Mechanism of beta 2 adrenergic receptor (β2AR) agonist in regulation of inflammation in Parkinson's disease

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Immunology

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by degeneration of dopaminergic neurons (DA-neurons) which are primarily present within a region of the brain called as substantia nigra (SN). Several studies suggest that the etiology of the PD includes destructive chronic inflammation within the mid-brain that also contributes to the progressive neurodegeneration. Microglia are the principle immune cells of the central nervous system (CNS) and play an important role in CNS homeostasis. The presence of activated glial cells, the participation of innate immune system, increased inflammatory molecules such as cytokines and chemokines, and increased oxidative stress and reactive oxygen species are the main neuroinflammatory characteristics present in neurodegenerative disorders including PD.

Previously, we have found that the use of a long-acting β2-AR (β2-adrenergic receptor) agonist, Salmeterol functions in part to inhibit the inflammatory response of activated microglial cells, and in this thesis, I have sought to discover the underlying mechanisms of the immunoregulatory effects of β2-AR agonists on microglial cells using several molecular approaches. β2-ARs belong to the G protein-coupled seven transmembrane receptor superfamily and microglial cells a high density of these β2-ARs on their cell surface. Activation of β2-AR by Salmeterol and other β2-AR agonists initiate intracellular signaling pathways either via G proteins or through β-arrestins. In the present set of studies that constitute my thesis, I have investigated the signaling pathways and anti-inflammatory effects of Salmeterol that regulates the response of microglial cells to inflammatory stimuli that can explain the ability of these β2-AR to protect DA-neurons against inflammation and progressive degeneration.

In initial studies, we have found that pre-treatment with Salmeterol suppresses production and release of pro-inflammatory mediators such as TNF-α, IL-1β and nitric oxide at both the protein and mRNA levels in LPS-stimulated BV2 cells. Salmeterol appears to mediate this anti-

inflammatory effect by inhibiting the NF-κB signaling pathway via suppressing the phosphorylation of TAK1 and degradation of IκBα. Furthermore, Salmeterol reduces nuclear translocation of NF-κB, thereby suppresses the production of these and other inflammatory mediators. Salmeterol appears to function by increasing the expression of β-arrestin2, suppressing TAK1/TAB1 interaction, and enhancing the interaction between TAB1 and β-arrestin2. Conversely, the silencing of β-arrestin2 abrogates the anti-inflammatory effects of Salmeterol in LPS-stimulated BV2 cells. These findings suggest that the anti-inflammatory properties of Salmeterol are dependent on the non-canonical or β-arrestin2-dependent pathway of GPCR signaling.

In further studies, we found that Salmeterol inhibits the production of other LPS-induced pro-inflammatory mediators of M1 phenotype such as IL-(interleukin) 18, IL-6, pro-inflammatory chemokines (CCL2, CCL3, CCL4) and reactive oxygen species from BV2 cells. Interestingly, treatment with Salmeterol and other β2-AR agonists robustly enhanced the production of the regulatory anti-inflammatory cytokine IL-10 from LPS-activated BV2 cells. In addition, Salmeterol upregulates the expression of arginase-1 and CXCL14. In contrast, the silencing of the transcription factor *Creb* abrogates the Salmeterol-mediated production of IL-10 in LPS-activated BV2 cells, but silencing of β-arrestin2 with *Arrb2* siRNA did not. In conclusion, these findings suggest that the Salmeterol-induced conversion of LPS-activated microglial cells from an M1- to M2-like phenotype by β2-AR agonists involves activation of the classical cAMP/PKA/CREB signaling pathway. Furthermore, we have performed RNA-sequencing to determine the effect of Salmeterol on global gene expression in LPS-activated BV2 microglia. Results from differential gene expression analysis show that pattern of inflammatory genes up-regulated by LPS stimulation, including cytokines, chemokines and transcription factors, was significantly altered

by pre-treatment with Salmeterol. In addition, other neurodegenerative disease-related genes were modified by treatment with Salmeterol in activated microglia.

Finally, I also tested whether the anti-inflammatory properties of Salmeterol can be used to target other chronic inflammatory diseases such as periodontitis (an oral-inflammatory disease). Results from this study showed that Salmeterol exerts similar anti-inflammatory effects on murine macrophages and human monocytes stimulated by *Pg*LPS (LPS from *Porphyromonas gingivalis*). Similar to our studies on microglia, Salmeterol suppresses production and release of pro-inflammatory mediators via inhibiting the NF-κB and MAPK signaling pathway. My results suggest the efficacy in using Salmeterol and other β2-AR agonists in the treatment of chronic inflammatory disorders.

Preface

This is an original work completed by Monika. No animal and human samples were used in this study. Content of this thesis has been modified form the following published articles.

1. Flood, P., Arbabzada, N., and, Sharma, M., (2016). **Inflammation: Role in Parkinson's disease and Target for Therapy.** *Challenges in Parkinson's. Disease.* This article is represented as Chapter 1 of this thesis. Sections 1.1, 1.2, 1.7 and 1.8 of this review article were written by M Sharma and sections 1.3-1.6 were written by N Arbabzada and P Flood.

2. Sharma, M., Flood, P.M., (2018). **Adrenergic Receptors as Pharmacological Targets for Neuroinflammation and Neurodegeneration in Parkinson's disease.** *Neuroprotection.* This article is represented as Chapter 2 of this thesis. Review article was written by M Sharma and edited by PM Flood.

3. Sharma, M., Flood, P.M., (2018). **β-arrestin2 regulates the anti-inflammatory effects of Salmeterol in lipopolysaccharide-stimulated BV2 cells.** *J. Neuroimmunol*. 325, 10–19. This article is represented as Chapter 4 of this thesis. M Sharma and PM Flood designed the experiments and all experiments were performed by M Sharma. Manuscript was written by M Sharma and PM Flood.

4. Sharma, M., Arbabzada, N., Flood, P.M. (2019). **Mechanism underlying β2-AR agonist-mediated phenotypic conversion of LPS-activated microglial cells**. *J. Neuroimmunol.* 332, 37-48. This article is represented as Chapter 5 of this thesis. M Sharma and PM Flood designed the experiments and all experiments were performed by M Sharma except ELISA experiments in Figure 5.4 were performed by N Arbabzada. Manuscript was written by M Sharma and PM Flood.

5. Sharma, M., Patterson, L., Chapman, E., Flood, P.M., (2017). **Salmeterol, a long-acting β2-adrenergic receptor agonist, inhibits macrophage activation by lipopolysaccharide from *Porphyromonas gingivalis*.** *J. Periodontol*. 88, 681–692. This article is represented as Chapter 7 of this thesis. M Sharma and PM Flood designed the experiments. All experiments were performed by M Sharma except the ELISA for TNF-α was performed by L Patterson and E Chapman. Manuscript was written by M Sharma and edited by PM Flood.

Dedication

To my Grandmother

Acknowledgement

First and foremost, I am extremely grateful for my dissertation advisor Prof. Patrick Flood for giving me the opportunity to conduct my research in his lab. You are so generous with your support, your mentorship and your time, and the experiences you have provided to me over the years. Your endless belief and confidence in me accelerated my motivation and was highly influential towards the completion of this body of work. Thanks a lot for never letting me give up. Thank you for giving me time from your busy schedule. I feel very fortunate to have had a mentor like you and it has been an absolute pleasure being one of your students.

I would like to acknowledge my committee members, Prof. Maria Febbraio, and Dr. Rob Ingham for their mentorship, encouragement, and research guidance throughout my program. I would like to thank past members of the lab and the department, Grace Garen, Tracy James and undergrad project and summer students for their support and help. To Dr. Kim Ellison and Judy, thank you both for giving me the opportunity to teach and it was a wonderful experience.

Thank you to the Department of Medical Microbiology and Immunology for their support over the years. I am highly grateful for the Parkinson's Alberta Association for providing me the graduate research fellowship for three years. In addition, I also thank the Faculty of Graduate Studies and Research and Graduate Student Association for the travel awards.

This would not have possible without the tremendous support of my family. I am highly grateful to my parents who have encouraged and supported me throughout my academic pursuit. I am also thankful to uncles and aunts, my sisters Sonika, Priyanka, Jyotika, Geetika, Vidhi and brother Dhruv for always being there to talk me when I was stressed, make me laugh, make me feel like I'm capable of anything and you all made the years fly by.

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List of Abbreviations

6 OHDA: 6-hydroxy dopamine
AD: Alzheimer's disease
ALS: Amyotrophic Lateral Sclerosis
ALS: Amyotrophic Lateral Sclerosis
BBB: Blood-Brain-Barrier
BDNF: Brain –derived Neurotrophic Factor
C/EBP: CCAAT-enhancer-binding protein
cAMP: Cyclic adenosine monophosphate
CCL2/MCP-1: C-C motif chemokine ligand 2/ Monocyte Chemoattractant Protein
CCL3: C-C motif chemokine ligand 3
CCL4: C-C motif chemokine ligand 4
CCL5/RANTES: C-C motif chemokine ligand 5/ Regulated on Activation, Normal T-cell Expressed and Secreted
cDNA: Complementary deoxyribonucleic acid
CNS: Central Nervous System
COMT: Catechol-O-methyl transferase
COPD: Chronic Obstructive Pulmonary Disorder
CREB: cAMP Response Element Binding Protein
CSF: Cerebrospinal Fluid
CXCL10: C-X-C motif chemokine 10
CXCL14: C-X-C motif chemokine 14
DA: Dopamine
DCFDA: 2,7 Dichlorofluoresceine diacetate
DEG: Differential Gene Expression
EDTA: Ethylene diamine tetra acetic acid
ELISA: Enzyme-linked immunosorbent assay
ERK1/2: Extracellular signal Regulated Kinase 1/2
FGF: Fibroblast Growth Factor
FPKM: Fragments Per Kilobase of transcripts per Million mapped reads
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GDNF: Glial-deriver Neurotrophic Factor
GO: Gene Ontology
GPCR: G-protein coupled protein receptor
IFN: Interferon
IL: Interleukin
iNOS: Inducible nitric oxide synthase
IκBα: Nuclear factor kappa-light-chain-enhancer of activated B cells inhibitor alpha
JNK: c-Jun N-terminal Kinase
KEGG: Kyoto Encyclopedia of Genes and Genomes

