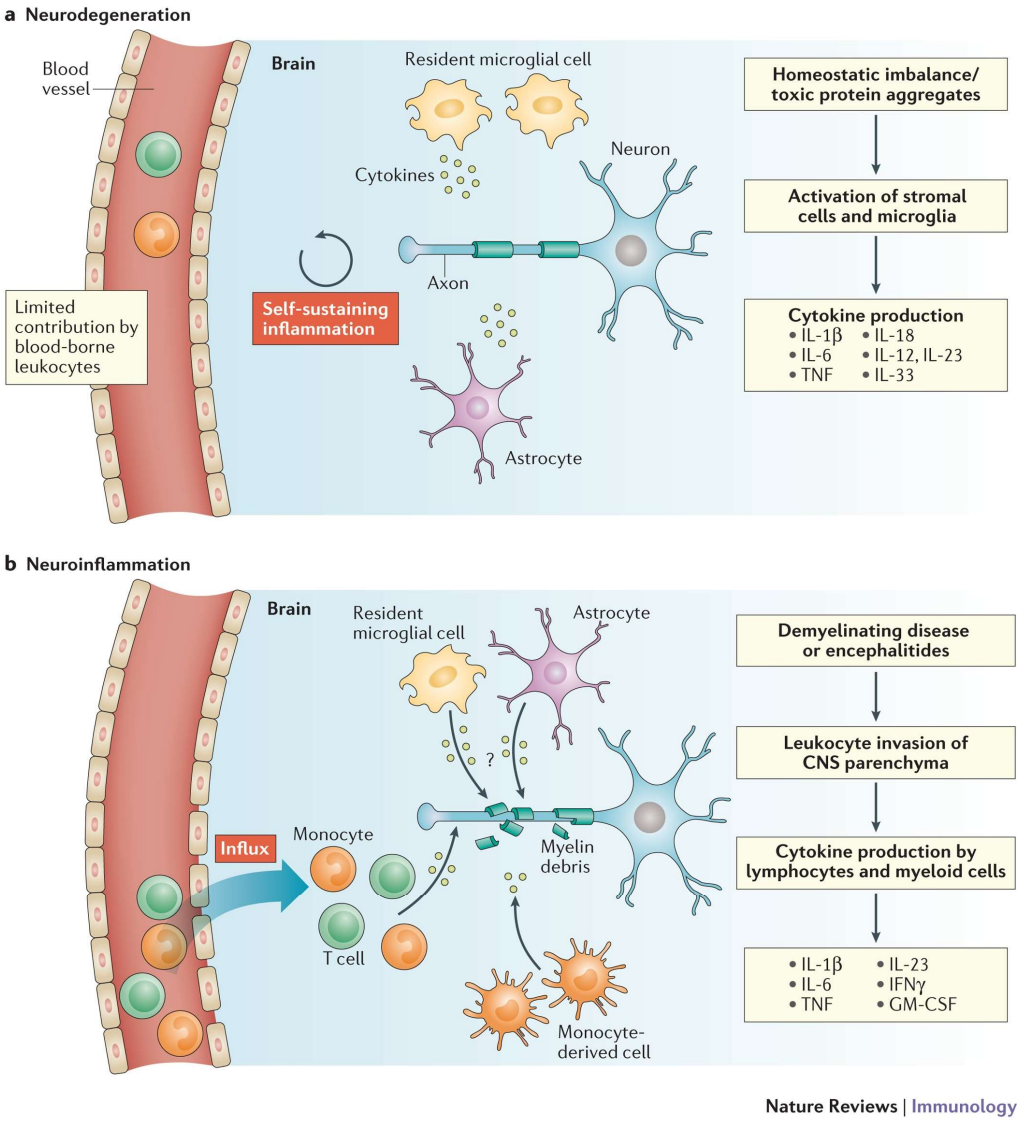

Figure 2.3: Cytokine Release during Neuroinflammation and Neurodegeneration (Becher, Spath et al. 2017)

2.7 Neuroinflammation Outcomes:

2.7.1 Microglial Priming:

Research from the recent years has reiterated that activation of microglia plays a significant the translation of peripheral inflammation to the progression of neurodegenerative diseases. Immune cells produce a response that is inflammatory in nature when activated by an insult. This happens after the immune cells get sensitized to

primary mechanical or chemical insults. This mechanism, principally a characteristic of macrophages, is also exhibited by microglial cells as well. Microglial priming in A.D occurs by the presence of the Aβ plaques. They produce NADPH mediated, reactive oxygen species leading to neuronal death and toxicity. (Murray, Sanderson et al. 2012)

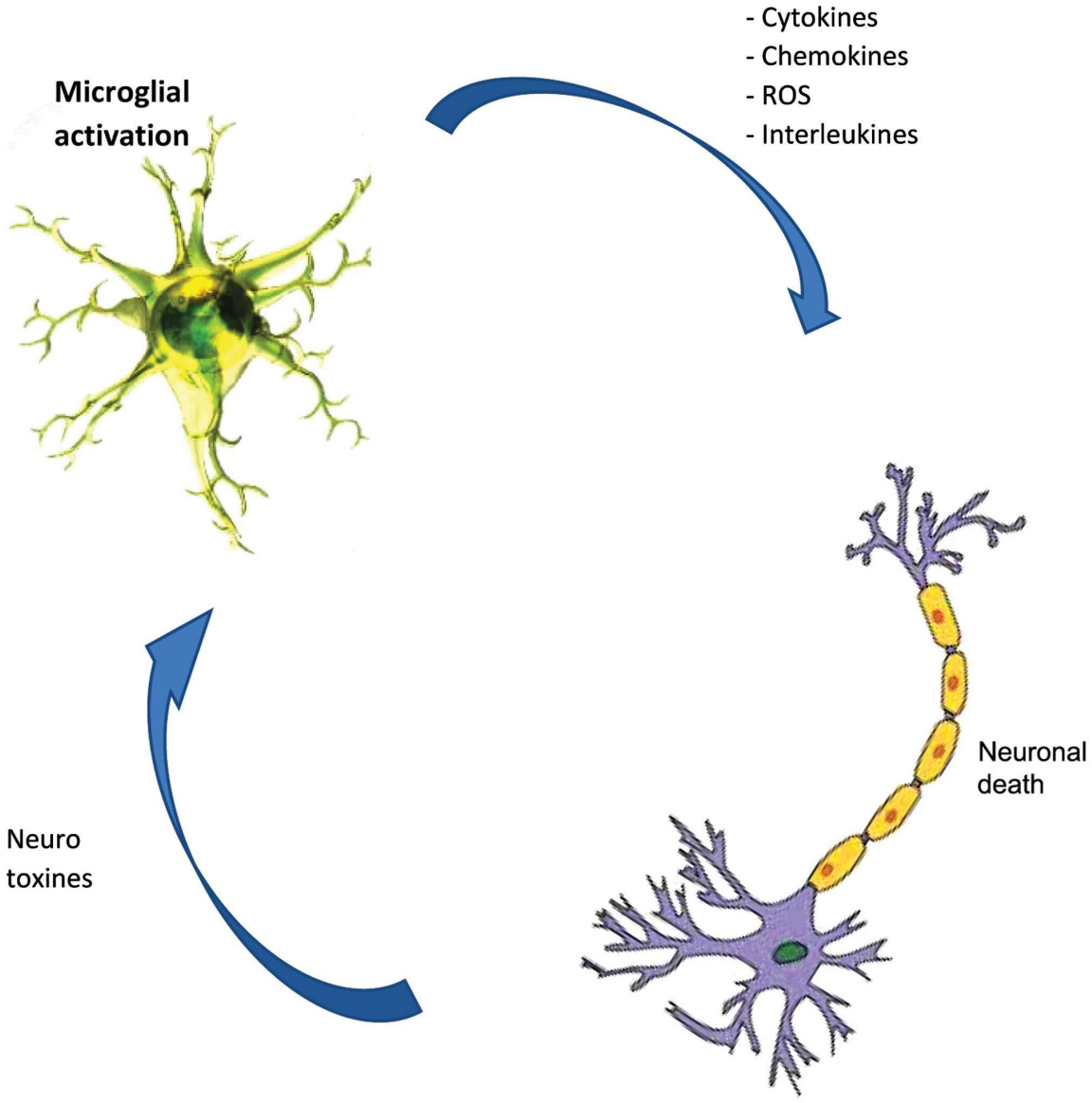

Figure 2.4: Mechanisms of Microglial Priming (Chen, Zhang et al. 2016)

2.7.2 Synaptic Impairment:

Synaptic dysfunction is a hallmark of both chronic and early stages of neurodegenerative disease. Synaptic impairment is a process that occurs long before cell death and makes the cell incapable of proper neurotransmission due to compromised connections. It manifests as either loss of function or synaptic plasticity. Plasticity is related with the process of memory formation and consolidation. Under physiologic conditions TNF-α and IL-1β levels maintain synaptic plasticity.(Carrero, Gonzalo et al. 2012) However, the overexpression of these cytokines under neuroinflammation results in the sensitizing of NMDA receptors in pre and post synaptic terminals to glutamate. This mechanism inducts COX-2 genes which are also implicated in NMDA sensitization through prostaglandin.(Stark and Bazan 2011) CNS insults such as mechanical injuries result in synaptic impairment, where a partial loss of function is observed. Synaptic function and integrity loss occurs before conditions such as taupathy. The exact underlying mechanism is still unclear but microglia assisted loss of neurons is involved. This highlights the role of neuroinflammation from the beginning in the progression of disease. The exact mechanism by which microglial cells confer synaptic plasticity is still confusing but it is understood that altering activity at the synapse, results in synaptic damage which manifests as loss of function and impairment both. (Vinet, van Weering et al. 2012)In tri-partite synaptic mode of transmission, in addition to two pre and post synaptic terminals, there is a third astrocytic process involvement as well. As discussed above as well, astrocytes are mainly responsible for maintaining the synapses. Inflammation mediated by astrocytes affects the nearby synapses, significantly decreasing synaptic function along with a number of other long-term effects. Cytokines determine the role of astrocytes e.g. TNF-α results in synaptic functional loss. Presence of Aβ plaques, results in activation of astrocytes, which s initiated by the cytokines TNF-α and IL-1β. Changing the status of resident microglia in the hippocampal cells tips off the fine balance between neuroprotection and neurotoxic attributes of microglia. Neurodegenerative diseases do not exhibit synaptic impairment only at the later stages but in the very early stages of pathology as well. This highlights the role of neuroinflammation in digressing the synapses from

healthy neuronal contacts to impaired, dysfunctional connections.(Faissner, Pyka et al. 2010)

2.7.3 Inhibition of Neurogenesis:

In the hippocampus, lateral ventricles and amygdala, the process of neurogenesis takes place. Studies relate the decline in cognitive abilities with the decline in neurogenesis in these areas, both in early and late stages of neurodegenerative disease like dementia. Disruption in neurogenesis mechanisms begin very early on, indicating that this process is implicated in progression of disease from the start. The extent of the impact of neurogenesis inhibition in neurodegenerative diseases is not conclusive, but this is established that neuroinflammation modulates it in a negative manner. Microglia stimulated by LPS produce cytokines such as IL-18,6 and TNF-α which act to inhibit differentiation of the Neuron Progenitor Cells.(Liu, Lin et al. 2005) Hippocampus is the prime region of Neuronal differentiation and there exists a direct proportionality in the number of activated microglia and inhibition of neurogenesis. Research also suggests that the nature of the microglia stimulant decides whether neurogenesis is inhibited or increased. Toll-like receptors of type 2 and 4 are mediators of this alteration in neurogenesis patterns.(Rolls, Shechter et al. 2007)