LABA: Long-acting beta agonist
LC: Locus Coeruleus
L-DOPA: Levodopa
LPS: Lipopolysaccharide
LRRK: Leucine Rich-Repeat Kinase
MAPK: Mitogen-activated protein kinase
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS: Multiple Sclerosis
MYD88: Myeloid differentiation primary response 88
NADPH: Nicotinamide adenine dinucleotide phosphate
NE: Norepinephrine
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NO: Nitric Oxide
NSAID: Non-steroidal anti-inflammatory drug
PAMP/DAMP: Pathogen-associated Molecular Pattern/ Damage-associated Molecular Pattern
PD: Parkinson's Disease
***Pg*LPS:** LPS form *Porphyromonas gingivalis*
PI3K: Phosphoinositide-3-kinase
PKA: Protein kinase A (cAMP-dependent protein kinase)
PMA: Phorbol 12 Myristate 13-acetate
PRR: Pattern recognition Receptor
ROS: Reactive Oxygen Species
SABA: Short-acting beta agonist
SBMA: Spinal and Bulbar Muscular Atrophy
SMA: Spinal Muscular Atrophy
SNCA: Alpha synuclein gene
SNpc: Substantia Nigra pars compacta
STAT: Signal Transducer and Activator of Transcription
TAB1: TAK-binding protein-1
TAK1: transforming growth factor-beta Activated Kinase-1
TGF-β: Transforming Growth Factor-beta
TH: Tyrosine Hydroxylase
TLR: Toll-like receptor
TNFα: Tumour necrosis factor alpha
TRAF: TNF-receptor Associated Factor
VEGF: Vascular Endothelial Growth Factor
β2-AR: Beta 2- Adrenergic Recepto

GENERAL INTRODUCTION

Chronic inflammation takes a significant toll on the human body and is the prevalent mechanism in several diseases including a number of neurodegenerative diseases. More than 100,000 Canadians are estimated to have PD, the second most common neurodegenerative disorder in humans after Alzheimer's disease. The estimated costs of treatment, disability payments, and lost productivity of this disease and its consequences are over $560 million annually, which is a pressing burden on our society. The symptoms of PD such as tremor, rigidity and impaired movements are caused by the insufficient formation and action of dopamine, a chemical produced by the human body which is critically important for allowing normal muscular movements. Currently, there is no such treatment to stop the diseases completely, but traditional treatment such as L-dopamine replacement therapy can effectively relieve symptoms. However, even with these treatments the disease still progresses and eventually these treatments are no longer effective.

β2-AR agonists are a class of FDA and Health Canada-approved drugs for in use in Canada as well as in the rest of North America and are widely accepted as a safe, highly effective drugs. One such drug is Salmeterol (Sal), which is an inhaled long-acting highly selective β2-AR agonist which is the active ingredient in Advair®, and used as a bronchodilator for the treatment of COPD (chronic obstructive pulmonary disease) and asthma. The re-tasking of this drug for the treatment of PD and other neurodegenerative disorders would be an important therapeutic approach for the treatment, as it would not require any additional development as a new investigational drug.

Research studies have found that β2-AR activation regulates the gene expression of the protein α-synuclein, which is a hallmark for PD pathology. Interestingly, it has now been found by looking a population of over 4 million patients in Norway and patients in Israel who regularly use the β2-

AR agonist Salbutamol that its long-term use is associated with reduced risk of developing PD. Conversely, the regular use of a β2-AR antagonist Propranolol has been linked with the increased risk of developing PD. Our lab previously has found that Salmeterol is a highly effective therapeutic for the inhibition of progression of disease in established mouse models of PD, and my current research indicates that Salmeterol is a highly effective anti-inflammatory in part because of its ability to switch predominantly type1 inflammatory responses (degenerative phase) to type2 inflammatory responses (regenerative phase). We believe that findings of this study will help in developing new, safer and more effective therapies against PD.

CHAPTER 1

Inflammation: Role in Parkinson's disease and target for therapy

This chapter is modified from the published article:

Flood, P., Arbabzada, N., and Sharma, M., 2016. **Inflammation: role in Parkinson's disease and target for therapy.** *Challenges in Parkinson's. Disease.*

1.1. Abstract

Evidence is now overwhelming that inflammation is a central process in the pathogenesis of progressive PD. The hallmark of this neuroinflammation is the activation of microglial cells and the secondary role of adaptive immunity in both the familial and idiopathic forms of PD, leading to the loss of dopamine-producing cells within the Substantia nigra. This activation is characterized by the oxidative stress response, production of inflammatory mediators, recruitment and activation of immune effector cells which create a toxic environment for dopaminergic neurons, and in forming a continuous cycle of inflammatory responses that result in chronic neuroinflammation and progressive neurodegeneration. This chapter focuses on the different components of the inflammatory response that are involved in Dopamine-neurodegeneration, evidence for inflammation in different forms of PD, and the role of inflammation in the various animal models of PD. Finally, we provide current evidence that targeting this inflammation with a number of anti-inflammatory therapies can be an effective way to halt the progression of chronic neuroinflammation-induced PD.

1.2. Introduction

Recently accumulated evidence suggests that neuroinflammation and chronic inflammation of the CNS may play a critical role in the development of a number of neurodegenerative diseases. Particularly, in PD, neuroinflammation has been proposed as a major contributing factor that plays a role in the initiation and progression of the dopaminergic neuronal loss that is the hallmark of the disease. Evidence to support neuroinflammation as the mode of pathogenesis for PD originates from postmortem studies n patients and animal models. The proliferation and activation of microglial cells, as well as increased levels of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, nitric oxide (NO), and reactive oxygen species (ROS),

are present in postmortem analysis of brains and in the cerebrospinal fluid (CSF) of PD patients (1). These findings suggest that pro-inflammatory cytokines, specifically TNF-α, may be involved neuronal cell death. Likewise, neuronal cell death can release mediators that activate microglial cells— thereby potentiating a vicious cyclical inflammatory-mediated neuronal cell death. PD is unique in that the clinical symptoms appear after a loss of approximately 70–80% of striatal nerve terminals and 50–60% of dopaminergic cells in the Substantia nigra pars compacta (SNpc), the region of the brain that is responsible for controlling movement (2,3). This recent scientific understanding is vital to developing potential early biomarkers and/or therapeutic strategies to help with better diagnosis and disease management. We discuss various components of neuroinflammation, focusing on the role of the innate and adaptive immune responses as they relate to PD. In addition, we briefly summarize the inflammatory pathology seen in the genetic and toxin-induced models of this disease, as well as discuss several anti-inflammatory therapies currently being used or tested as potential treatments for PD.

1.3. Innate immune response and PD

The innate immune response serves as the first line of defense to both infiltrating pathogens and/or endogenous insults. As such, it primarily functions to initiate an immediate and nonspecific response to any compound it deems unnecessary and/or a potential threat. Pathogen-associated molecular patterns (PAMPS) and/or endogenous damage-associated molecular patterns (DAMPs) can trigger an innate immune response. In the case of CNS, the innate immune system has several components: cells that mediate an immune response, such as microglia and astroglia, the complement system, and the physical obstruction imparted by the blood-brain barrier (BBB). For centuries, the CNS was thought to be immune privileged because the BBB did not allow various compounds to enter the CNS through the circulatory system. However, as we are beginning to

appreciate the intricacy of the immune-nervous system interaction, the notion of immune privilege no longer holds (4).

In PD, the various components of the innate immune system are activated and the integrity of the BBB is compromised, allowing for the innate-mediated recruitment and activation of the adaptive arm of the immune system (5). While PD is not among other immune-dependent degenerative diseases, Parkinsonian symptoms have been shown to develop after infectious inflammatory diseases such as Epstein-Barr virus (EBV)-induced encephalitis (6). Likewise, many anti-inflammatory therapeutic agents have served protective functions in PD models (5). As such, while the role of the immune system is not clear and/or extensively studied in the etiology of the disease, it is well established that the immune system is critical for the progression of the disease. Initial activation of the innate immune cells as well as the complement proteins may serve protective roles, but when these innate defense mechanisms become unregulated and maladaptive, it leads to disease progression. As immediate responders, cells of the innate immune system play an important role in initiating an inflammatory response against various nonspecific components of endogenous DAMPS and/or PAMPs (7). The innate cells, astrocytes and microglia, play an active role in the pathological mechanism responsible for the progression of the disease.

1.3.1. Astrocytes

Astrocytes make up about 20–40% of the glial cell population in the CNS. Their functions include, but are not limited to, maintaining the integrity of the BBB, facilitating repair and scar formation, and maintaining the extracellular ion homeostasis. The expression of receptors that are critical for innate immunity such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domains, double-stranded RNA-dependent protein kinase, scavenger receptors, mannose-binding lectin receptor, and complement system components has implicated a role for astrocytes in innate

immunity (8). The role of astrocytes in PD is debatable and not well understood. Studies are inconclusive as to whether astrocytes have a neuroprotective effect and/or a neurotoxic effect in PD. However, astrocytosis, the activation of astrocytes, has been reported in some cases of PD as demonstrated by an increase in the glial fibrillary acid proteins (GFAP) (9,10). GFAP is an intermediate filament needed by astrocytes to synthesize cytoskeletal structures and is a well-established biomarker for astrocytosis (10).

Furthermore, activated astrocytes are reported in postmortem brains of PD patients (11). In contrast, astrocyte activation is not only well documented in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) models of PD, but it has been reported to precede neuronal cell death (12–14). In the MPTP model, astrocytosis and dopaminergic cell death are synchronized. In the 6-OHDA model, several laboratories demonstrate that astrocytosis occurs in a time-dependent manner, peaking at 4 days post injection and remaining in the brain for about a month (15). There are also studies that contradict the presence of astrocytosis post 6-OHDA injection (16). Therefore, while astrocytes are an important cell type in the CNS, their role in inflammation and PD is not yet well established.

1.3.2. Microglia

In contrast to astrocytes, consistent microglial activation and the accompanying inflammatory response have been reported in both patient and animal models of PD. As the resident CNS macrophage, microglia cells are responsible for scavenging the CNS milieu for potential infiltrating pathogens and/or endogenous insults. Consequently, the phagocytic, cytotoxic, and antigen-presenting capabilities of these cells enable them to protect the CNS from various insults. Activated microglia targets infiltrating pathogens and damaged cells by releasing toxic ROS, free radicals, and phagocytosis. Evidence of microglial activation and its role in PD pathogenesis is

indisputable. Knott et al. (11) and others (17) have reported activated amoeboid-shaped microglia in postmortem brain of PD patients. These activated microglia cells are densely located and confined to the SNpc with a limited presence in the vasculature of the caudate and putamen regions. Additionally, in determining the role of microglia in PD pathogenesis, our results as well as those from other groups (18–20) have shown that in various PD models, microglial cells are needed for the pathogenesis of PD. For example, we have shown that high doses of the β2-adrenergic receptor agonist Salmeterol can induce dopaminergic cell toxicity. However, microglial cells are indispensable in the β2-AR-mediated toxicity, as high-dose Salmeterol has no effect on neuron-only cultures (18). In PD, the expression of ROS, free radicals, and the enzymes responsible for the production of these species such as NADPH oxidase (NOX or PHOX), induced nitric oxide synthase (iNOS), and myeloperoxidase (MPO) are elevated in the SNpc. These reactive species can activate microglial cells, and these activated microglia release pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 to recruit additional lymphocytes in the process of inflammation. Additionally, these cytokines can cause cytotoxicity in a direct, receptor-mediated manner, and/or in an indirect manner by inducing the further production of ROS and pro-inflammatory cytokines (21). For example, dopaminergic cells express receptors for TNF-α, which, upon binding the TNF-α ligand, can cause cell death through Fas ligand-mediated apoptosis, and Tumor Necrosis Factor Receptor (TNFR) knockout is protective in the MPTP model of PD (22). Indirectly, TNF-α can also activate additional microglia to release ROS and a variety of pro-inflammatory cytokines. This leads to a cyclical pathway whereby activation of a few cells can amplify the initial insult to a greater magnitude. This process has been termed reactive microgliosis and is now a leading working model for understanding and targeting neuroinflammation in PD (23).

1.4. Adaptive immune response and PD

The adaptive immune response is a highly specific response to injurious agents mediated by B and T-lymphocytes, which is characterized by the humoral and cell-mediated response, respectively. In PD, innate immune activation leads to an increased BBB permeability, allowing for the infiltration of peripheral T-cells and B-cells (24). These infiltrating cells are activated by active microglia expressing MHC 1/II through presentation of endocytosed peptides to the respective cells. Evidence to suggest the involvement of adaptive immune system is that single nucleotide polymorphism (SNP) in the MHC Class II predisposes individuals to PD, implying the role of both the innate and adaptive immune response in PD pathogenesis. Recent genome wide association studies (GWAS) have highlighted alleles HLA-DRA and HLADRB5 as risk factors for PD (25). Furthermore, MHC Class I proteins are typically used by CD8 T-cells and require β2-microglobulin, a protein required for the structural stability of MHC Class I, and in PD, the expression of β2-microglublin is found to be increased on microglial cells (24). Additionally, an increase in the number of cytotoxic CD8 and CD4 T-cells infiltrating into the SNpc of PD patients is accompanied by a decrease in the cytotoxicity suppressing capacity of regulatory T-cells (Treg) (26,27). Therefore, it suggests that toxicity of these effector T-cells is not properly regulated and can exacerbate neuronal cell death in the SN. With regard to B-cells, antibodies (Ab) to dopaminergic neurons have been found in the CSF of a proportion of the PD patients, thus implicating the involvement of the peripheral humoral arm of the adaptive immune response (28,29). Furthermore, immunization, which uses B-cells to generate antibodies against an antigen, with bovine mesencephalic homogenates (30) and hybrid dopaminergic cell line homogenates (28), can cause selective DA neuron damage in a microglia-dependent manner. The adaptive immune system has a delayed contribution to the pathology of PD but, nevertheless, is important

to understand in order to develop therapies that can mitigate and counter the pathology induced by this system.

1.5. Neuroinflammation and overlapping vulnerability of Substantia nigra (SN) neurons

The oxidative stress hypothesis focuses on the role that reactive oxygen and nitrogen species play in the neurodegeneration seen in PD. Reviews by Fahn and Cohen (31) as well as by Zigmond and Burke (32) discuss four characteristics of SNpc dopaminergic neurons that support the oxidative stress hypothesis as one of the major mechanisms responsible for the pathology of PD. However, understanding these characteristics can help explain the chronic, self-perpetuating inflammatory pathology that is responsible for disease progression in PD.

While inflammation involves activated immune cells and the release of a multitude of pro-inflammatory cytokines, the cycle does require a start point. The etiology of PD is unknown as is what gives rise to the chronic inflammatory pathogenesis seen in PD. Two different explanations of the chronic etiology of PD suggest that neuronal cell death leading to activated immune cells and the resulting uncontrolled inflammation further exacerbates cell death, or that activated immune cells cause cell death which results in the activation of additional immune cells resulting in a vicious cycle of immune cell-mediated inflammation and neuronal death (33,34). The process of innate and adaptive immune response depicted as schematic in **Figure 1.1.**

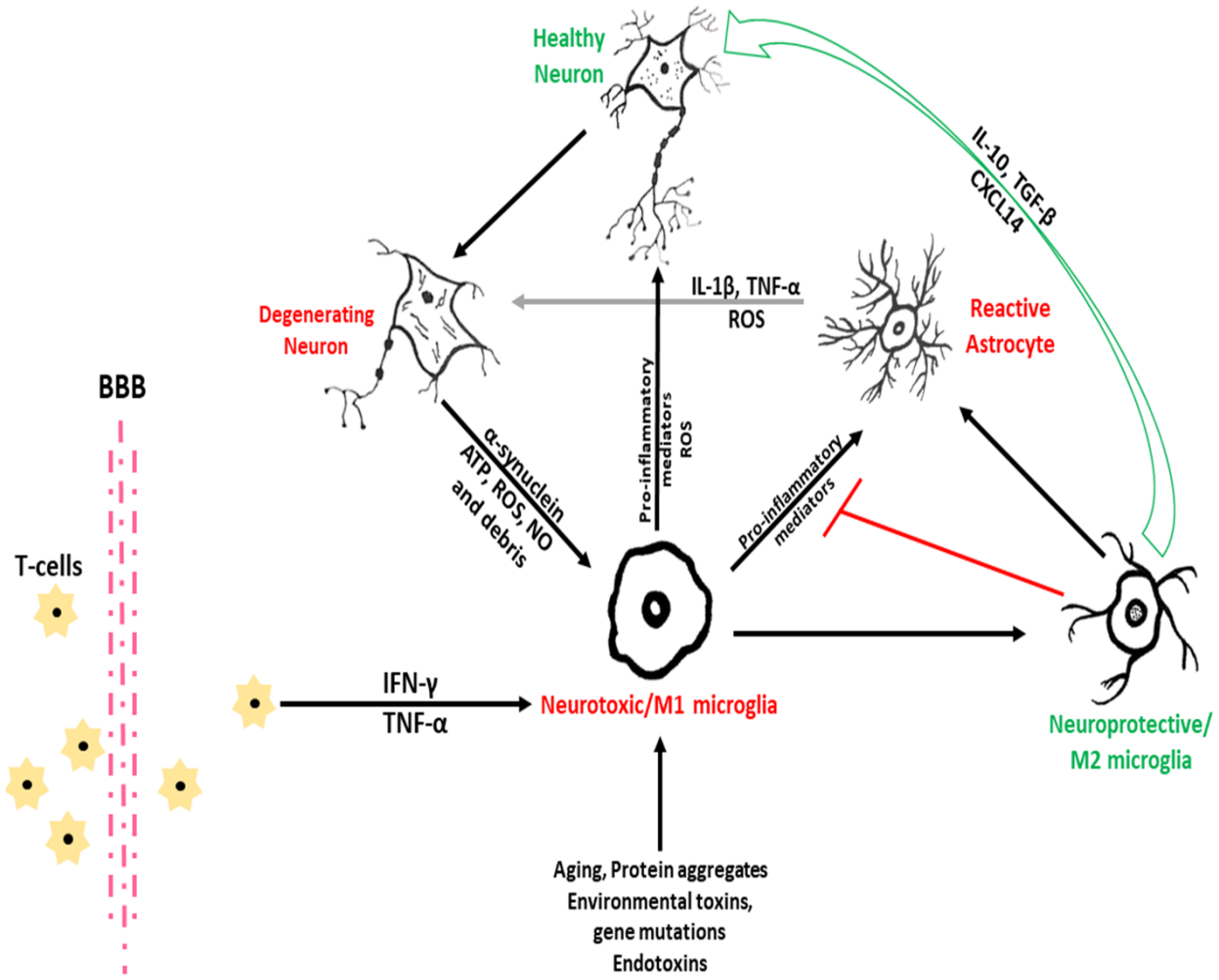


Figure 1. 1: Schematic representation of inflammatory mechanisms involved in PD pathogenesis.

Under pathological conditions such as α-synuclein protein aggregation, gene mutations, environmental factors and cytokines released from infiltrated T cells, microglia become activated called M1 phenotype. The pro-inflammatory mediators from M1 microglia further activate astrocytes leading to elevated production of pro-inflammatory factors, nitric oxide and superoxide radical, contributing to degeneration of dopaminergic neurons. The degenerating DA-neurons release many toxic factors that activate microglia and these degenerating neurons are vulnerable to inflammatory insult and co-localize or attract a large population of microglia in SN which suggest a mechanistic basis for the progression of PD. At certain stage of PD, subpopulation of microglia may become activated M2 phenotype releasing anti-inflammatory factors, including TGF-β, and exert a neuroprotective effect in PD.

The characteristics of SNpc dopaminergic cells make them vulnerable to ROS, subsets of which are important pro-inflammatory cytokines. First, dopamine degradation occurs by oxidative deamination, resulting in the production of H_2O_2 that then react with iron present in the neurons to form reactive radicals. Second, superoxides and free radicals are byproducts of the reaction between dopamine and the readily available oxygen to form reactive quinones. Third, the SNpc particularly rich in iron and hence the neurons found therein are more vulnerable to cell death via oxidative stress. Fourth, the SNpc neurons contain neuromelanin, which is formed by the auto-oxidation of DA, and the by-product of this reaction is ROS. These characteristics make SNpc neurons particularly sensitive to a cyclical process of oxidative stress contributing to inflammation that that leads to furthermore neuronal damage (33).

1.6. Genetic causes of PD and neuroinflammation

Although PD is typically a sporadic disease, approximately 10% of PD cases have been linked to several specific genes. These genes are α-synuclein, Parkin, UCH-L1 (ubiquitin C-terminal hydrolase L1), PINK1 (PTEN-induced kinase 1, NB for mitochondrial function), DJ-1, LRRK2 (leucine rich repeat kinase2), Pael-R, and glucocerebrosidase (24,35,36). These genes and their products have a role in the degradation of α-synuclein and/or in the control of the oxidative milieu. Mutations in genes encoding α-synuclein, Parkin, and/or UCH-L1 result in the accumulation of misfolded α-synuclein protein and are accompanied by neuronal cell death (24). Additionally, a pathological feature of PD is the Lewy body cytoplasmic inclusion bodies, which primarily consist of α-synuclein, tau, ubiquitin, and Parkin. These genes were identified in familial PD, as risk factors for sporadic PD, and further verified by a GWAS. Therefore, understanding the role of these genes and their products in mediating inflammation can help not only in developing more holistic model(s) of PD but also for therapy development.