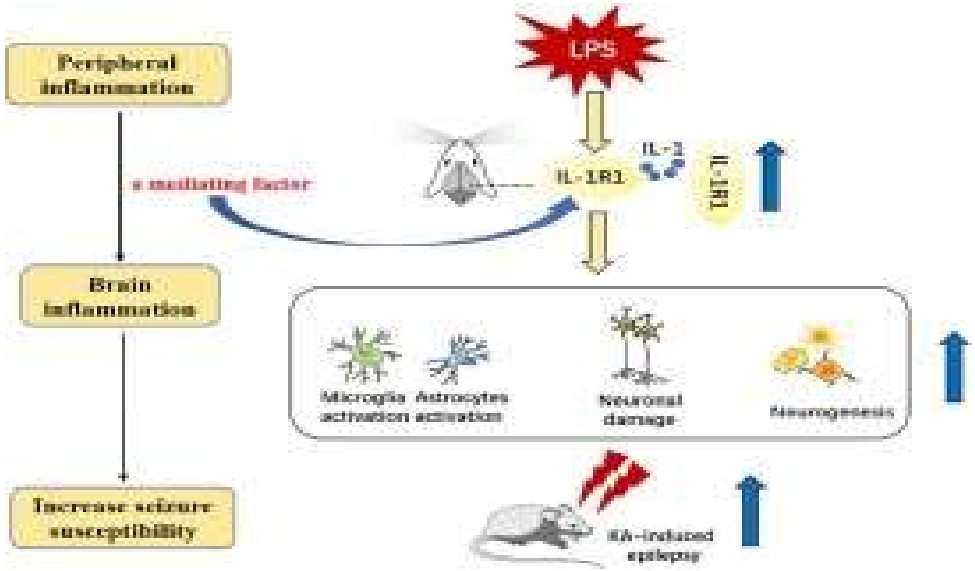

Figure 2.5: Mechanism of Neurogenesis inhibition (Hu, Yuan et al. 2022)

2.7.4 Neuronal Death:

Neuronal loss may occur as a consequence of programmed cell death; apoptosis or as a result of acute injury or trauma leading to necrosis. Apoptosis occurs by the activation of apoptotic pathways mediated by cytokine burst during neuroinflammation, suggesting the degenerative role of apoptosis in chronic diseases. Among the cytokines, Tumor Necrosis Factor -Alpha activates the apoptosis cascade by interacting with membrane receptors; Tumor Necrosis Factor Receptor-1 and 2 and the induction of adaptor proteins such as FADD. Together, TNF-α, TNF-R1/R2 and FADD form a complex activate caspase 8, which ultimately results in apoptosis. An increase in the expression levels of TNF-α is directly corelated to the signaling in apoptotic pathways. (Harry, Lefebvre d'Hellencourt et al. 2008)

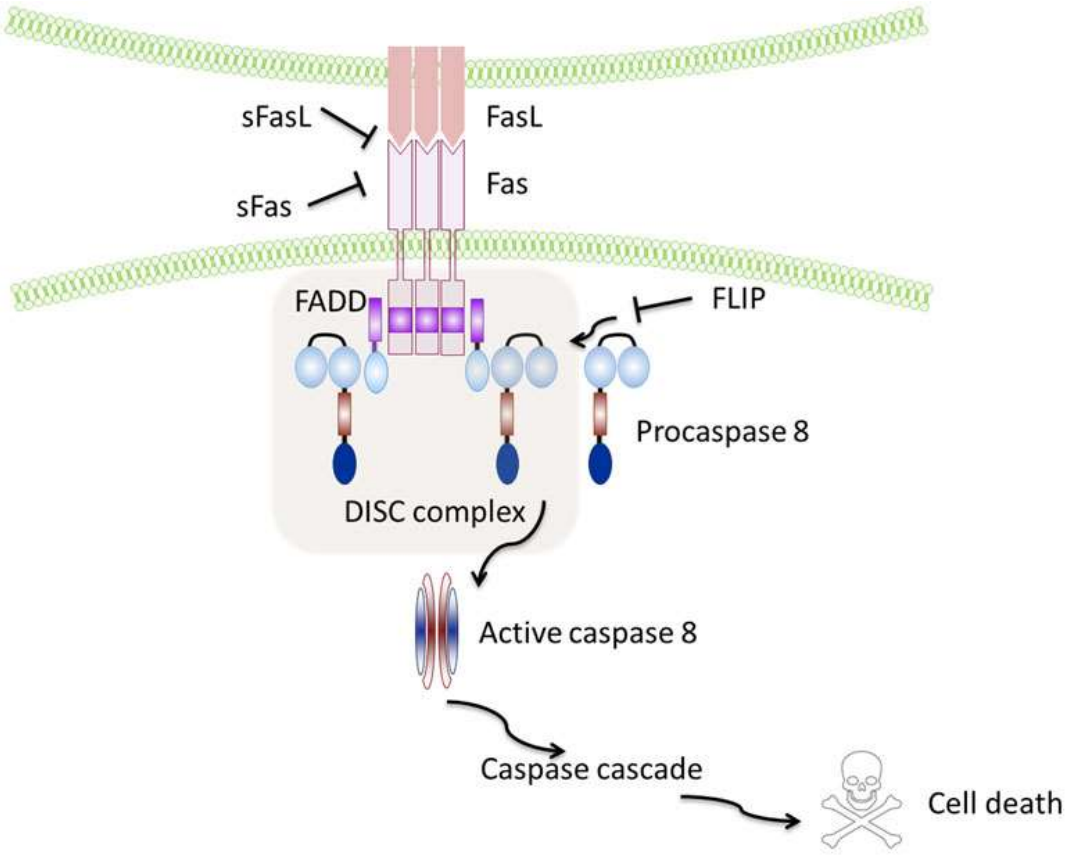

Figure 2.6: Tumor necrosis Factor- Alpha mediated Apoptosis(Volpe, Sambucci et al. 2016)

Another way in which microglial and astrocyte activation contributes to neuroinflammation and ultimately apoptosis is through the release of NO by inducing iNOS. Following increased glutamate release mediated by reduced respiration of neuronal cells, the NMDA receptors get activated and lead to cell death due to excitotoxity. NO produced from iNOS and superoxide contributed by the NADPH oxidase from microglial cells, together cause death of neurons by the formation of peroxynitritre. Where on one hand it is attractive to target the apoptotic pathways, the therapeutic prospects of this approach are not decisive yet as the cytokines released during neuroinflammation engage with and activate a number of separate apoptotic pathways by employing different adaptor proteins. Hence the interplay of the signaling molecules, together with the complex cross-talk of different pathways make it difficult to individually target the death pathways. That being said, the balance between anti and pro-apoptotic factors is an important aspect of the apoptosis cascade that can be exploited therapeutically.(Mander and Brown 2005)

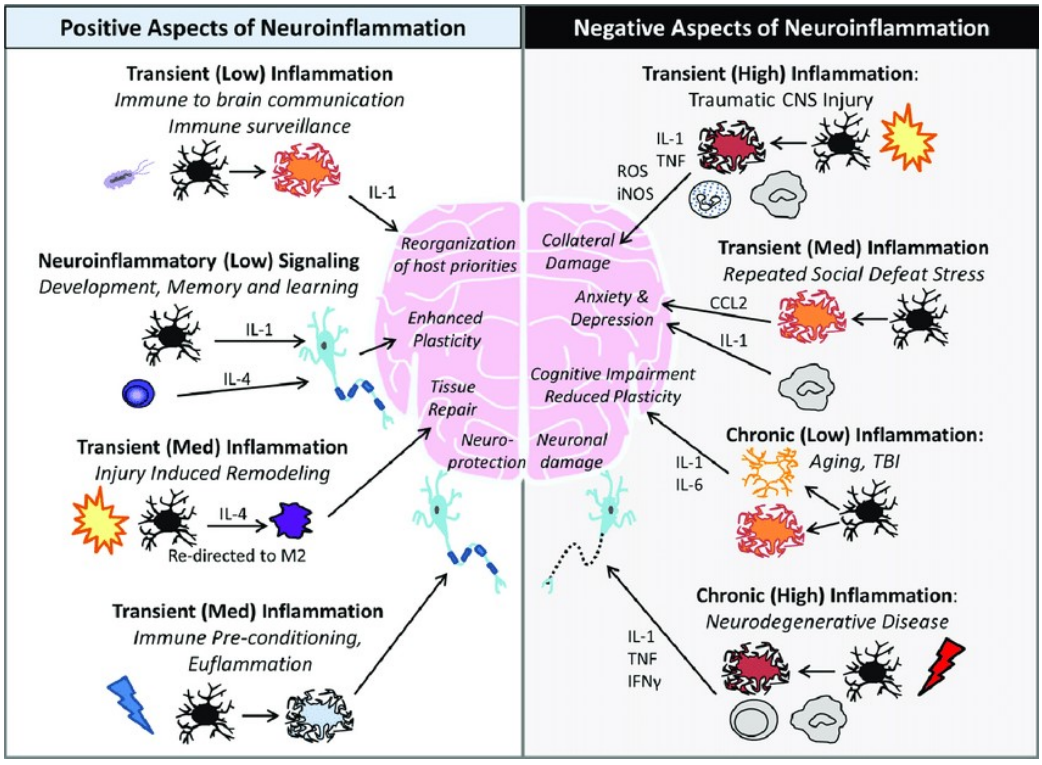

Figure 2.7: Positive Vs. Negative aspects of Neuroinflammation(DiSabato, Quan et al. 2016)

2.7.5 GSK-3 regulation:

GSK-3 is a protein kinase with either serine or threonine; an enzyme that mediates inflammation and is involved in a number of biological activities. Research on this molecule established that it is involved in the neuroinflammation process and its inhibition leads to up-regulated expression of the anti-inflammatory IL-10 and down-regulated expression of pro-inflammatory IL-6, IL-1 and TNF-α. GSK-3 is actively involved in microglial priming and release of pro-inflammatory mediators from these cellular compartments.(Green and Nolan 2012) Studies also point to the role played by GSK-3 in increased permeability, decreased integrity of BBB and migration of monocytes. These studies together show the promise this protein holds as being a potential target for chemotherapeutics in neurodegenerative disease.(Ramirez, Fan et al. 2010)

2.7.6 Cognitive Impairment:

The exact mechanism as to how memory is formed and stored, remains a mystery even now therefore, the specific role played by neuroinflammation in cognitive decline is still not theorized. Long-term potentiation (LTP) was a concept coined in 1973 which stated that, synaptic firing from two synapses simultaneously and repetitively increases the length of the synapses. Behavioral tests such as Morris Water Maze are considered as a standard in measuring cognitive decline and LTP effects in the context of neuroinflammation. These tests are not conclusive about the whole picture of LTP, cognitive decline and neuroinflammation because the whole spectrum of cognition and memory are not examined.(Buchanan, Sparkman et al. 2008) Cytokines such as IL-1β decrease spatial memory when assessed by Morris water maze. JNK and p38 MAPK pathways are activated by IL-18 and free radicals contributing to the formation of ROS. However normal levels of IL-1β, promote LTP of synapses and contribute positively to cognition and memory. This is supported by findings which show that age-related decrease of IL-1β is associated with decreased LTP. Same is the case with TNF-α, whose exaggerated levels in neuroinflammation reduce LTP, but under physiological conditions their basal levels help the same process. Age is a significant factor in the etiology of the progression of neurodegenerative disease. (Belarbi, Jopson et al. 2012)

2.8 Alzheimer's Disease:

Marked by classical misfolding of proteins, hyperphosphorylation and over exaggerated expression of pro-inflammatory cytokine mediators, Alzheimer's Disease is a disorder with progressive degeneration of neurons with subtle symptoms in the early pathology followed by decline in cognition and memory deficits in a progressive manner.