1.6.1. LRRK2

Leucine-rich repeat kinase 2 (LRRK2) is an enzyme that is commonly expressed on multiple immune cells such as B-cells, monocytes, dendritic cells, and microglia (37). Mutations in LRRK2 are associated with autosomal dominant form of PD with high resemblance to the idiopathic PD phenotype and other inflammatory-mediated diseases as Crohn's disease (38) with a high predisposition to leprosy infection (39). LRRK2 is a member of the receptor interacting protein kinase (RIPK) family. The RIPK family has important roles in immunity as well as regulating of cell death (40). Furthermore, TLRs are an important activator of microglial cells. In the TLR-signaling pathway, LRRK2 is phosphorylated (41), and Kim et al. (42) as well as other groups (43) have reported a decrease in NF-κB-mediated transcription, specifically of TNF-α, post LRRK2 phosphorylation. NF-κB is a major transcription factor for many of the pro-inflammatory cytokines that are reported to play a role in the pathogenesis of PD, such as TNF-α, IL-1β, and IL-6; LRRK2 modulation of NF-κB will have important cellular effects on the inflammatory state of the activated microglial cells. Furthermore, a mutation in LRRK2, specifically R1442G, is reported to alter the phenotype of activated microglial cells to produce higher amounts of inflammatory cytokines with a decrease in the production of anti-inflammatory cytokines (43). Gillardon et al. tested the neurotoxicity of these microglial cells on cortical neurons by exposing neurons to conditioned medium from LPS-activated microglial cells, compared to conditioned medium from LPS-activated wild-type (WT) microglia, conditioned media from LPS-activated LRRK2 mutant significantly increased cell death (44). In addition, Kim et al. have shown that LRRK2 deficiency mitigates LPS-mediated increase in the mRNA of iNOS, TNF-α, IL-1β, and IL-6 (42). By itself, overexpression of the mutant LRRK2 *in vivo* and *in vitro* causes neurotoxicity (45). These data

support a role for LRRK2 in regulating the inflammatory response of microglial cells and the resulting effect on neuronal viability.

1.6.2. Parkin

Parkin is an important component of the multi-protein E3 ubiquitin ligase complex that is responsible for the ubiquitin-proteasome-mediated degradation of α-synuclein in the brain. Mutations resulting in loss of function of Parkin are responsible for autosomal recessive form of juvenile PD (46). Parkin not only regulates mitochondrial health but also is involved in the regulation of the NF-κB signaling pathway (47). Parkin ubiquitinates damaged mitochondria and subjects it to mitophagy and allows clearance from the cell (48). Similarly, activated Parkin catalyzes ubiquitination of the IκB kinase (IKK) subunit IKKγ, resulting in the downstream activation of NF-κB (47,49). In this NF-κB signaling pathway, TNF-receptor-associated factor-6 (TRAF6) also plays a role in regulating IKK activity. Loss-of-function mutation in Parkin increases the expression of TRAF6 (50), thereby activating transforming growth factor-1 (TAK1) that activates IKK and ultimately NF-κB and its associated transcriptional activity (49). With regard to Parkin and mitochondria, while mitophagy and PD have not yet been linked, damaged mitochondria are a source of ROS that can activate microglial cells through TLR-PAMP/DAMP pathways (36).

1.6.3. α-Synuclein

α-synuclein is an 18-kDa protein found in high concentrations in the CNS compared to other areas. While the function of α-synuclein is unclear, it is thought to be important for the release of neurotransmitters and vesicle trafficking (51,52). Mutations in the *SCNA*, gene coding for α-synuclein, is implicated in inherited forms of PD. Similarly, α-synuclein aggregation is a critical component of Lewy bodies in both sporadic and genetic PD. With regards to inflammation, α-

synuclein is thought to activate microglial cells through the nonspecific DAMP TLR2/ 4 pathway (53,54). An emerging link between gut microbiota and peripheral inflammation and PD is of interest to note. A study by Forsyth and colleagues (55) reported increased gut permeability and *Escherichia coli (E. coli)*, a Gram negative bacterium, staining in early onset PD patients. The implication of this study is that *E. coli*-dependent inflammatory processes resulted in an increased iNOS that then nitrosylated α-synuclein. WT, mutant, aggregated forms of α-synuclein can all trigger microglial activation by acting as a TLR-ligand. Conditioned media from dopaminergic cell line SH-SY5Y that either overexpressed WT or A53T mutant α-synuclein activated BV-2 microglial cell line, with the conditioned media from the neurons overexpressing mutant α-synuclein caused a more robust increase in TNF-α, IL-1α, and IL-1β (56). More importantly, mutant and aggregated fibrils of α-synuclein are reported to have cell-to-cell transmission capacity, thereby causing neuronal toxicity in a prion like mechanism as well (57). Moreover, nitrated α-synuclein can activate peripheral immune activation, especially T-cells and initiate the involvement of the adaptive immune response (58). Lastly, Tran and colleagues (59) have recently reported that antibodies to α-synuclein can offer a promising protective effect by inhibiting the entry of α-synuclein fibrils into neurons and causing neuronal death.

1.6.4. PINK1

PINK1 is a mitochondrial serine/threonine protein kinase implicated in providing cellular protection against mitochondrial-associated oxidative stress. As such, it is reported to regulate stressed mitochondria by enabling the binding of Parkin to stressed mitochondria and inducing autophagy (60). The role of PINK1 in inflammation is somewhat unclear; as evidence suggests that in PINK1 null animals injected with LPS, IL-1β, IL-12, and TNF-α are increased (61). However, in PINK1-deficient embryonic fibroblasts, there is no increase in pro-inflammatory

cytokine production post LPS injection because of decreased NF-κB activity (61,62). As such, experiments aimed at understanding the role of PINK1 in inflammation should be investigated in microglial cells which are known to propagate the inflammatory response in PD.

1.6.5. DJ-1

DJ-1, or Parkinson's disease Protein 7, inhibits the aggregation of α-synuclein, thereby acting as an oxidative stress sensor. PINK1 and DJ-1 deletion causes disruption of other genes involved in mitogen-associated protein kinase (MAPK)/NF-κB signaling pathway and thereby alters the innate immune response of the microglia and other inflammatory cascades (8). MAPKs are signaling proteins that mediate various intracellular signals in response to external stimuli. Several important MAPKs play an essential role in the integrity of the cell as well as modulating inflammation such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal regulated kinases (ERKs). In astrocytes, DJ-1 loss of function primes astrocyte to release increased pro-inflammatory cytokines post LPS challenge (63). This response was mediated through p38 and JNK, thereby DJ-1 may have a pivotal role in regulating TLR4-MAPK signaling and downstream transcriptional responses (64). LPS-mediated activation of macrophages increases DJ-1 expression (65), which is aligned with the associated TLR/MAPK mediated signaling in microglial cells challenged with LPS.

1.7. Inflammation and PD models

There are several models of PD, both toxin based and gene based, used to study disease progression and/or therapeutic development. In many of these models, inflammatory mechanisms are reported to play roles in the pathogenesis and manifestation of the disease in various animal models. In the remainder of this chapter, we will focus on characterizing the inflammatory response seen in the various models.

1.7.1 Toxin-based models

1.7.1.1. MPTP

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a dopaminergic neurotoxin selective for the dopaminergic cells of the SNpc. It is a lipophilic compound capable of crossing the BBB, and once it has crossed the BBB, it is oxidized to MPP+ by MAO-B. MPP+ interrupts the mitochondrial complex I of the electron transport chain (ETC) and results in cell death and release of ROS (26). The MPTP model is a widely used model to develop animal models of the disease, as PD progression post MPTP administration is similar in both humans and monkeys (66). In the MPTP model, microglial activation accompanied with an increased endothelial expression of adhesion molecules on the BBB to enable infiltration of T-cells is reported (24). Additionally, there are an increased number of activated microglial cells which are amoeboid in shape representative of activated cells (26). Infiltrated CD8 and CD4 T-cells have lymphocyte function-associated antigen-1 (LFA-1), a protein expressed for recruitment and these infiltrated T-cells are primarily located it the SNpc and striatum (67). Furthermore, an increased expression of MHC I and MHC II and microglial iNOS expression are observed (26). In conjunction with anti-inflammatory therapies, minocycline, a potent inhibitor of microglial activation and iNOS knockout mice, is protective against MPTP-induced neuronal cell death (68).

1.7.1.2. 6-OHDA

Oxidopamine, commonly known as 6-hydroxydopamine (6-OHDA), is another dopaminergic neurotoxin capable of inducing PD symptomatology in animal models. In the 6-OHDA model, inflammatory pathology is propagated by activated microglial cells which occurs 1–3 days post intra nigral injection of 6-OHDA and DA neuronal loss occurring 1 week post injection (69). As

part of the inflammatory milieu, there is an increase in TNF-α, a pro-inflammatory cytokine capable of inducing cell death through TNFR.

1.7.1.3. LPS/rotenone

Lipopolysaccharide (LPS) is an endotoxin derived from Gram negative bacteria and another widely used toxin to induce PD in animal models. The LPS model is different from MPTP and the 6-OHDA toxin-based models, as LPS will activate microglial cells through the TLR2/4 receptors. Activated microglial will upregulate NO, superoxide, TNF-α, and IL-1β production and release (70). These pro-inflammatory mediators can cause neuronal cell death. In animal models, intra nigral injection of LPS induces microglial activation prior to neuronal loss (37). Rotenone is another lipophilic herbicide that disrupts the mitochondrial complex 1 causing cell death and associated upregulation in ROS. In the rotenone animal models of PD, fibrillary cytoplasmic inclusions equivalent of Lewy bodies is found in the SNpc. In addition, rotenone injection in neuronal-only culture does not cause DA cell death, but in a mixed neuronal-microglial culture, DA neuron cell death is observed. This suggests that rotenone requires microglial cells for its toxicity (26,71). Lastly, inhibition of superoxide is protective against rotenone-induced DA neuron degeneration. These two models suggest that while the etiology of disease is unknown, microglial cells are indispensable for the progression of disease and the resulting neuronal degeneration is seen in PD.

1.7.2. Gene-based models

1.7.2.1. α-synuclein

While many genes are implicated in the PD etiology but α-synuclein is the most widely used gene-based model so far (72). The α-synuclein models include a transgenic knockout and overexpression of mutant or WT α-synuclein (73). Viral vectors expressing human α-synuclein injected into adult

brains have also been used to increase α-synuclein in the respective brain regions. α-synuclein models have been developed in monkeys, rats, mice, and in flies. Bae et al. (73) as well as Watson et al. (74) reported astroglia and microglia activation accompanied with increased mRNA transcripts of TNF-α and several TLRs (1, 4, and 8) in SNpc (75). α-synuclein can also act as a DAMP and activate microglia via TRLs, thereby suggesting a primed microglial sensitivity. In the transgenic models of α-synuclein null mice, little neuronal loss and behavioral changes are reported. In addition, transgenic null mice can offer a degree of protection against MPTP intoxication and cell death (76,77). In contrast, viral overexpression models of α-synuclein in brain of adult animals show DA neurotoxicity accompanied by the activation of both the innate and adaptive immune response (78). Learning from these models includes and further verifies a gain of function of α-synuclein as ablation of α-synuclein features no neuropathological changes (72). Furthermore, in a recent study, Van der Perren et al. (79) reported the immunophilin ligand FK506 in a rAAV2/7 α-synuclein overexpression rat model to have anti-inflammatory therapeutic potential. Specifically, the group (79) reported a decrease in the infiltration of CD4+ and CD8+ T cells as well as in the number of activated microglial cells. This further supports neuroinflammation as a key to the progression of the disease and efficacy for therapeutic development.

1.7.2.2. LRRK2

LRRK2 mutations are implicated in an autosomal dominant form of PD with similar phenotypic expression as idiopathic PD. To study the role of LRRK2 in PD pathogenesis, LRRK2 knockout animals were developed. Several groups (80,81) report no DA degeneration in LRRK2 deficient rat and mice models. Lee et al. (82) developed herpes simplex virus (HSV) amplicon-based mouse model of LRRK2 dopaminergic neurotoxicity. Overexpression of the LRRK2 G2019S resulted in

significant loss of tyrosine hydroxylase (TH+) neurons. Thereby these data suggest that knockout of LRRK2 may provide neuroprotection, and similarly, Lee et al. (82) used LRRK2 inhibitors and found that it protected the overexpressed LRRK2 mice from developing PD (82). Most notably, the mechanism of protection seems to be dependent on the activation and proliferation of microglial cells (80), implicating LRRK2 in the inflammatory etiology for PD. Therefore, it appears LRRK2 is critical in PD pathology and plays a significant role in regulating cellular inflammation, thereby supporting the notion that neuroinflammation is critical to PD pathogenesis.

1.8. Anti-inflammatory therapies in PD

While evidence strongly suggests that inflammation plays a major role in the etiology of a number of different forms of PD, emerging evidence also demonstrates that therapies used to lessen inflammation, including those directed against immune cells or inflammatory mediators, can play a positive role in halting the degeneration of DA neurons in several models of PD. Many studies suggest that inflammatory mediators such as TNFα, PGE2, NO, free radicals, and other immune mediators play a role in the pathogenesis of PD and degeneration of dopamine-producing neurons, and that the use of specific reagents that target these mediators, inhibition of cellular signaling mechanisms that regulate the production of these mediators, or the use of neurotrophic factors that help protect against the neurotoxicity induced by these mediators hold significant promise as therapeutic treatments for PD. In addition, epidemiological and observational studies already suggest that use of anti-inflammatory drugs lower the risk of developing PD (83). Observations which demonstrate that inflammation in SNpc plays a role in PD led many investigators to initially study the potential use of steroidal and nonsteroidal anti-inflammatory drugs for the treatment of PD. Steroidal anti-inflammatory drugs (SAIDs) such as dexamethasone showed neuroprotective effects and LPS-induced neurotoxicity in Substantia nigra (84). Nonsteroidal anti-inflammatory

drugs (NSAIDs) are used as analgesics and anti-pyretics to suppress the adverse effects of inflammation. NSAIDs as a group normally reduce the production of prostaglandins by inhibiting cyclooxygenase (COX, an enzyme that catalyzes specific prostaglandin synthesis) and also reduce the synthesis of nitric oxide. In addition, it has been found that a subset of NSAIDs called as selective Aβ42 lowering agents (SALAs) reduces the risk of Alzheimer dementia (AD) (85) and consequently may be effective in PD as well. Neuroprotective effects of ibuprofen have been studied in PD pathogenesis, and these studies show that it can protect dopaminergic neurons against glutamate toxicity *in vitro* (86,87). It is interesting to note that some neurologic drugs used to treat PD have been found to result in changes to immune system. One such drug, amantadine (Symmetrel, Endo Pharmaceuticals) LPS induced mice and 6-OHDA induced mesencephalic culture which functions as an antagonist of the NMDA-type glutamate receptor leading to increased dopamine release and dopamine reuptake, also increases the CD4:CD8 ratio (88) and enhances IL-2 levels in PD patients. In contrast, L-DOPA monotherapy does not show similar effects (89). In the next sections, we discuss the effectiveness of a number of anti-inflammatory treatments in preventing dopaminergic cell death in animal models of PD (section summarized in **Table 1.1**).

Table 1. 1: Neuroprotective effects of anti-inflammatory therapies. Content of this table summarizes the various therapies used against neuroinflammation and neurodegeneration in PD models *in vitro* and *in vivo.*

Therapy	Compounds	Study design	Outcomes	Ref
SAIDs and NSAIDs	Dexamethasone	Intra-nigral injection of LPS in rats	↑ TH activity in striatum.	(83)
	Ibuprofen	Primary mesencephalic culture from rats treated with ibuprofen	↑dopamine uptake, protected neurons against excitotoxicity.	(86)
	Amantadine	Peripheral blood mononuclear cells from idiopathic PD patients	↑ $CD3^+$ $CD4^+$ cells, improves T-cell mediated immunity.	(88)
Antibiotics	Rifampicin	LPS induced BV2 microglia	↓ in NFκB activation, ↓expression of iNOS, COX2, TNF-α, IL-1β ↓ Production of NO, PGE_2.	(90)
		MPP induced PC12 cells	↓MPP induced neuronal death, ↓expression of α-syn multimer.	(91)
	Ceftriaxone	PC12 cells exposed to neurotoxin 6-OHDA	Binds with α-syn and blocks its polymerization *in vitro*.	(92)
		6-OHDA induced PD rat model	↑GLT-1 expression, reduces striatal TH loss.	(93)
	D-Cycloserine	MPTP induced PD rat model	Improved behavioral deficits restored IL-2 level ↓microglial activation.	(94)
	Rapamycin	In PC12 cells and MPTP induced mouse model	↓neuronal death, regulate Akt phosphorylation at Thr308.	(95,96)

	Minocycline	In MPTP induced mouse model, primary mesencephalic culture	↓expression of iNOS, caspase-1, inhibited NO induced neurotoxicity, ↓phosphorylation of p38.	(97)
		In differentiated LUHMES cells induced by reactive oxygen species	Specific scavenger of peroxynitrite, ↓microglia activation.	(98)
Neuropeptide	PACAP	MPP^+ induced SHSY-5Y neuroblastoma	↓cell death, ↑ cAMP	(99)
		Oxygen and glucose deprived BV2 microglia	↓TRL4, MyD88, NFκB ↓production of pro-inflammatory cytokines	(100)
		MPTP induced mice	↑TH expression, ↓caspase-3, IL-6, TNFa	(99)
	VIP	LPS induced mice and mesencephalic culture	↓pro-inflammatory cytokine, ↓NO	(101)
Polyphenols	Pinocembrin	6-OHDA induced SHSY-5Y	↓apoptosis, ↓ROS,	(102)
	Naringenin	6-OHDA induced mice and SHSY-5Y	↑Nrf2.↑neuroprotectio, ↓ROS	(103)
	Flavonoids from *Selaginella* extract	Rotenone induced Drosophila PD model	↑anti-oxidant enzyme activity, ↓oxidative stress	(104, 105)
	Curcumin	6-OHDA induced SHSY-5Y	↓p-p38, ↓caspase-3	(106)
	Baicalein	MPTP induced mice and primary rat astrocytes	↓NFkB nuclear translocation, ↓microglia and astrocyte activation, ↓COX2, ↓phosphorylation of JNK and ERK	(107)
	Theaflavin	MPTP with probenecid induced mice	↑TH expression, ↓caspase-3,8,9, ↓COX2, IL-1β, TNF-α, IL-6	(108)
	Resveratrol	MPTP induced mice	↑TH protein and mRNA, ↑SOCS-1, ↓TNF-α, IL-1β,IL-6,	(109)

			↓TNF-αR1, IL-1βR1, IL-Rα	
		Rotenone or MPP^+ induce primary rat astrocytes/microglia, mesencephalic culture, BV2	↓NO, ROS, MPO, ↓iNOS, COX2, TNF-a, IL-1b mRNA, ↓phagocytic activity	(110)
Anti-inflammatory Cytokines	IL-10	LPS induced mesencephalic culture	↓TNF-α, NO, ↓extracellular superoxide, inhibit PHOX activity	(111)
		Hu-IL-10 gene transfer in 6-OHDA induced rats.	↑TH-positive neurons, ↓ glial activation	(112)
	TGF-β	Retrograde model of PD induced with 6-OHDA and treated with TGF-β alone and in combination with GDNF.	Reduce dopamine receptor hypersensitivity, ↑TH-positive neurons, induce synaptogenesis	(113, 114)
		LPS and MPTP induced mice	↓ROS, ↓PHOX $p47^{phox}$ activity, ↓LPS activated phosphorylation of ERK and $p47^{phox}$	(115)
Insulin therapy	Pioglitazone	MPTP induced mice	↓microglial activation, ↓GFAP positive cells in SN, ↑ IκBα, ↓NFκB activation.	(116)
		LPS induced rat microglial cells	↓NO, iNOS, TNF-α, IL-1β, IL-6, ↓phosphorylation of p38.	(117)
	GLP-1 receptor agonist: Exenatide	MPTP mice and 6-OHDA induced mesencephalic culture	↓neurotoxicity, improve motor functions, ↑TH-positive cells, ↑cAMP ↓TNF-α, superoxide, NO	(118)
β2-AR agonists	Salmeterol	LPS and MPTP induced mice	↓phosphorylation of MAPK, p65 NFκB.	(20)
Morphinan compounds	L-morphine	LPS and MPP^+ induced rat mesencephalic culture	↓PHOX activity, ↓pro-inflammatory	(119)

			cytokines, ↓ERK phosphorylation.	
	Sinomenine	LPS and MPP^+ induced rat mesencephalic culture	↓TNF-α, PGE_2, ROS ↓PHOX activity.	(120)
	3-HM	Rat mesencephalic culture stimulated with LPS	↓NO, TNF-α, PGE_2, ROS, ↓microglia activation	(121)
		LPS and MPTP models	↑expression of neurotrophic factors ↑acetylation of histone H3 ↓reactive microgliosis ↓ROS	(122)
Therapy against NFκB	NBD	MPTP induced mice	↓NFkB activation, ↓iNOS, ↓TNF-α, IL-1β ↓CD11b, ↓neuronal death	(123)
	Compound A (IKK inhibitor)	LPS induced rats and mesencephalic culture	↓neurotoxicity, ↓TNF-α, IL-1β, ↓NO, iNOS, ↓IKKβ phosphorylation and NFκB activation ↓phosphorylation of ERK, p38, JNK, ↓ROS, ↓superoxide	(124)
Anti-oxidants	DPI (NADPH oxidase inhibitor)	LPS induced midbrain neuron-glia culture	↓neurotoxicity ↓NOX2 activation.	(125)
		LPS induced rats and mesencephalic culture	↓ROS, ↓TNF-α, NO ↓ERK phosphorylation, ↓PHOX activity	(126)
	Coenzyme Q10	MPTP model	↓ROS, ↓free radicals production, neurotoxicity	(127)
	NAC	Intravenous infusion of NAC in PD patients	↑glutathione level in blood	(128)
	Edaravone	MPP^+ induced PC12 cells	↓oxidative stress, ↓ROS ↑Heme-oxygenase-1 expression	(129)