2.8.1 Two-way interactions in A.D:

It was recently discovered that all these mechanisms of neuroinflammation that enable A.D contribute to the neuroinflammation as well. For example, the Aβ monomers and hyperphosphorylated tau proteins are capable of secreting pro-inflammatory mediators. This confirms that A.D pathology is a series of self-replicating and vicious cycles. Another example of this phenomenon is the APP synthesis mediated by IL-6 and later the implication of APP and Aβ in the production of IL-6 for more microglial priming through the TLR 2 pathway.(Vukic, Callaghan et al. 2009) Microglial cells responsible for contributing to ROS generation also follows this principle, where the cytokines from Aβ proteins activate and mobilize microglia to produce NO by inducting iNOS. TNF-α as discussed before is higher in expression in neuroinflammatory models, both in-vivo and in-vitro.(Gabbita, Srivastava et al. 2012) IL-1β, TNF-α are considered reliable biomarkers for A.D detection. As in the case of neuroinflammation, A.D is significantly associated to impairment of synapses. The presence of Aβ peptides and related exaggerated expression of APP hinders the synaptic connections and causes damage to the pre-synaptic terminals. The deposition of Aβ in the neuronal cell bodies depends upon the synaptic transmission mechanisms and the synapses take the feedback from Aβ plaques. This two-way interaction results in a synaptic dysfunction. Also referred to as the "Loss of synaptic gain"; reflective of the loss in function of synaptic transmission and decrease in the number of post-synaptic connections.(Kotilinek, Westerman et al. 2008)

2.8.2 COX-2 Pathway:

The implication of COX-2 genes in the Aβ mediated synaptic impairment and inflammation mediated loss of memory signifies its role in AD progression. It is the long-term impairment of synapses and changes in the morphology of glia in CNS that manifests as chronic neuroinflammation in AD. Studies are underway to determine the role of acute mechanical injury and surgery in synaptic transmission defects.(Ricoy, Mao et al. 2011)

2.8.3 BBB in A.D:

Blood Brain Barrier facilitates neuroinflammation very early on in A.D pathology and modulation of its permeability is a big deciding factor for the progression of disease. LPS stimulated release of cytokines such as Interleukin-6, Interleukin-10 and Interleukin-13 increases the cellular concentration of Aβ peptides and associated cleavage of APP. Another study has confirmed that following LPS induction, prostaglandins are formed and they mediate the migration of Aβ plaques across the BBB.(Pflanzner, Janko et al. 2011) This transport of Aβ products is seemed to be facilitated by receptor modulation of Advanced Glycation End Products (RAGE). In addition, the transport of these peptides is aided by the binding of small lipid-based molecules. An example includes the LRP 1(Lipoprotein-receptor related Protein 1). In some cases, in the presence of an ongoing chronic condition is topped up by an acute condition. This involves a time-barred exacerbation of otherwise subtle symptoms and may lead to serious conditions in patients with a strong neuroinflammatory stimulus that activates mechanisms of acute inflammation.

2.8.4 Aβ plaques in A.D:

Amyloid precursor protein is present in all neuronal cells and its proteolytic cleavage gives rise to one of the two isoforms of 40 to 42 amino acid long amyloid-beta plaques. These Aβ proteins and their derivatives play central roles in neuroinflammation and progression of neurodegenerative disease. (Pearson and Peers 2006)These peptides are found under normal physiological conditions as well. Their precise roles under normalcy

are not yet determined. In neuroinflammation, Aβ is involved in modulation of membrane permeability, Ca++ increase in microglia and death by either necrosis or apoptosis. Aβ acts as a neurotoxin and stimulates the microglia and astrocytes to change into phagocytes and start the inflammation cascade entering a repetitive cycle of neuronal loss. Aβ formation in neuronal cells is a characteristic of AD. Induction of inflammatory cytokines over a longer time results in increased synthesis of APP and phosphorylation of Tau proteins. Studies show that through and upregulation of IL-1β, TNF-α and IFN-γ, APP cleavage is increased through the MAPK pathway.(Ghosal, Vogt et al. 2009) Deposition of Aβ plaques and progression of neuroinflammation does not follow a linear proportionality or even an inverse one. Studies on the same points towards the existence of a complex relationship and the nature of stimulant, its duration of exposure determine the way neuroinflammation progresses and its effect on the pathology of neurodegenerative diseases.(Herber, Roth et al. 2004)

2.8.5 Tau hyperphosphorylation:

A hallmark of A.D and related neurodegenerative diseases is the hyperphosphorylation of the Tau protein followed by the formation of tangles known as "Neurofibrillary Tangles". Originally related to the stability of microtubules, Tau protein when hyperphosphorylated leads to a disintegration of the microtubule and the formation of tangles. It is speculated that neuroinflammation contributes to exacerbated phosphorylation of tau protein. This mechanism is observed in LPS-mediated and age-related microglial activation.(Lee, Rizer et al. 2010) The hyperphosphorylation of Tau protein is facilitated by kinases of different types, for example Cyclin-dependent kinase 5 (CDK 5). Upregulation of this pathway results from overexpression of p 35. IL-6 activates p 35 which in turn activates CDK-5.(Kitazawa, Oddo et al. 2005) IL-1β mediates tau hyperphosphorylation through p 38 MAPK pathway.(Quintanilla, Orellana et al. 2004) NO also contributes to tau hyperphosphorylation through kinases. There exists a cross-talk between the hyperphosphorylated tau protein and the deposited Aβ plaques and this synergy in neuroinflammation is an important area of research.(Saez, Pehar et al. 2004)

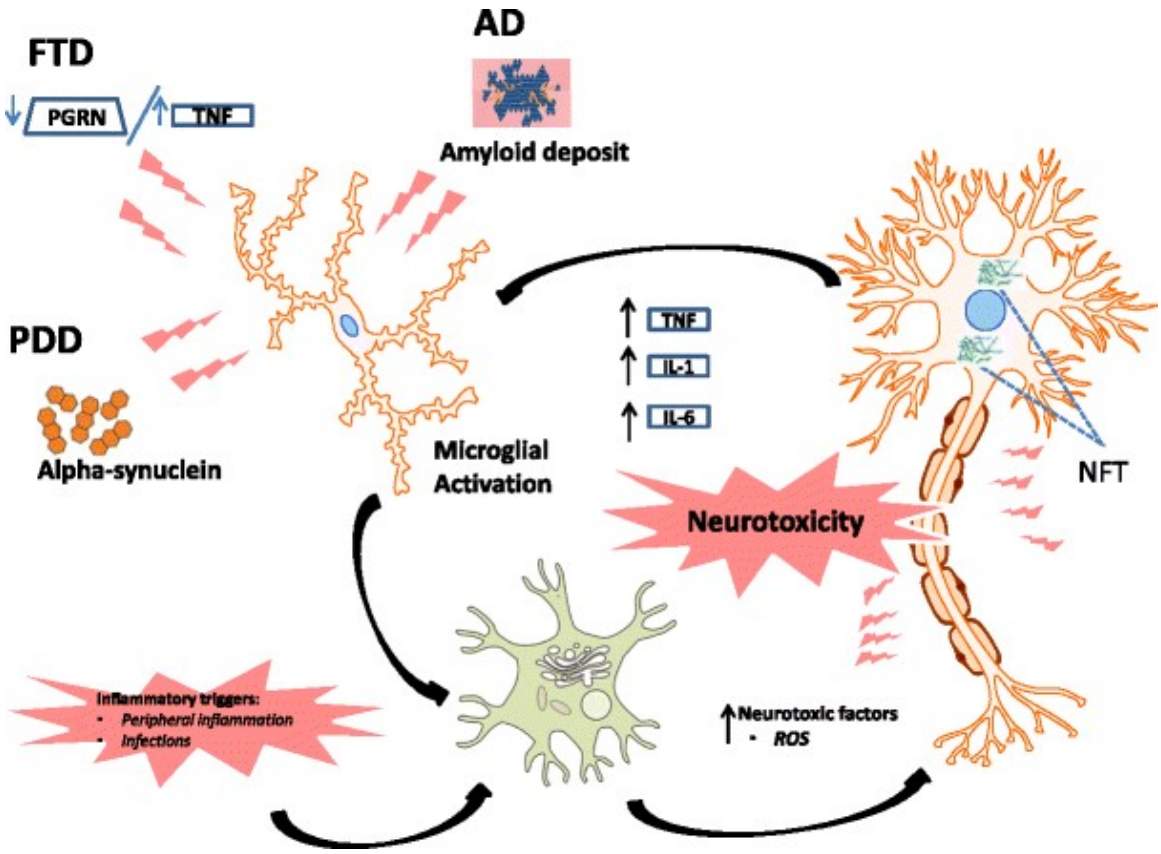

Figure 2.8: Common Mechanisms of Neurodegenerative Diseases (Pasqualetti, Brooks et al. 2015)

2.9 Oxidative Stress:

A concept in Redox Biology and cancer, Oxidative Stress is defined as the imbalance of pro and anti-oxidative factors. Reactive Nitrogen and Oxygen Species (RONS) are themselves free radicals or are compounds that give free radicals when in interaction with different molecules.(Sies 2020) High concentration of these free radicals in cellular compartments results in damage. This damage is attributed to a number of diseases including but not limited to cancer. Oxidative Stress, resulting either as more free radicals or a decrease in the concentration of anti-oxidants, has been considered to be an active player in many pathologies.(Lichtenberg and Pinchuk 2015)

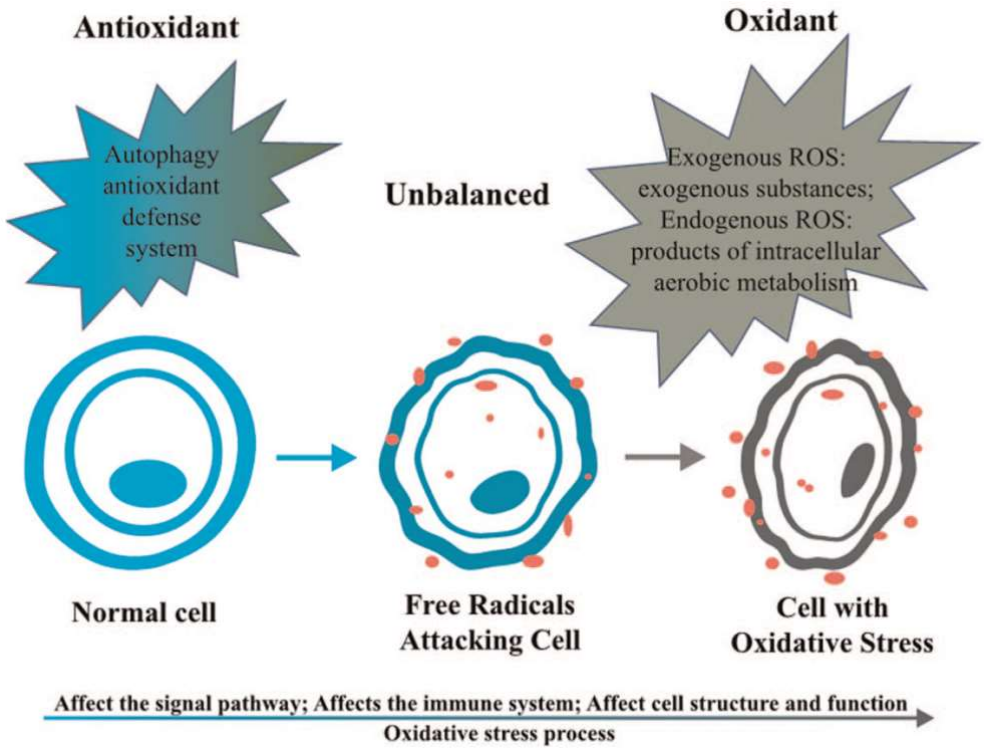

Figure 2.9: Oxidative Stress Mechanism (Dong, Zhang et al. 2021)

2.9.1 Free Radicals:

Free radicals have unpaired electrons that makes them reactive and unstable. These, go on to form interactions with DNA, proteins and lipids, resulting in more unstable and highly reactive free radicals. This chain reaction is halted by the activity of bi-radicals. Free radicals such as hydroxyl (OH). Superoxide anion (Oz-), Iron (Fe++) and Copper (Cu++), Nitric oxide (NO), Peroxynitrite (ONOO-).(Preiser 2012)

OH, a potent free radical as an oxidant is short-lived, reacts at the site of its formation resulting in the initiation of chain reactions of more free radicals. Superoxide is a weak oxidizing agent but a potent reducing agent for Iron complexes; it gives rise to hydrogen peroxide and hydroxyl radicals.(Jones and signaling 2006)

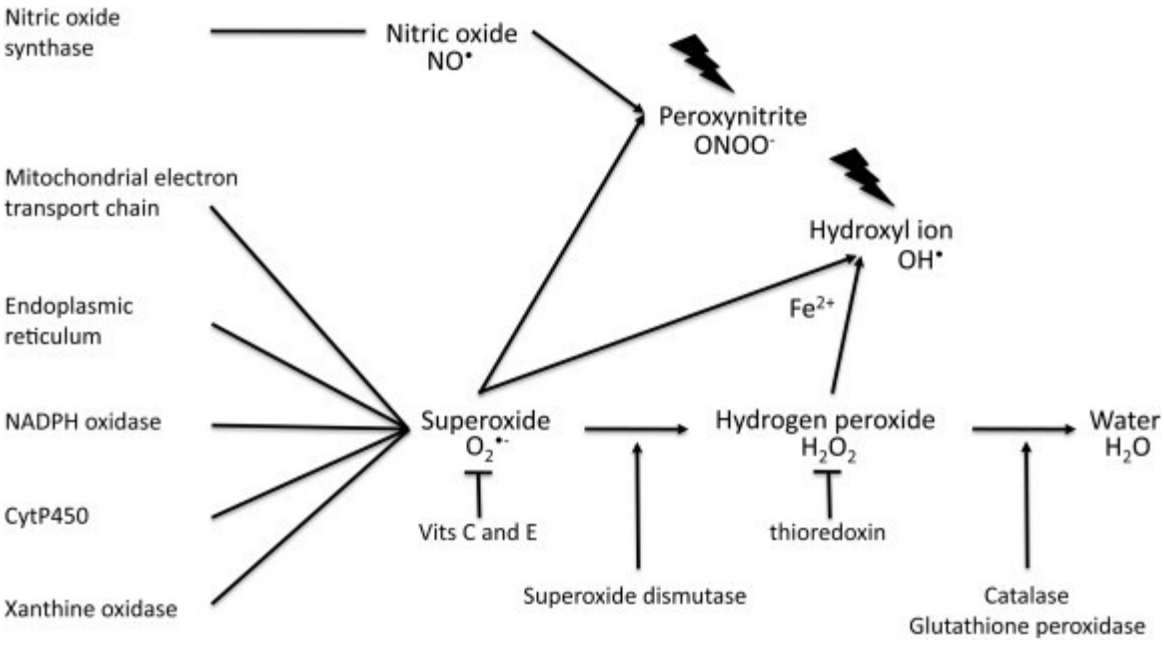

Figure 2.10: Reactive Oxygen Species (Burton and Jauniaux 2011)

2.9.2 Tissue Damage:

Arising from either aerobic respiration that yields hydroxyl and superoxide radicals or Arachidonic Acid cascade activation resulting in reduction of molecular oxygen, or activation of phagocytes that result in production of hypochlorous acid (HOCl), or Nirtic Oxide resulting from microglia; whatever the mechanism employed for the production of free radicals, the end result is always tissue damage.(Betteridge 2000)

2.10 Saikosaponin B2:

Traditional Chinese Medicine (TCM) has made use of the Radix Bupleuri (Chai Hu) for more than 2000 years as reported in “Shen Nong Ben Cao Jing”, the earliest record of medical history. It has been used extensively therapeutically for ailments including liver related disorders and cold.(Yuan, Yang et al. 2017)

2.10.1 Physical Structure:

Mainly using the root of this herb, this medicinal plant is exploited for its medicinal benefits. Chinese Pharmacopeia recognizes Bupleurum Chinese DC (Apiaceae) and Bupleurum scorzonerifolium Willd as the main plants of Radix Bupleuri. A number of Bupleurum species are being used in countries such as Korea, China , Japan, Eurasia and North Africa but they are abundantly found in the northern hemisphere.(Jia, Yang et al. 2022)

Figure 2.11: Dried Roots of Radix bupleuri (Yuan, Yang et al. 2017)

Radix Bupleuri species are perennial herbs with simple, long slender leaves. Their flowers are yellowish or purple bisexual that have five stamens, compound umbels and cremocarps.

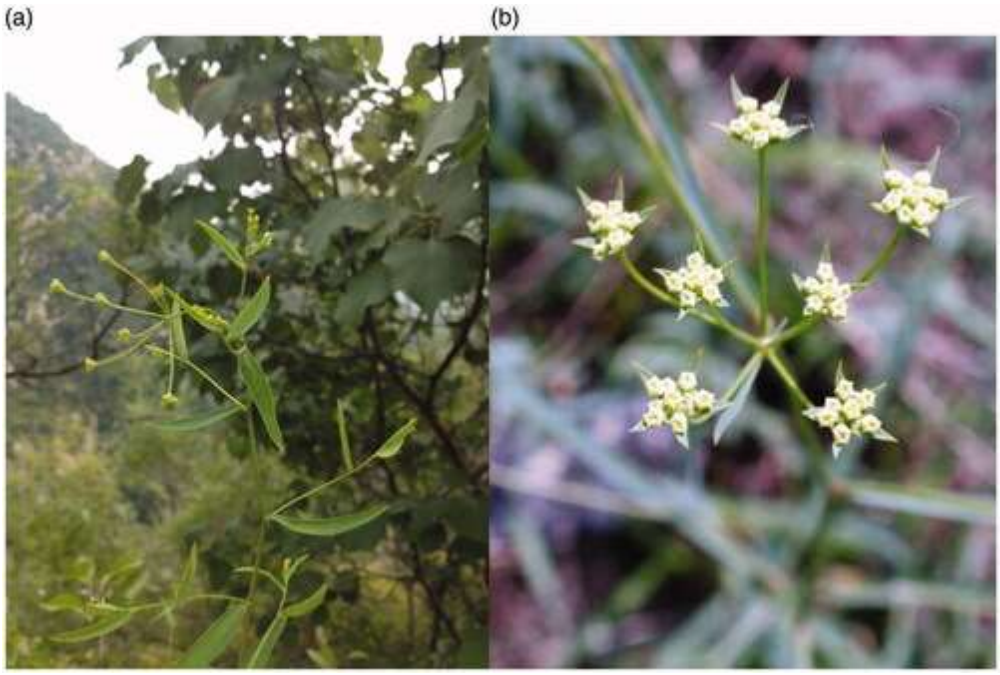

Figure 2.12: Flowers of Bupleurum Chinense DC (Yuan, Yang et al. 2017)

2.10.2 Chemical Structure:

Extraction of secondary metabolites followed by their identification and isolation from RB gives flavonoids, coumarins, fatty acids, polyacetylenes, polysaccharides, essential oils and saponins that are steroidal and triterpenoid in nature.(Li, Li et al. 2018)

Analysis and investigation based on the presence of aglycones present among the different Bupleurum species categorizes the Saponins into seven types. Triterpenoid saponins having epoxy ether oleanane structures are SSa, SSc and SSd whereas others having different aglycones or chains of sugars are SSb1, SSb2, SSb3 and SSb4 among others, these are heterocyclic dienes.(Lin, Chung et al. 2015)

More than one hundred different types of Saponins, including glycosylated oleananes and glycosylated ursanes have been isolated and characterized from the roots of Bupleurum plants.(Liu, Gu et al. 2023) Being the major bioactive constituents of the extracts of RB, Saikosaponins have been reported for a number of biological activities showing antagonism to immunity, cancer, neurology and viral related diseases in addition to being hepatoprotective and neuroprotective.(Xu, Li et al. 2016) Saikosaponins are

basically pentacyclic triterpenoids composed of aglycones and sugar and belong to the oleanane class of compounds. The aglycones differ in their position and number of double bonds at their C and D rings and the oxygen atoms present at Carbon number 16, 23 and 28. The sugar backbone is composed of galactose, rhamnose, xylose, glucose of fructose. SSb2 belongs to type II of the seven classes of saponins. They have double carbon bonds in either 11, 13(18)-diene or 12,16(17)-diene carbon rings.(Jia, Yang et al. 2022)

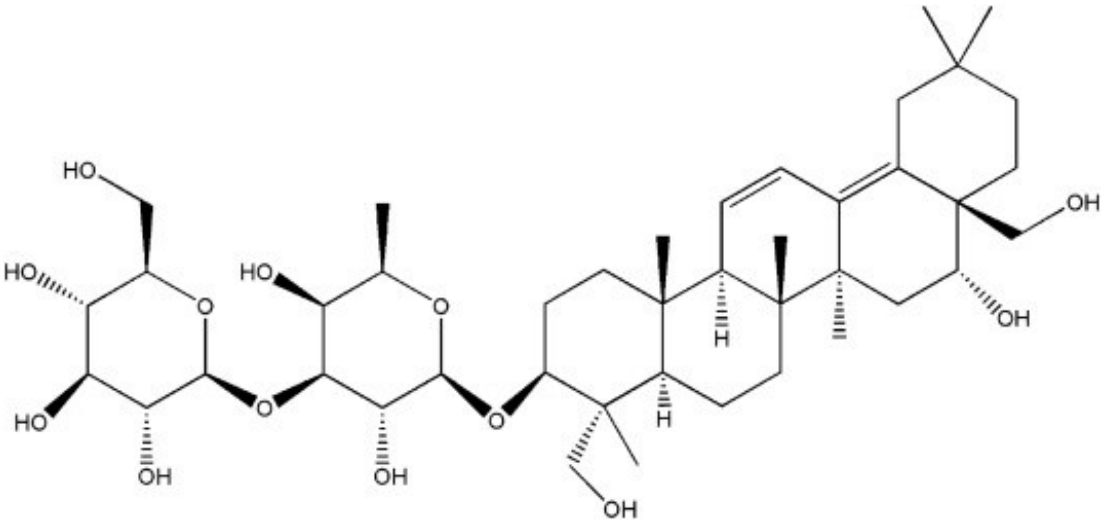

Figure 2.13: Structure of Saikosaponin B2 (Zhao, Feng et al. 2019)

For extraction, conventional processes were used such as Ultrasonic assisted, Solvent partitioning and reflux. But with the advancement in research these methods are being replaced by Accelerated Solvent Extraction, which gives better yield and is faster than the other methods.(Li, Li et al. 2018)

2.10.3 Pharmacological Effects:

Sr. No.	Animals / Cells	Model	Dosage	Time	Effects	Reference
1.	Mouse spleenocytes	Con A	0.5- 2.5 µg/ml	48 hr	IFN-γ & TNF-α ↓, IL-4 & IL-10 ↑	(Li, Li et al. 2018)
2.	B16 melanoma cells	NA	10 µM	48 hr	G1 phase accumulation, apoptosis induction	(Zong, Fujikawa-Yamamoto et al. 1996)
3.	B16 melanoma cells	NA	5 mM	30 days	Differentiation of melanoma cells	(Zong, Fujikawa-Yamamoto et al. 1998)
4.	Human hepatoma HuH7 cells, HuH7.5 and S29 cells	HCV	50 µM	7 days	Virus counteraction & attachment	(Lin, Chung et al. 2015)
5.	Human Fetal lung fibroblasts	Human coronavirus 229E infection	0.25 – 25 µM	8 hr	Viral attachment and penetration ↓, SSb2 IC50 1.7 µM	(Ren, Luo et al. 2020)
6.	HEK293 cell/ Mast cell	IgE	2.5 – 10 µM	NA	TAS2R14 ↑ with IC50 4.9 µM, mast cells degranulation	(Zhang, Wang et al. 2017)

Table 2.1: Pharmacological Activities of SaikosaponinB2

2.11 Toll-Like Receptors in LPS-induced inflammation:

Essentially important elements of the early immune responses, Toll-like Receptors are signaling proteins and take part in inflammatory responses as well. These are receptors for molecules like PAMP's which are involved in pathogenic insults. On the recognition of a foreign entity, they get activated and prompt appropriate intracellular cascades involved in response. In the scenario of inflammation, TLR-4 is of utmost importance.; activated by LPS.(Warren, Fitting et al. 2010) LPS is a toxin of the Gram -ive bacteria,

found in its extracellular membrane, causing sepsis and inflammatory responses of systemic scale in animals and humans are particularly susceptible to it action. LPS-induced inflammation is a well-established protocol used to study the effects of inflammation. It is the major receptor involved in neuroinflammation and its activation leads to up-regulated IL-1β, IL-6 and TNF-α. TLR's are also present on the microglia and Astrocytes and help launch a full neuroinflammatory response. Major Histocompatibility Complex I and II are expressed by microglia which are mainly involved in response to infection. These complexes are also reported to play roles in progression of neurodegenerative diseases. (Al Nimer, Beyeen et al. 2011)

CHAPTER 3 : MATERIALS AND METHODOLOGY

3.1 In-Vitro Analysis:

3.1.1 Cell Lines:

Cell lines; HT22 were provided by Cell Culture Lab of Atta-Ur-Rehman School of Applied Biosciences, NUST Islamabad.