1.8.1. Antibiotics in neuroprotection in PD

Antibiotics are routinely used to kill or inhibit the growth of microorganisms at low concentrations. In addition to their antimicrobial activity, antibiotics can either directly or indirectly regulate the expression of many inflammatory gene transcripts (130), and a number of antibiotics such as tetracycline and β-lactams have been shown to have significant anti-inflammatory properties (131). Antibiotics now appear to have protective effects against neurodegeneration and the neuroinflammatory process (90). These properties of antibiotics make them suitable for the development of effective therapies against neurodegenerative diseases such as PD. Rifampicin, a macrocyclic antibiotic, reduces amyloid β aggregation in brain, and it is also neuroprotective in other chronic neurodegenerative diseases and cerebral ischemia (132). Pretreatment with Rifampicin increases cell viability and reduces α-synuclein expression and its aggregation. Moreover, in MPP+-induced PC12 cells, Rifampicin prevents the formation of α-synuclein oligomer (91). It can also block the release of pro-inflammatory cytokines such as NO, PGE2 TNF-α, and IL-1β from LPS-stimulated BV-2 microglial cells (133).

Similarly, β-lactam also has protective role against neurodegeneration and can cross BBB. β-lactam antibiotic ceftriaxone has demonstrated neuroprotective activity as well as high binding affinity with α-synuclein and can block its *in vitro* polymerization (92). Ceftriaxone also increases the expression of glutamate transporter-1 (GLT-1) which enhances glutamate uptake and therefore reduces excitotoxicity in 6-OHDA model of PD (93). D-cycloserine (DCS), an antibiotic prescribed for Mycobacterium tuberculosis, also acts as an NMDA receptor antagonist that prevents excitotoxicity damage induced by MPTP (94) and inhibits the production of MMP3 and MMP9 in LPS stimulate microglial cells. In addition, Rapamycin was able to prevent mitochondrial dysfunction in PINK1/Parkin Drosophila mutants (95). Furthermore, it enhances

the expression of neuronal survival promoting kinase Akt, antioxidant enzymes and anti-apoptotic markers (96). Similarly, Minocycline has shown neuroprotective effects in PD models (97). Minocycline suppresses α-synuclein aggregation and its toxicity (98), as well as microglial activation of p38 the MAPK signaling pathway resulting in the suppression of pro-inflammatory mediator release (134). These results support the potential of antibiotics as neuroprotective and therapeutic agents in PD.

1.8.2. The role of anti-inflammatory compounds in neuroinflammation and PD

Neurotrophic factors are essential for neural growth and development, and these factors normally signal through Trk receptors. Adenosine and pituitary adenylate cyclase-activating peptide (PACAP) act as ligands and induces activation of Trk receptors through adenosine (A2A) receptor and PAC1 receptors, respectively (135). Recent studies reported the antioxidant and anti-inflammatory properties of PACAP (100,136). It can inhibit the release of several pro-inflammatory mediators from LPS-activated microglial cells by inhibiting the transcriptional activity of NF-κB (137), as well as the production of several chemokines like MIP-1α, -1β, MCP-1, and RANTES (137). A synthetic analog of PACAP showed neuroprotection in MPP+-induced SHSY-5Y cells and MPTP-injected mice. It restored the expression of tyrosine hydroxylase in Substantia nigra and modulated the inflammatory response (99). Another peptide, called vasoactive intestinal peptide (VIP), can also inhibit the expression of pro-inflammatory cytokines from LPS-activated cultured microglia (101). These studies suggest these peptides can serve as promising molecules for the development of anti-inflammatory and neuroprotective drugs in the treatment of PD.

1.8.2.1. Neuroprotective and anti-inflammatory role of polyphenols

Several traditional medicinal plants and herbs are rich in polyphenol, and their neuroprotective effects have been studied extensively. These compounds have neuroprotective properties against oxidative stress, neuroinflammation, mitochondrial dysfunction, and protein fibrillization. Kong et al. reported that polyphenols reduce the intracellular level of ROS in DA neurons (138). Recently, it has been found that pretreatment with flavonoids such as pinocembrin (102) and naringenin (103) reduces the formation of ROS, in 6-OHDA-challenged human neuroblastoma SHSY-5Y cells. This effect was due to an increase in Nrf2 protein level and by activating ARE pathway genes. In addition, flavonoids from *Selaginella* species have the ability to increase the expression and activity of anti-oxidative enzymes endogenously (104), and the aqueous extract of *Selaginella* suppresses rotenone induced neurotoxicity, attenuated locomotor dysfunction, oxidative stress, and mitochondrial dysfunction in *Drosophila melanogaster* (105). Polyphenols may also target MAPK pathways and apoptosis, since phosphorylation of MAPK and expression of cleaved caspase 3 were reduced in 6-OHDA induced SHSY-5Y cells by curcumin (106). Similarly, the phosphorylation of NF-κB, JNK, and ERK was inhibited by flavone baicalein in MPP+-induced primary astrocytes and indicated its implication in the treatment of PD (107). Several other polyphenols have been shown to reduce the expression of pro-inflammatory cytokine such as IL-1β, TNF-α, and IL-6 (139,140).

Furthermore, theaflavin treatment in MPTP mice model of PD increases the expression of anti-inflammatory cytokines such as IL-4 and IL-10 by the modulation of the suppressor of cytokine signaling 1 (SOCS1). Oral administration of resveratrol in MPTP mouse model upregulated the expression of SOCS1 in striatum and Substantia nigra and suppresses the production of pro-inflammatory cytokines (108) and also improved cell survival in rotenone-induced primary

mesencephalic culture. Resveratrol also diminished the level of MPO (MPO; an enzyme produces hypochlorous acid and tyrosyl radical during microglial respiratory burst) and ROS in MPP+-induced BV2 microglia cells (109,110). Many polyphenol compounds have been studied to test their neuroprotective and anti-inflammatory properties, but further research will be needed to understand the signaling mechanism of how these compounds act to offset neuroinflammation.

1.8.2.2. Anti-inflammatory cytokine therapies in PD

The use of anti-inflammatory cytokine serves as a potent approach for the development of antiparkinsonian drugs. Two major anti-inflammatory cytokines, IL-10 and transforming growth factor beta 1 (TGFβ1), produced by Treg cells, have been studied in PD models. Pre- and Post-treatment of rat mesencephalic neuron glia culture with IL-10 showed neuroprotective effects against LPS-induced neurotoxicity by inhibiting the production of TNF-α, nitric oxide, and extracellular superoxide (111). Gene delivery of human IL-10 by using adeno-associated viral type-2 (AAV2) in 6-OHDA rat model of PD also showed neuroprotection by suppressing the 6-OHDA-induced loss of TH-positive neurons (112). Similarly, TGFβ1 also shows protective effects against neurotoxicity. TGFβ1 in combination with GDNF reduces progressive cell death and enhances the expression of TH in surviving nigral neurons in retrograde model of Parkinsonism in rats (113). It has also been shown that TGFβ1 protects from neuronal death induced by glutamate excitotoxicity (114). The neuroprotective effect of TGFβ1 is primarily due to its ability to inhibit the production of ROS from microglia during activation. Additionally, after LPS activation, ERK phosphorylation and subsequent serine phosphorylation on p47phox were significantly inhibited by pretreatment with TGFβ1 (115). Recently, it also has been reported that overexpression of fractalkine (CX3CL1) reduces neuronal loss in 6-OHDA model of PD and suppresses α-synuclein-

mediated neurodegeneration (141). The use of these anti-inflammatory mediators therapeutically to suppress represents a new therapeutic avenue for the treatment of PD.

1.8.2.3. Regulatory T-cell therapy

Treg cells have the capability to mitigate inflammation and serve as an attractive therapeutic target. Treg cell therapy can be used for neuroprotection in PD as these cells also utilize immunosuppressive mechanisms including the production of anti-inflammatory cytokines. The neuroprotective effects of bee venom is associated with the deactivation of microglia and suppression of CD4+ T cell infiltration, and it also increases the proportion of CD4+, CD25+ and Foxp3+ Treg cells in MPTP mouse model of PD. Several studies have been shown that Treg cell responses inhibit microglial activation and enhance neuronal survival in MPTP mouse model of PD (27,142) In addition, Th2 cells also inhibit microglial activation by the production of IL-4 and IL-10 against MPTP-induced neurotoxicity (142). What signals suppress the Treg cell functions and how to improve anti-inflammatory activity are yet to be determined.