Material	**Manufacturer/Provider**
Cell lines	ASAB
Dulbecco's Modified Eagle Medium (DMEM)	Solar Bio
Fetal Bovine Serum (FBS)	Sigma Aldrich
Trypsin	Sigma Aldrich
Penicillin/Streptomycin (Pen/Strep)	Sigma Aldrich
Phosphate Buffer Saline (PBS)	Sigma Aldrich
T25 Flask	Thermofisher Scientific

Table 3.1: Materials used for Cell Culture

3.1.2 Media Preparation:

In a Class II Biosafety cabinet, 50 ml aliquots of media were prepared at a time using 45 ml DMEM, 5 ml FBS and 1 ml Pen/Strep.

3.1.3 FBS heat-inactivation:

Fetal Bovine Serum was kept at 4 degrees Celsius overnight and divided into corning tubes of 20 ml. These were kept in the freezer and melted in water bath at 37 degrees Celsius before every use.

3.1.4 Cell Thawing:

Cells were taken out of the liquid nitrogen freezer set at – 80 degrees Celsius. Briefly the cells were thawed by keeping them in the incubator at 37 degrees Celsius for 3 to 5 minutes.

3.1.5 Cell Culturing:

After thawing of cells, the cryo vials were sprayed with 70 % ethanol and placed inside the laminar flow hood. The cryovial suspensions, after transference to eppendorfs and being spun at 1600 revolutions per minute for a duration of 10 min. Discarded supernatant using a micropipette and washed pellet with 1 ml PBS. After resuspension of cells in 1 ml media it was centrifuged at 1000- 1200 rpm, twice. The eppendorfs were were decanted and pellet was immersed in 5 ml Media and very carefully shifted to T 25 flask. The flask was then kept in a water jacketed humidified incubator, supplied at 5 % CO2 and 37^{o}C.

3.1.6 Sub-culturing:

After bringing the T-25 flasks to the laminar flow hood, Media and trypsin was kept in the incubator for atleast 20-30 mins. Media removal was followed by washing in 1 ml PBS twice by pouring it over the sides and then PBS was removed too to avoid cell attachment. Then 1 ml trypsin was poured over whole area of the flask, and kept in incubator for 3-5 minutes. Followed by shifting to eppendorfs containing media after tapping and spun at 3000 rpm (5 mins). The pellet was reconstituted in fresh medium containing 10 % FBS and 1 %Pen/Strep following supernatant removal. HT-22 cells were grown till they reached a confluency of 70-8-%, medium was changed every 3 days and the seeding density was kept at 1×105 cells/cm2.

3.1.7 Cell Freezing:

Freezing media was prepared by mixing 70 % DMEM, 20% FBS and 10 % DMSO. After bringing the T-25 flasks to the laminar flow hood, Media and trypsin was kept in the

incubator for at least 20-30 mins. Media removal and cell washing with PBS 1 ml was done once by pouring it over the sides and then PBS was removed too to avoid cell attachment. Then 1 ml trypsin was poured over whole area of the flask and kept in incubator for 3-5 min. Then the contents of the flask were shifted to eppendorfs containing media after tapping and centrifuged for 5 minutes at 3000 rpm. After centrifugation and suoernatent removal, 1-2 ml freezing media was added. Each iso-propanol coated cryovial received 1 ml of suspension containing freezing media. The cryo vials were then placed in Mr. Frosty, overnight at a temperature of -80 degrees Celsius. The cryo vials were shifted to a cryo box the next day for longer preservation in a liquid nitrogen freezer.

3.1.8 Trypan Blue Exclusion Assay:

Before plating cells for Cell Viability, a Trypan Blue exclusion assay was performed to plate cells consistently. For this, cells from the T25 flask were trypsinized, collected and centrifuged. This was followed by the resuspension of cells in 1 ml of prepared medium. The cells were vortexed and 10 µl was put on a paraffin strip. To this, 10 µl trypan blue was mixed to dilute it. By pipetting up and down, homogenization was achieved and 10 µl was injected on a haemocytometer. Cells that are non-viable absorb the dye in their damaged plasma membrane, whereas viable cells exclude the blue trypan dye and appear white.

3.1.9 Cell counting:

An accurate counting of cells per ml was achieved by carefully counting all the viable cells in the four grids of the haemocytometer. Using the following formula, an accurate number of cells was determined:

$$X = \frac{Grid\ A + B + C + D}{4} \times Dilution\ Factor \times 10{,}000$$

3.1.10 Drug Dilutions:

The compound Saikosaponin B2 was dissolved in DMSO to prepare a stock solution of 20 mM. It was then serially diluted in DMEM media supplemented with 10 % FBS. At each step, the dilution factor was kept the same to ensure that the concentration progresses in a geometric fashion, logarithmically. The 20mM stock solution was serially diluted as follows; 8 µl Saikosaponin B2 (20 mM) stock solution was added to 1 ml DMEM in an Eppendorf and mixed. This mixture was added to another Eppendorf containing 500 µl media. In this way starting from a concentration of 160 µM serial dilutios of 80µM, 40µM, 20µM, 10 µM and 5µM were prepared and used for Cell viability assay.

3.1.11 MTT Assay:

1×104 cells/ well were plated in a 96 well plate and kept in incubator overnight to allow the cells to adhere. Once the cells had adhered, 50 µl of different doses of Saikosaponin B2(5 µM, 10 µM, 20 µM, 40µM) were added to the wells in triplicates. 50 µl of untreated cells were treated with 0.1% DMSO in triplicate which was taken as the negative control. In addition, 100 µl culture medium was plated in triplicates, to remove any effects of the medium in the results. The plate was kept in the incubator for 24 hours at 37^{o}C and 5 % CO2. After the completion of the doubling time, in each well containing cells and drug doses, 15 µl of MTT dye was added. 5 mg of MTT dye was dissolved in PBS (1 ml). MTT dye checks the viability of cells after treatment with different doses of the drug. Once MTT was added, the 96-well plate was kept back in the incubator for a period of 3-4 hours, depending upon the formation of crystals. This colorimetric assay is based upon the principle that yellow colored Tetrazolium dye is converted into Purple colored formazan crystals by the viable cells' metabolic activity. After taking out the plate from the incubator, 100 µl medium was carefully pipetted out to not disturb the cells. This medium was replaced by 100 µl DMSO to dissolve the crystals. Bio Rad spectrophotometric microplate reader was used to determine the absorbance at 540 nm after complete dissolution of crystals had been achieved by pipetting up and down. The following formula was used to determine the percentage of viable cells:

$$Cell\ Viability = \frac{Sample\ Absorbance - Media\ Absorbance}{Control\ Absorbance - M \qquad Absorbance} \times 100\%$$

3.2 Mechanistic Studies:

3.2.1 Cell Treatment:

HT22 cell line grown at a density of 2×105cells per ml in T 25 flasks were subjected to 1µg/ml LPS for the induction of inflammation, after which a 24- hour treatment with SSB2 was done. Following treatment and media removal, washing with PBS was done. Following washing, trypsinization and centrifugation at 1500 rpm for 5 minutes, the resulting pellet was either stored at -20^{o}C or used for RNA extraction.

3.2.2 RNA Extraction:

Total RNA extraction was done using Trizol reagent. Trizol(1 ml)was used for lysis by repeated pipetting. Then the sample was kept on ice for a duration of 5 min. 200 µl of Chlorofrom addition was followed by vigorous shaking of the sample for 15 seconds and then incubation for 2-3 min, again on ice. After incubation and centrifugation at 12000 g at 4^{o}C for 15 min. This centrifugation gives three phases, the upper phase which was aqueous was separated in a tube and 500 µl, 100 % isopropanol was added to it and kept on ice for 15 min. The sample was again centrifuged at 12000 g for 10 minutes at 4^{o}C and the supernatant was discarded which left the RNA pellet at the bottom. To this 500 µl, 75 % ethanol was added and vortexed followed by centrifugation at 7500 g for 5 minutes at 4^{o}C, again the supernatant was discarded. The RNA pellet was air dried for 10 minutes and resuspended in 20 µl of RNase-free water. This was set aside for c-DNA synthesis at -80^{o}C.

3.2.3 Assessment of RNA Quality and Quantity:

For checking the quantity of extracted RNA, Nanodrop 2000 (Thermoscientific, USA) was used. 260/280 ratio was checked to determine the purity of RNA.

3.2.4 Complementary DNA synthesis:

For cDNA synthesis, 1000ng of RNA was used as the reverse transcription template. 1 µl Oligo dT20 (10µM) was taken in a 0.2 ml microtube. 1 µl DTT (100mM), 1 µl dNTP's (2.5 mM), 2 µl 10 X reaction buffer and 0.5 µl RNase inhibitor (40U/µl) was added to this microtube. To make 20 µl, 2µl template RNA and nuclease-free water was added. Kit Protocol was used to establish the reaction profile after placing the microtubes in the (Wizbio) thermocycler.

3.2.5 cDNA synthesis Confirmation:

PCR reaction mixture consisted of the following list of reagents:

Reagents	**Quantities**
cDNA	20 µl
$MgCl_2$(25mM)	2 µl
Reaction Buffer (10 X)	2.5 µl
dNTPs (10 mM)	1.5 µl
Taq DNA Polymerase	0.5 µl
Forward Primer (GAPDH)	1 µl
Reverse Primer (GAPDH)	1 µl
Nuclease Free water	9.5 µl

Table 3.2: Reagents used for Polymerase Chain Reaction.

The assembly was made where in Step I initial denaturation occurred at 95^{o}C for 5 minutes, this was followed by stage II in which 35 cycles were repeated. In each of these cycles, denaturation occurred at 95^{o}C for 1 minute, annealing lasted for 45 seconds at 60^{o}C followed by extension of cDNA strand for 45 seconds at 72^{o}C. In Stage III, extension occurred for 10 minutes at 72^{o}C. The prepared PCR product was stored at -20^{o}C to be used later on.

3.2.6 Gel Electrophoresis:

Confirmation of cDNA synthesis was done by running the PCR product on 2 % agarose gel. For agarose gel preparation, 1 g agarose was dissolved in 50 ml 1X TAE buffer. Preparation of 50 ml 1 X TAE buffer requires the dissolution of 1 ml 50X TAE Buffer in 49 ml of distilled water. The solution was mixed and kept in a microwave for 40 seconds. On complete dissolution, the solution became clear and was cooled down before the addition of 4 µl ethidium bromide. The gel was poured in the casting tray and combs were placed; gel was allowed to set. After setting, the combs were taken out and wells were loaded with 4 µl and 2 µl of PCR product and loading dye respectively. 1 kb DNA ladder (Thermo Scientific) was used as the marker. Gel was run at 500 Amperes for a duration of 40 minutes.

3.2.7 Real Time PCR:

For gene expression, real-time PCR was done. The primers of gene of interest were amplified at 10 mM (Applied Biosystems 7300). A specific PCR strip was specified for each primer and the entire process was done on ice. Reaction mixture was prepared as follows:

Reagents	**Quantities**
cDNA	5 µl
Forward Primer (10 mM)	1.5 µl
Reverse Primer (10 mM)	1.5 µl
SYBR Green Master Mix (2X)	12.5 µl
Nuclease Free Water	4.5 µl

Table 3.3: Reagents for RT-PCR.

The reaction consisted of three steps:

Step I: Reaction mixture was heated to 50^{o}C for 2 minutes

Step II: Reaction mixture was heated to 95^{o}C for 10 minutes

Step III: 40 cycles of PCR were conducted.

Each PCR cycle constitutes three incubation phases: i) incubation for 15 seconds at 95°C ii) incubation for 45 seconds at 60°C iii) incubation for 30 seconds at 72°C. The disassociation stage consisted of three incubation phases i) 95°C for 30 seconds ii) 60°C for 30 seconds and iii) 95°C for 30 seconds. Information gathering was done at 72°C. As a housekeeping gene, forward and reverse primers for GADPH were used. For data Analysis SDS software for ABI 7300 was used. For determining the relative quantification, 2-ΔΔCt technique was made use of.

3.3 ROS Detection:

10 mM stock solution of DCHF-DA dissolved in DMSO was used, diluted by PBS to give a working solution of 10µM. After seeding 5×105 cells per well in a 24well plate, once the cells had adhered; they were treated with 1µg/ml Lipopolysaccharide for 2-3 hours and then with the IC 50 value of SSB2 i.2. 10µM.Media removal and cell washing was done. 100µl of 10 µM DCFH-DA working solution was added to each well and the plate was immediately covered in foil and kept for 30 min in a humidified incubator. Once incubation period was over, dye removal followed washing. PBS was replaced with Hank's Balanced Salt Solution. 530/485 nm emission and excitation wavelengths were selected in a fluorescence microscope and images were captured and fluorescence intensity was measured using software for (BD FACSCalibur, USA).

3.4 Annexin V Staining:

1×10^5 cells plated in 6- well plate, were grown to confluency. After treatment the cells were collected after trypsinization and detached and then spun at 300-400 g for 5 mins. Supernatent was discarded and the pellet was two times washed in 100 µl prediluted binding buffer (10X). This was the suspended in 100 µl binding buffer. Afterwards, 2 µl V-FITC was added and incubated in dark for a duration of 30 minutes at room temperature. Flowcytometer (BD FACSCalibur, USA) was used to detect the conjugates of fluorophores and Annexin V. This was detected at of 525 nm (emission) and of 488nm (excitation). Data was analyzed using software to quantify different percentage of states.

3.5 Morphological studies:

4′,6′- diamidino-2-phenylindole (DAPI) was prepared as a stock solution by dissolving 1 mg in 1 ml of PBS. This was diluted to prepare a working solution of 1 µg/ml in PBS. 1×10^5 cells per well were cultured in 24-well plates until confluent. Media removal and washing was done. 200 µl DAPI working solution (1µg/ml) was added in each well. The plate was kept at R.T for 30 min in dark. After incubation cells were washed to remove excess dye. The Plate was mounted on a fluorescence microscope and images of stained cells were captured at 360 nm for excitation and 460 nm for emission.

3.6 Statistical Analysis:

Statistical Analysis was done using GraphPad Prism Software 8.0.2. One-way ANOVA and Two- way ANOVA were the statistical tests done to determine significance between the different groups and p values less than 0.05 were considered significant. Results were reported as Mean ± S.E.M

CHAPTER 4 CHAPTER 4: RESULTS

4.1 Cell-Viability Analysis:

4.1.1 Saikosaponin B2 affects cell viability of HT-22 cells in a dose-dependent manner:

IC 50 value or Half-maximal Inhibitory Concentration is the measure of a drug that is required to inhibit the proliferation or viability of cells by 50 %. MTT assay determines this IC 50 value to determine non-toxic doses of drugs, which selectively inhibit target cells. This colorimetric assay is based upon the principle that yellow colored Tetrazolium dye is converted into Purple colored formazan crystals by the viable cells' metabolic activity. Results showed that treatment of HT-22 cells with 5 µM of Saikosaponin B2 reduced cell viability down to 80.19 %± S.E.M. Whereas significant viability decrease was seen when cells were treated with 10 µM of SSB2. Cell viability dropped down to 36.09 ± S.E.M. Hence 10 µM was selected as IC 50, HT-22 cells were treated with this IC 50 value in all subsequent experiments.

Figure 4.1: Formazan Crystals dissolved in DMSO.

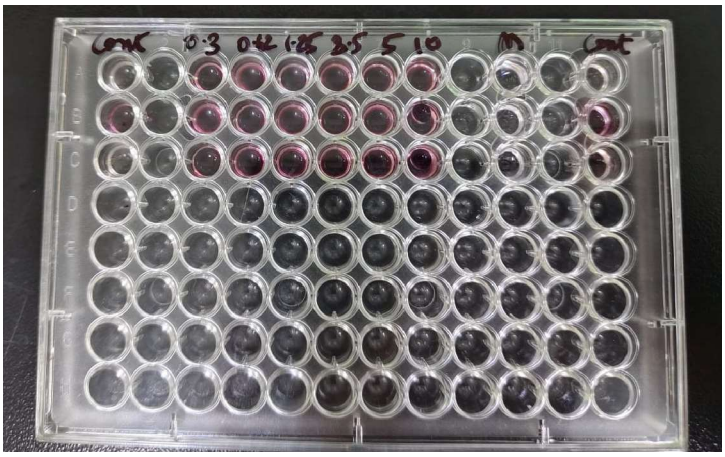

Figure 4.2: Formazan Crystals dissolved in DMSO.

MTT assay was performed to assess the viability of HT-22 cells after treating them with different doses of Saikosaponin B2 for 24 hours. Negative control was the control group treated with 0.01 % DMSO. Formation of Purple Formazan crystals is a result of the metabolic activity of the viable cells. They were dissolved completely to measure the absorbance at 540 nm.

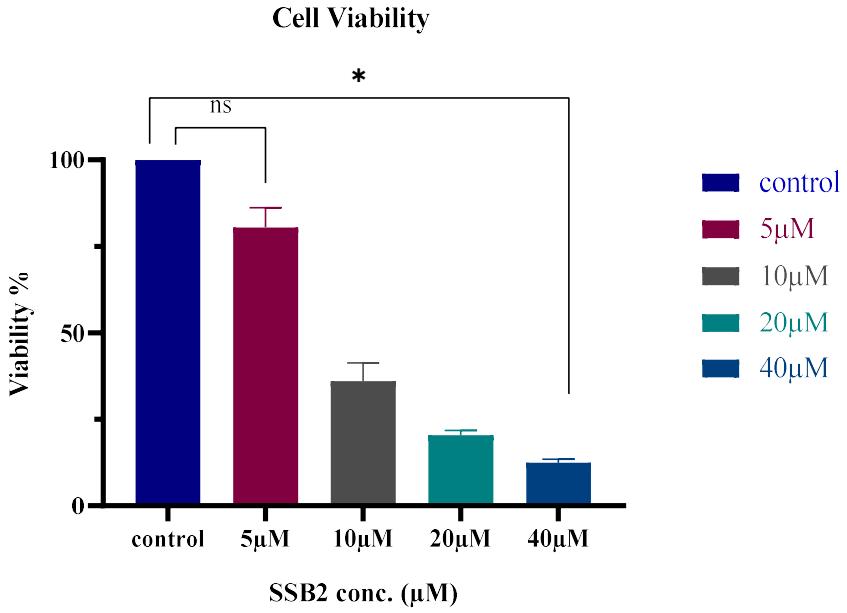

Figure 4.3: Saikosaponin B2 affects cell viability in a dose-dependent manner.

Each bar represents the Mean ± S.E.M (n=3). The Viability percentage decreased by more than 50 % for SSB2 concentration of 10 µM compared to the control and 5 µM doses. One Way ANOVA was used to check statistical significance. Significant difference ($p<0.05$) existed between group receiving DMSO and Group receiving 10 µM SSB2.

4.2 Gene-expression Analysis:

4.2.1 SSB2 regulates the expression of pro-inflammatory genes in LPS treated HT-22 cells:

In order to validate the neuroprotective effects of Saikosaponin B2 in HT-22 cell lines and identify the modulators of this mechanism, the expression levels of pro-inflammatory genes were checked. IL-6 and IL-1β are cytokines actively involved in neuroinflammation, having a plethora of roles in acute, chronic and transient inflammation. Following LPS treatment for 3 hours, HT-22 cells were treated with IC 50 of SSB2 i.e 10 µM. Total RNA was extracted using Trizol reagent. GAPDH was used as the endogenous housekeeping gene. Quantification of the relative gene expression was done using comparative Ct (2-ΔΔCt) method as described by Livak and Schmittgen.(Livak and Schmittgen 2001). Ct values of IL-6 and IL-1β were normalized to the Ct value of GAPDH gene. Changes in mRNA expression were expressed as fold changes relative to that of control.

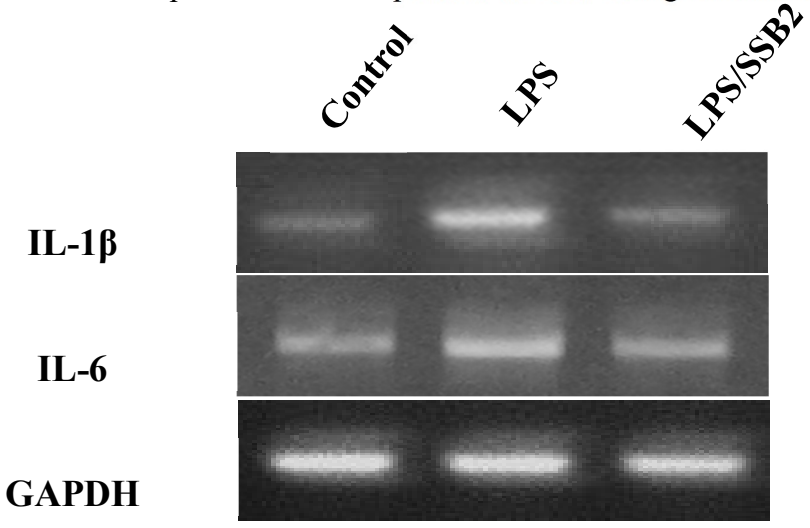

Figure 4.4: Gel Electrophoresis results of RT- PCR

Represents the image of Agarose gel when the cDNA was run on it. The intensity of the bands shows that the expression of both IL-1β and IL-6 was increased when the cells were exposed to LPS alone. When given SSB2 treatment, the intensity of the bands for IL-1β and IL-6 was significantly decreased. The intensity of the bands for the control group and LPS+SSB2 is following the same fashion, suggesting that the mRNA expression of genes, upregulated in case of LPS insult, was significantly reversed. The results of this image were supported with the relative expression values of both the genes, normalized to the expression of the control.

In case of IL-1β (Figure 4 A) the relative mRNA expression increased by more than 0.75 folds for the LPS treated group. For treatment group that received both LPS and SSB2, the mRNA expression remained almost the same as that of the control group. The relative expression was recorded at 1.1 which was negligible.