1.8.2.4. Insulin as potent therapeutic agent for treatment in PD

Insulin is a peptide hormone and most responsible for lowering the blood glucose, but it has also been found to have potent anti-inflammatory effects. Insulin signaling regulates a number of cellular processes such as neurotransmission, vesicle trafficking cell survival, and inflammatory mediator production. Recent evidence has shown that insulin signaling is impaired in Alzheimer and, to some degree to, Parkinson's patients. Preclinical studies suggest that the application of insulin or long-lasting analogs of incretin peptides in transgenic animal model of PD, and AD reduces neurodegeneration and neuronal and synaptic functionality (117,143). Pioglitazone is generally prescribed for type 2 diabetes mellitus and reduces insulin resistance and acts on peroxisome proliferator-activated receptor γ (PPARγ) receptors. It also functions to reduce

microglial activation and induction of iNOS positive cells by enhancing inhibitory protein kappa B (IκBα) and inhibition of NF-κB subunit p65 in MPTP mouse model of PD (116). In LPS model of PD, Pioglitazone showed neuroprotection by inhibiting microglia- mediated oxidative stress (117). Another anti-diabetic agent, GLP-1 (glucagon-like peptide), is a hormone which maintains homeostasis between insulin and glucose. Exenatide is a synthetic agonist for the GLP-1 receptor and shows significant promise as neuroprotective in PD animal models (118). These studies describe the potent impact of insulin or antidiabetic treatments as possible anti-inflammatory neuroprotective therapies for PD.

1.8.2.5. Use of β-adrenergic receptor agonists as an anti-inflammatory agent in the treatment of PD

β2AR are seven transmembrane G-protein-coupled receptors found on numerous cell types, including inflammatory cells and neurons. β2AR agonists are FDA approved for the treatment of chronic obstructive pulmonary disorders (COPD), and their use as treatment for neurodegenerative diseases such as PD represents a new and potentially very productive therapeutic approach. In the CNS, microglia expresses high levels of β2AR, and it has been demonstrated that long-acting β2-AR agonists such as Salmeterol (Advair, GlaxoSK) protect against DA neuronal death from microglia-mediated neuroinflammation (20). In addition to inhibiting the production of inflammatory mediators and oxidative stress responses by microglial cells, several *in vivo* studies also reported neuroprotective roles of long-acting β2AR agonists by inducing neurotrophic growth factors and astrocyte activation (144,145). Long-acting agonist such as Salmeterol showed neuroprotective effects by pretreatment in LPS-stimulated long-term mouse model and also by the treatment with Salmeterol after MPTP injection. Low dose of Salmeterol treatment in these models suppressed the NF-κB activation and its nuclear translocation. Similarly, it also reduces phosphorylation of MAPK such as ERK1/2, p38, and JNK (146). Furthermore, it has been shown

that low dose of Salmeterol also inhibits TGF-beta-activated kinase 1 (TAK1), which is a common upstream regulatory molecule for MAPK and NF-κB activation, and involves in various inflammatory signaling pathways (147). This suggests the anti-inflammatory effects of Salmeterol by reducing phosphorylation of MAPK and NF-κB activation via inhibition of TAK1. The activation of β2-AR stimulates MAPK signaling also via β-arrestin-dependent and G-protein-independent mechanism (146). Overall, these agonists can inhibit inflammatory response and have potential to regulate inflammation in chronic inflammatory disorders of CNS. These results suggest that β2AR agonists can be developed as anti-inflammatory therapy to subside the progressive loss of dopaminergic neurons in PD patients.

1.8.2.6. Use of morphinan-related anti-inflammatory compounds in PD

Several morphinan analogs such as naloxone, dextromethorphan, or naltrexone have been described as anti-inflammatory and neuroprotective. Morphine isomers (L-morphine and its D stereo enantiomer) can inhibit microglial activation and LPS- or MPP+-induced neurotoxicity in rat primary mesencephalic cultures. Furthermore, it also suggests that morphinan compounds bind to the catalytic subunit of PHOX and inhibits its activity leading to the reduced production of superoxide and other pro-inflammatory cytokines (119). Similar results were observed with sinomenine, a dextrorotatory isomer of morphine and protective effects of sinomenine mediated through the inhibition of microglial PHOX activity (120). Similarly, 3-hydroxymorphinan (3-HM), a metabolite of dextromethorphan, recently emerged as a potent therapeutic agent for the treatment of PD. These compounds show neuroprotection by two different pathways; one through a neurotrophic effect mediated by astrocytes and another by their anti-inflammatory effect mediated by the suppression of microglial activation. When the 3-HM compound was studied for its mechanistic effects *in vivo*, it was found that it attenuated the depletion of striatal levels of

dopamine and showed neuroprotection against LPS- and MPTP-elicited neurotoxicity (121). These effects were observed even when drug was administered post MPTP injections (122). Collectively, these findings offer a different yet highly potent new therapeutic direction for the treatment of neuroinflammation in PD.

1.8.2.7. Pro-inflammatory transcription factor, NF-κB as a therapeutic target in PD.

Nuclear transcription factor NF-κB plays an important role in inflammation. It regulates the expression of various genes involved in immune function and cell survival. NF-κB activation has been reported in Substantia nigra of PD patients and in animal models of PD. The inhibition of NF-κB activation can suppress oxidative stress and production of pro-inflammatory cytokines and chemokines in microglia (148). Ghosh et al. reported that intraperitoneal injection of NBD (NF-κB essential modifier-binding domain) peptide reduces nigral activation of NF-κB, inhibits microglial activation in Substantia nigra, and improves motor function in MPTP mouse model of PD (123). Selective inhibitors against IKK-β also reduce microglial and neuronal death in SNpc in MPTP-intoxicated PD mice (123) and in LPS-induced neurodegeneration by inhibiting NF-κB activation and decreasing the production of pro-inflammatory cytokines. It also suppresses the activity of microglial NADPH oxidase and reduces the production of ROS (124). These reports suggest the suppression of NF-κB signaling pathway in microglia is neuroprotective and represent NF-κB as a strong potential target for anti-inflammatory therapy in the treatment of PD.

1.8.2.8. Antioxidants as neuroprotective agents in PD

Oxidative stress and generation of free radicals have been reported to be a major effector of neuronal death seen in neurodegeneration in PD. This can also be linked to other processes such as nitric oxide toxicity, excitotoxicity, mitochondrial dysfunction, and inflammation. Oxidative stress impairs cell viability by damaging lipid, proteins, and nucleic acids (149). The development

of therapies against oxidative stress and free radicals may be beneficial in PD by inhibiting the onset of apoptotic cell death and degeneration of nigrostriatal dopaminergic neurons. The neurotoxin MPTP inhibits the mitochondrial electron transport chain and suppresses the activity of mitochondrial complex I and eventually elevates oxidative stress within DA neurons. MPTP also increases the production of free radicals and ROS by microglial cells, ultimately leading to the death of dopamine producing neurons. It has been found that mice lacking the NADPH oxidase complex do not exhibit DA neurotoxicity from MPTP or LPS-induced neurodegeneration, and that the administration of NADPH oxidase inhibitor DPI can prevent DA neurotoxicity (125,126). Several other antioxidants have been investigated in the treatment of PD, and it has been found that coenzyme Q10 is a potent antioxidant and electron acceptor for mitochondrial complex I and II, can enhance activity of complex I, and reduce oxidative stress (150).

Clinical trial with randomized, parallel group, placebo controls, and double-blind with multiple doses of CoQ10 (300, 600, or 1200 mg/day) in 80 early PD patients showed that CoQ10 is well tolerated at doses up to 1200 mg/day, less disability was developed in PD subjects, and symptomatic relief was higher in subjects receiving the highest dose (127). In contrast, a recent phase III, randomized, double blind, placebo-controlled clinical trial concluded that CoQ10 is safe and well tolerated but showed no evidence of clinical benefits (151). Another antioxidant and a pro-drug of amino acid cysteine called N-acetyl-cysteine (NAC) also showed neuroprotective effects. Preclinical data suggest NAC is neuroprotective and can reduce oxidative stress and ROS accumulation. Recently, a clinical trial with NAC intravenous infusion concludes that NAC enhances the level of glutathione (a potent antioxidant) in blood and brain in PD patients (128). Similarly, Edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrozolin-5-one) is a neuroprotective antioxidant, generally prescribed for recovery of acute brain ischemia and cerebral infraction

(152). It showed neuroprotective effects in MPP-induced PC12 cells by reducing oxidative stress and enhancing expression heme oxygenase-1 expression (a cellular stress response protein) (129), but clinical trials are yet to be done.

In summary, inflammation plays an important role in the etiology of a number of different forms of PD, and anti-inflammatory drugs hold much promise as a therapeutic treatment for patients with mild and moderate forms of PD. The continued evaluation of these drugs, including their efficacy, target, and mechanism of action, hold much promise for the future treatment of PD.

CHAPTER 2

Adrenergic Receptors as Pharmacological Targets for Neuroinflammation and Neurodegeneration in Parkinson's disease

This is modified from the published article:

Sharma, M., Flood, P.M., 2018. **Adrenergic receptors as pharmacological targets for neuroinflammation and neurodegeneration in Parkinson's disease.** *Neuroprotection.*

2.1. Abstract

Inflammation is a key component of the dopaminergic neurodegeneration seen in progressive Parkinson's disease (PD). The presence of activated glial cells, the participation of innate immune system, increased inflammatory molecules such as cytokines and chemokines, and increased oxidative stress and reactive oxygen species are the main neuroinflammatory characteristics present in progressive PD. Therapeutic targets which suppress pro-inflammatory responses by glial cells (mainly microglia) have been shown to be effective treatments for slowing or eliminating the progressive degeneration of neurons within the substantia nigra. In this chapter, we will detail a specific anti-inflammatory therapy using agonists to β2-adrenergic receptors that have been shown to be effective treatments for models of dopaminergic neurodegeneration and that have had efficacy in patients with progressive PD. We will also detail the possible molecular mechanisms of action of this therapeutic in stopping or reversing inflammation within the CNS.

2.2. Introduction

There are a number of neurological disorders that fall under the umbrella of neurodegeneration, with the major ones including Alzheimer's disease, PD, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal dementia, spinal cord injury, and others. Currently, there are no generally effective treatments available to slow down or reverse the debilitating effects of these diseases, and the long-term effects of these diseases are the progressive degeneration and death of neurons. A majority of the neurodegenerative diseases are linked with inflammation in CNS (153), and the presence of activated glial cells, infiltration and activation of adaptive and innate immune cells, increased presence of inflammatory molecules such as cytokines and chemokines, and increased oxidative stress and reactive oxygen species (ROS) are the main neuroinflammatory characteristics present in lesions associated with these neurodegenerative disorders. Recent approaches found to

be effective in the treatment of PD involve the use of anti-inflammatory agents and cytokines such as agonists to the β2-adrenergic receptors (β2-AR) to inhibit neuroinflammation and the progression of dopaminergic neurodegeneration. In this chapter, we will address the current understanding of therapeutic approaches targeting neuroinflammation linked with PD and the use of β2-AR agonists as an effective treatment for PD.