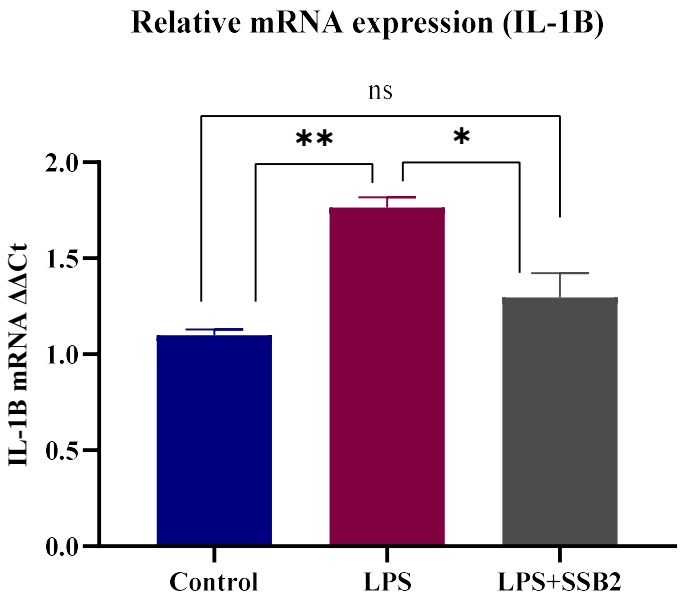

Figure 4.5: SSB2 downregulates the expression of IL-1 β in LPS treated HT-22 cells

Each bar represents the Mean ± SD (n=3). The relative expression of IL-1β increased in treatment group receiving only LPS. Relative expression of IL-1β decreased for treatment group receiving both LPS and SSB2. One Way ANOVA analyzed the results

and significant difference (P<0.05) existed between the Control and LPS groups and between LPS and LPS+SSB2 groups.

For mRNA expression of IL-6 in LPS treated group, there was a 4.2-fold increase relative to the control group. Cells receiving both LPS and SSB2 showed only a minor fold increase of 0.3. This fold increase is evidence that Saikosaponin B2 protects HT-22 cells against the inflammatory effects of LPS and employs mechanisms to reverse them. This also suggests that Saikosaponin B2 has anti-inflammatory role in the context of neuronal cells.

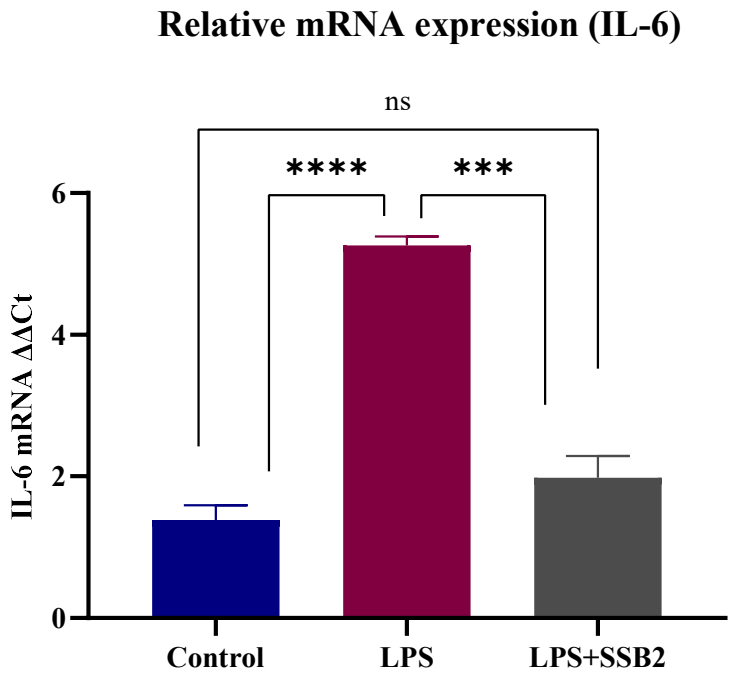

Figure 4.6: SSB2 downregulates the expression of IL-6 in LPS treated HT-22 cells

Each bar represents the Mean ± SD (n=3). The relative expression of IL-6 increased in treatment group receiving only LPS. Relative expression of IL-6 decreased for treatment group receiving both LPS and SSB2. One Way ANOVA analyzed the results and significant difference (P<0.05) existed between the Control and LPS groups and between LPS and LPS+SSB2 groups.

4.3 Apoptosis Analysis:

4.3.1 Saikosaponin B2 inhibits LPS-induced apoptosis in HT-22 cells:

To establish the effects of SSB2 on apoptosis, Annexin V staining was done. Once the cells were effectively stained with Annexin V /Propidium Iodide (PI) stains and incubated they were assessed using a Flow Cytometer that determined the percentage of early apoptotic, late apoptotic, necrotic and viable cells. Figure 4.7 and 4.8 represents the distribution of cells as being in different states. In Control group the percentage of viable cells was 96.6 %, with 1.4 % cells were undergoing early apoptosis and 1 % cells undergoing late apoptosis. In LPS treatment group the percentage of early and late apoptotic cells increased to 16.3 % and 14.1% respectively, whereas the percentage of viable cells was 67.7 %. Treatment group that received both LPS and SSB2 showed a reversal of percentage of late and early apoptotic cells. 4.9 % and 3.5 % cells were in their early and late apoptosis and 90.9 % cells were viable. This implies that SSB2 treatment of HT-22 cells significantly protects against inflammation induced apoptosis.

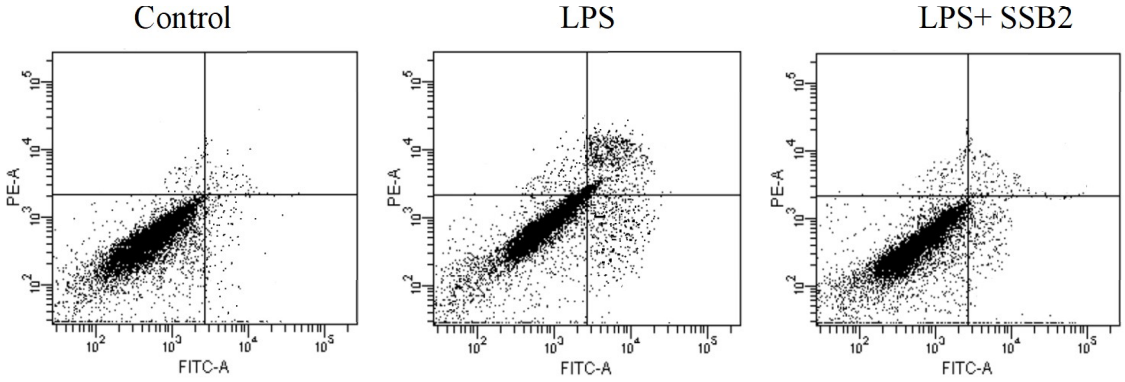

Figure 4.7: Saikosaponin B2 inhibits LPS-induced apoptosis in HT-22 cells.

Lower left grid (An V-/ PI -) shows percentage of viable cells, lower right grid (An V+/ PI-) shows percentage of early apoptotic cells, upper right grid (An V+/PI+) shows late-apoptotic cells and upper left grid (An V-/PI+) shows necrotic cells.

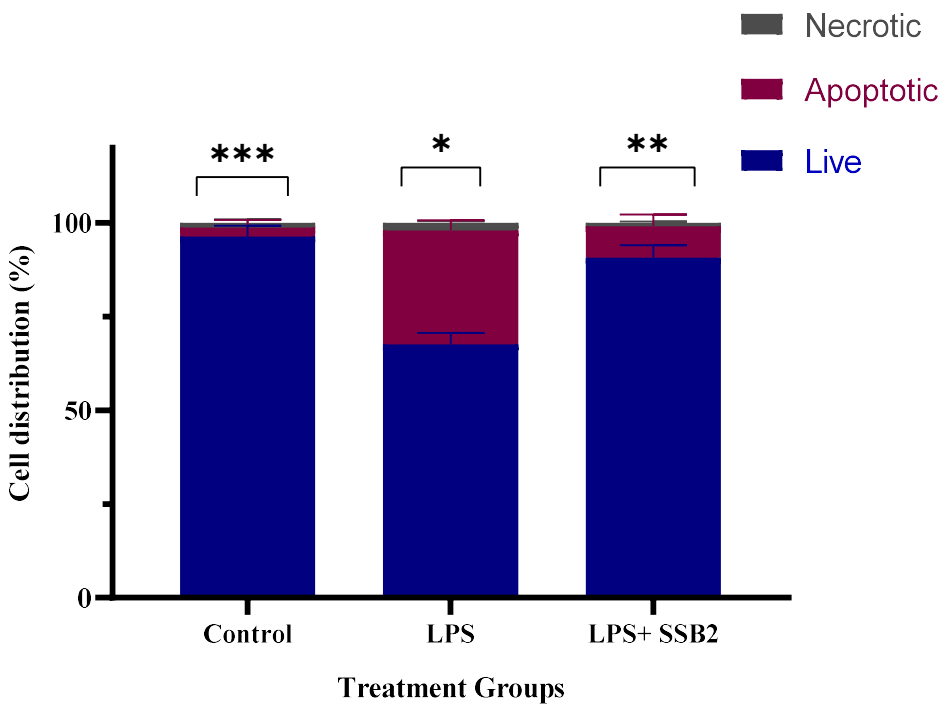

Figure 4.8: Saikosaponin B2 decreases LPS-induced apoptosis in HT-22 cells

Each bar represents Mean ± S.D (n=3). Apoptosis percentage of LPS receiving group increased compared to control group($p<0.05$) Early and late apoptosis was decreased once cells were given SSB2 after LPS injury ($p<0.05$). Two Way ANOVA was used to compare group means. $P<0.05$ was significant among group means.

4.4 Morphological Analysis:

4.4.1 SSB2 protects against characteristic nuclear fragmentation in LPS-induced inflammation in HT-22 cells:

To validate the findings of Annexin V staining, HT-22 cells were stained with DAPI. 4′,6′- diamidino-2-phenylindole is a compound with high affinity for A-T rich regions in DNA. Bound to this stain, apoptotic cells appear brightly stained with characteristic features of apoptosis i.e condensation of chromatin, fragmentation of nuclei and aggregated apoptotic bodies. Under a fluorescence microscope they appear bright blue. 20-30 cells were chosen for analysis at a time in each experiment. As can be seen in Figure

4.9, cells of control group, are regular in shape with consistent size and uniform distribution of cellular contents i.e. without nuclear fragmentation and chromatin condensation. In LPS treated group there is a visible increase in disrupted membranes, signature staining; chromatin condensation and nuclear fragmentation. In the treatment group receiving both LPS and SSB2, there is a significant decrease in the number of cells having apoptotic characteristics.

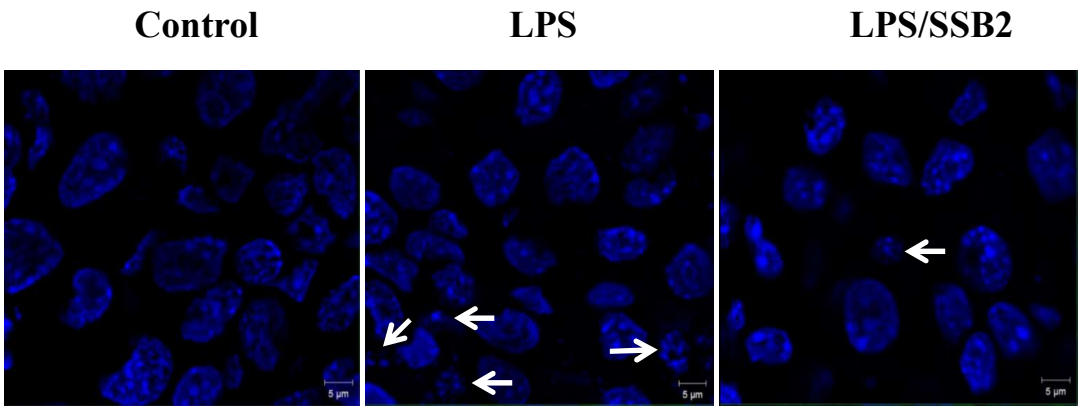

Figure 4.9: SSB2 protects against characteristic nuclear fragmentation in LPS-induced inflammation in HT-22 cells

Figure 4.10 represents the calculated Apoptotis Index in the treatment groups. The number of apoptotic cells was computed by dividing the total number of cells to determine the percentage in each sample. In Control group, the mean apoptotic index was 5.33%, whereas it was recorded at 71.33 and 16 % for LPS and LPS+SSB2 groups respectively.

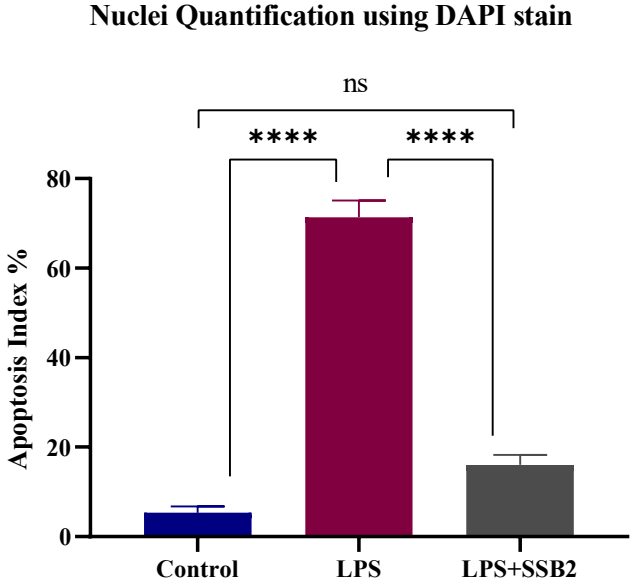

Figure 4.10: SSB2 protects against characteristic nuclear fragmentation in LPS-induced inflammation in HT-22 cells.

Each bar represents Mean±S.D (n=3). Apoptosis Index increased for LPS treated group significantly, compared to the control group ($p<0.001$). Significant decrease in apoptotic index was seen for HT-22 cells treated with both LPS and SSB2 ($p<0.05$) compared to LPS group. One Way ANOVA was used to analyze the results.

4.5 ROS Generation:

4.5.1 SSB2 reduces ROS generation during LPS induced inflammation in HT-22 cells:

ROS mediated oxidative stress plays a pivotal role in a number of neurodegenerative diseases including Alzheimer's Disease. To understand the relationship between ROS generation and neuroinflammation DCFH-DA assay was done. A widely used method for the detection of Reactive Oxygen Species (ROS) utilizes the DCFH-DA, which is a stable compound that readily enters the cell and interacts with the intracellular esterases to yield DCFH, which gets trapped inside the cell. Many ROS including peroxides and hydroperoxides with low molecular weight oxidize this DCFH to give the highly

fluorescent 2′,7′-dichlorofluorescein (DCF). Hence the fluorescence produced is directly propotional to the generation of ROS inside the cell. Figure 4.11 shows the images captured of cells after they were dyed with the DCFHDA dye. Cells with increased production of Reactive Oxygen Species belong to the treatment group that only received LPS, this indicates that LPS injury successfully initiated injury in HT-22 cells; as increased ROS is a responsive mechanism to oxidative stress. Figure 4.12 is illustrative that SSB2 treatment, reduces ROS production in cells once they were exposed to LPS. It can be clearly seen that fluorescence is significantly reduced after treatment with SSB2.

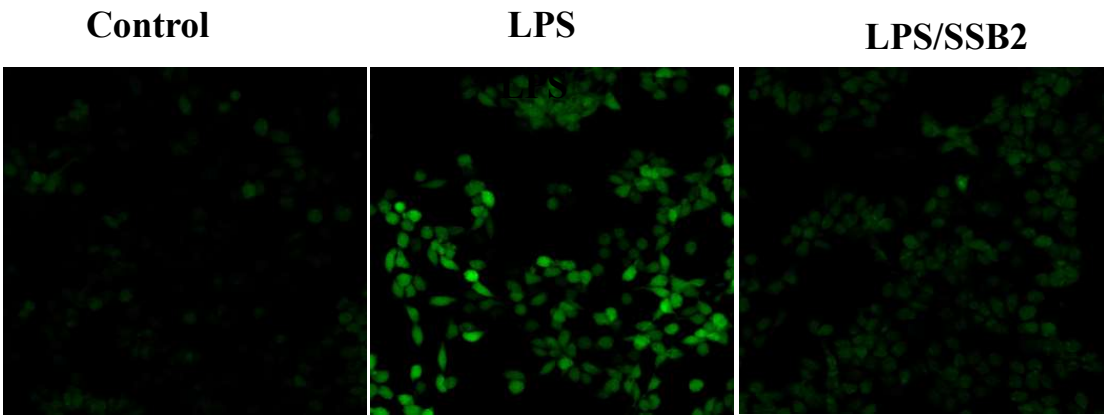

Figure 4.11: SSB2 reduces ROS generation during LPS induced inflammation in HT-22 cells.

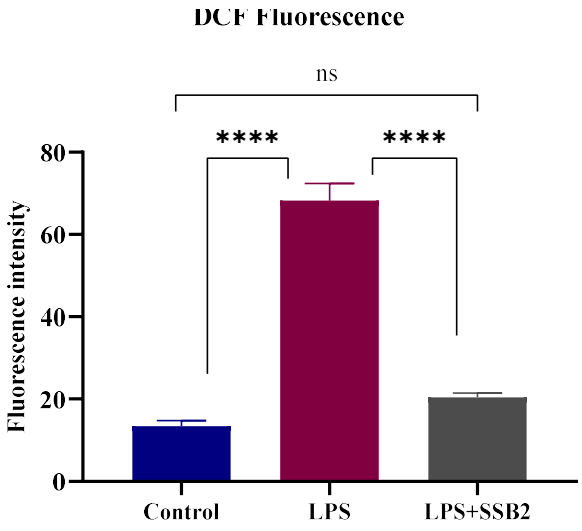

Figure 4.12: SSB2 reduces ROS generation during LPS induced inflammation in HT-22 cells.

Each bar represents Mean ± S.D(n=3). The intensity of fluorescence in the LPS treated group is recorded at 68.2 which signifies that the ROS generation has increased from the control group by more than 50 units ($P<0.01$). Treatment with SSB2 decreases intensity of fluorescence to 20 units ($p<0.01$). One way ANOVA was used to analyze group means.

CHAPTER 5 : DISCUSSION

The focus of this study was to examine the impact of Saikosaponin B2 on neuroprotection in LPS induced neuroinflammation in mouse hippocampal cells (HT-22). LPS is extensively used to incite inflammation owing to its pro-inflammatory properties and well-studied TLR-4 mediated activation of immune system. The need of advanced medical and veterinary research employs in-vivo and in-vitro models of LPS-induced inflammation and oxidative stress in a number of diseases, particularly in neurodegenerative diseases as neuroinflammation is an important pathomechanism in them. Neuroinflammation plays a pivotal role in the progression of A.D; a unifying concept in LPS-induced prompting of TLR-4 signaling as well as upregulated expression of pro-inflammatory cytokines and the progression of Alzheimer's disease. Hence LPS is an established stimulus of inflammation both in-vivo and in-vitro. HT-22 cell line is derived from mouse hippocampal cells which house microglia involved in pathogen detection mediated by Toll-like receptors. A model involving neuronal cells, treated by LPS to induce neuroinflammation creates conditions where it becomes ideal to research, analyze and come up with strategies of therapeutic grade for diseases like A.D (Skrzypczak-Wiercioch and Sałat 2022)

There has been a shift in research in the past years, the focus being neuroinflammation; which is considered as the main hallmark or pathogenesis of degenerative diseases of the neurons. A number of studies have confirmed that the progression of degenerative diseases is always initiated and accompanied by neuroinflammation.(Mansur, Delgado-Peraza et al. 2020, Zhou, Wang et al. 2021) In this study, mouse hippocampal cell lines, HT-22 were stimulated with LPS, which is a well-established mode for studying effects of Alzheimer's in-vitro. The results of the cell-viability analysis showed that the proliferation and viability of neuronal cells was decreased by 50 % at a concentration of 10 μM (SSB2). It was derived from this finding that using Saikosaponin B2 at this concentration would be non-toxic to other healthy microglia and would specifically target glia expressing pro-inflammatory cytokines. It can

be inferred from these findings that SSB2 under in-vivo conditions would inhibit the proliferation of activated microglial cells.

The Central Nervous System consists of many components, microglia are an important part of which. They play significant roles in neuroinflammation.(Woodburn, Bollinger et al. 2021) Important bio-markers of microglial activation are IL-6 and IL-1β which are proinflammatory cytokines and play pivotal roles in progression of A.D and P.D . Results of our study showed that increase in pro-inflammatory cytokines was effectively decreased after treatment with SSB2, suggesting that SSB2 relieves neuroinflammation and employs neuroprotective mechanisms in hippocampal cells.

Chronic neuroinflammation which is characterized by a long-term activation of the immune components such as microglia, create conditions which results in neuronal damage and death. (Kim, Wong et al. 2019) Cell death from chronic neuroinflammation differs from the patterns of Apoptotic death and Necrosis. In order to determine if there was a change in the percentage of viable cells a consequence of chronic neuroinflammation, we stained the cells with Annexin V and Propidium Iodide stains. Our results showed that in response to LPS insult, SSB2 considerably reduced the cells undergoing cell death as a result of neuroinflammation.

The hippocampus houses centres for advanced mental activities such as emotional control and integration, cognition and memory (Royero, Higa et al. 2020) Located in the ventral region of hippocampus , the CA1 region regulates emotional behaviours.(Huang, Coupland et al. 2013) Under normal conditions, microglial cells are in the resting phase and they participate in promoting neuronal survival, neuronal differentiation, increasing synaptic plasticity and formation of neuronal networks.(Colonna and Butovsky 2017) In the presence of a chemical injury, these resting microglia are activated and they start secreting pro-inflammatory cytokines resulting in reduced function of hippocampus and loss of structural integrity.(Cserép, Pósfai et al. 2021) In this series of experiments, one focused on the morphological analysis of the cells under stress. This was done by staining the cells with DAPI stain, a dye that binds A-T rich regions in the DNA and fluoresces a bright blue when illuminated. This was done to confirm if the cell death was a consequence

of neuroinflammation or not. Classical feature of apoptosis i.e. increased membrane blebbing, nuclear fragmentation and cell shrinkage were observed as a result of neuronal insult. SSB2 visibly reduced Nuclear Pyknosis and significantly prevented the nuclear condensation and fragmentation observed when the cells were treated with LPS alone, suggesting that SSB2 plays neuroprotective roles in neuronal cells under conditions of inflammation.

Mitochondrial Dysfunction entails a number of disruptions and mechanisms including swelling and fragmentation, the cristae show a disruption in structure. But the mechanism of utmost importance is the generation of ROS that accelerates neuronal damage.(Urrutia, Bórquez et al. 2021) Production of ROS activates the microglia which leads self-promoting vicious cycles of imflammatory responses. This upregulation festers more production of ROS and the neuroinflammation becomes chronic. Our results from DCFH-DA staining showed that SSB2 reduced ROS generation in LPS-induced Neuroinflammation, suggesting that SSB2 plays potent neuroprotective roles in chronic diseases like A.D.

To summarize this study; Saikosaponin B2 is a potent neuroprotective agent against LPS-induced neuroinflammation. It substantially decreases pro-inflammatory mediators in the event where injury or chemical insult is present. Neuroinflammation led decline in neuronal survival is potentially reversed by SSB2. In addition, ROS generation, a contributing factor to chronic neuroinflammation is decreased in cells treated with SSB2 after LPS treatment.

CHAPTER 6 : CONCLUSION

The current study evaluated Saikosaponin B2 as a neuroprotective agent against LPS induced inflammation in mouse hippocampus cells. LPS successfully induced inflammation in HT-22 cells. Saikosaponin B2 significantly down-regulated gene expression levels of IL-6 and IL-1β. It also markedly reduced inflammation steered neuronal death and production of ROS in the microglia. Nuclear morphology of the LPS-treated cells was also significantly recovered. These results favor the possible utilization of Saikosaponin B2 as a neuroprotective agent and can help in the management of neurodegenerative diseases like A.D as a potent therapeutic agent.

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