2.3. Parkinson's disease: a chronic neurodegenerative and neuroinflammatory disease

PD is a progressive neurodegenerative disorder which leads to impaired motor skills. The major pathological feature of PD is the degeneration of dopaminergic (DA) neurons which project from substantia nigra (SN) to the striatum in the midbrain (nigro-striatal pathway) (154). Another neuropathological feature of PD is the cytoplasmic inclusion of misfolded α-synuclein protein in degenerating dopaminergic neurons called Lewy bodies (155). The primary motor symptoms of PD, such as tremor, rigidity, and bradykinesia, are caused by inadequate formation and neurotransmission of dopamine within the nigro-striatal pathway (156,157). Dementia is reported in 28% of PD cases with the prevalence rising to 65% in those aged 85 years and above. Patients with PD also show non-motor related symptoms such as olfactory deficits, depression, cognitive deficits, sleep disorders, and autonomic dysfunction (158). The majority of PD cases are idiopathic Parkinson's, and the disease mechanism that ultimately causes idiopathic PD is largely unknown. In the remainder of the cases of PD, about 10–15% of patients do have a family history and those patients are referred to as having the *familial* form of PD. For these patients, their PD appears to be caused by a mutation in one of a few selected genes (such as *SNCA*, *Parkin*, *LRRK2*, *DJ-1*, etc.) (159,160). Although the etiology of the idiopathic form of the disease remains elusive, there are some risk factors associated with the development of the disease. These risk factors include exposure to environmental toxins, severe cranial trauma, systemic or localized infections, and

inherited genetic risk factors. These genetic and non-genetic risk factors have the potential to initiate neurodegeneration and subsequent chronic inflammation in the brain which eventually contributes to the pathophysiology of PD (161). In addition, several cellular and molecular pathways such as oxidative stress (162), proteosomal dysfunction (163), excitotoxicity (164), and mitochondrial dysfunction (165) have also been identified which contributes to neuronal death.

The presence of activated glial cells, increased inflammatory molecules such as cytokines/chemokines, and increased oxidative stress and ROS are the main neuroinflammatory characteristics present in PD (166). PD is now not only characterized as loss of DA-neurons and motor impairment, but also recognized to have an inflammatory component which plays a crucial role in the progression of the disease. Several inflammatory mediators such as TNF-α, IL-1β, ROS, and NO, released from non-neuronal cells exacerbate the disease pathology (155,167). It has been suggested that α-synuclein released from dying neurons also activate the microglia via TLR2 activation (54). Furthermore, the elevated levels of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been reported in serum, cerebrospinal fluid (CSF), and striatum of PD patients (168). The influx of peripheral macrophages has been reported in brains of patients with PD (169), but the role of these cells in disease pathology remains to be tested. Additionally, activation and increased number of glial cells and infiltrating peripheral lymphocytes such as cytotoxic CD4+ and CD8+ cells in SN also support the role of adaptive immunity in the etiology of the disease (160). Overall, these studies and others suggest the contribution of the immune system in the pathophysiology of PD.

2.4. Microglial activation and Neuroinflammation in PD

Microglia originate from erythromyeloid progenitors in the yolk sac which migrate and differentiate during development to form the central nervous system (CNS). Fully differentiated

microglial cells are also considered to be the resident macrophages of the CNS (170), although some phenotypic and functional differences between microglia and macrophages have been found (171). Growing evidence suggests that the activation of microglia in CNS plays an important role in the pathogenesis of PD. It is not well understood how microglia activation is either beneficial or detrimental to the neuron or how microglial activity is regulated. It has been found that microglial activation is required for neuronal survival by the removal of toxic substances through innate immunity (172). On the other hand, it has been found that *over*-activated microglial cells are detrimental and neurotoxic (173). Research studies of post-mortem brain tissue from patients with PD and related parkinsonian syndromes suggest the presence of activated microglia around degenerating DA-neurons in the SN (17) and these activated microglia are not only limited to the SN but also present in extended brain areas such as hippocampus, putamen, trans-entorhinal cortex, cingulate cortex, and temporal cortex (174). Imaging of activated microglia in the striatum could be used as a biomarker for detecting neuroinflammation in neurodegenerative parkinsonian disorders (175). The resting microglia switches to an activated microglia phenotype in response to pathogen invasion or release of toxic or inflammatory mediators and thereby promotes an inflammatory response (153). Once activated, microglial cells produce a wide range of inflammatory mediators which serve to initiate an innate immune response or glial cell-propagated inflammation termed as neuroinflammation (176). Also, the degenerating DA-neurons release many toxic factors that activate microglia and these degenerating neurons are vulnerable to inflammatory insult. Degenerating neurons will co-localize or attract an even larger population of microglia in the SN (33). Collectively, these activated microglia and damaged neurons form a repetitive and vicious cycle that leads to chronic inflammation and continued extensive DA neurodegeneration over time, leading to the progression of PD **(Figure 2.1)**. These findings

confirm neuroinflammation as a pivotal process in the progression of neurodegenerative disorders and the central role of microglia in this process (173). Targeting neuroinflammatory pathways within microglia could be a significant step in the development of new therapeutics for neurodegenerative diseases, including PD.

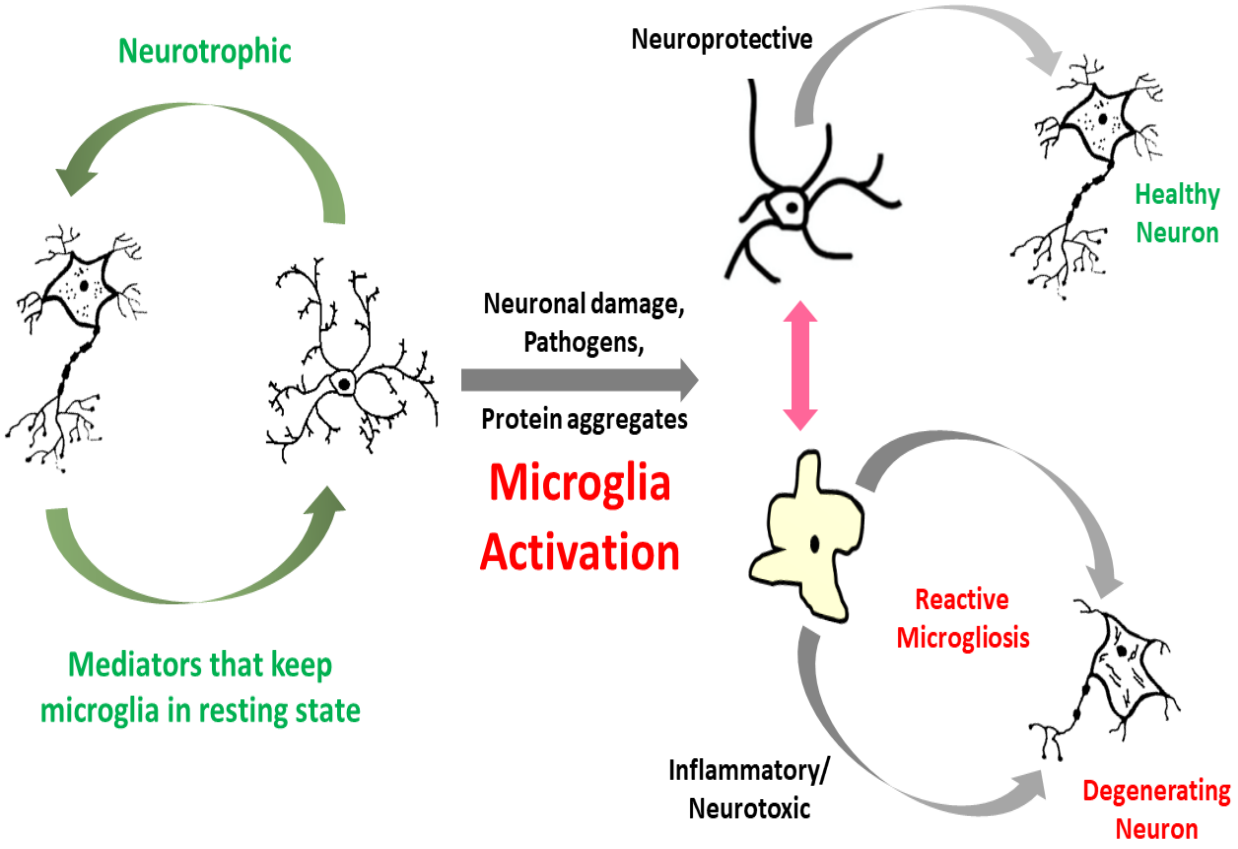


Figure 2. 1: Neuron-microglia homeostasis and Microgliosis.

Under normal conditions, microglia state and activity are regulated by neuron-microglia communication where microglia plays diverse beneficial roles in support of neuronal survival including homeostasis maintenance, production of neurotrophic factors. Inflammatory triggers, neuronal damage or abnormal protein aggregation can activated microglia (Neuroprotective) which secrete inflammatory mediators and protects neurons from further damage. In contrast, under over activation state microglia (neurotoxic) promotes neuronal death and this results into reactive Microgliosis which is an underlying mechanism of progressive neurodegeneration.

2.5. Therapies targeting neurodegeneration/neuroinflammation in PD

Treatment for PD normally involves medications such as Levodopa to enhance the dopamine levels and deal with movement symptoms (177). While none of our current treatments are able to stop the disease, medication and surgery can be helpful for managing the symptoms (178). These treatments work well in patients initially, but they are also associated with unwanted side-effects and reduced efficacy over time (179). On the other hand, many studies suggest that inflammatory mediators such as TNF, PGE2, NO, free radicals, and other immune mediators play role in the pathogenesis of PD and degeneration of dopamine-producing neurons and that targeting these mediators can be an effective treatment for PD. This opens up the potential of using anti-inflammatory drugs as an effective and long-term treatment in PD. These anti-inflammatory drugs can act by arresting the disease onset (primary prevention) or by interrupting or even reversing the disease progression (secondary prevention). Epidemiological and observational studies suggest that the use of anti-inflammatory drugs lower the risk of developing PD (180). Observations which demonstrated that inflammation in SN plays a role in PD have led many investigators to initially consider the potential use of both steroidal and nonsteroidal anti-inflammatory drugs for the treatment of PD. Steroidal anti-inflammatory drugs (SAIDs), such as dexamethasone, have shown neuroprotective effects in LPS-induced neurotoxicity in the SN in LPS models of PD (84). Nonsteroidal anti-inflammatory drugs (NSAIDs) have also been used as analgesics and antipyretics to suppress the adverse effects of inflammation (83). The neuroprotective effects of Ibuprofen have been studied in PD pathogenesis and these studies demonstrate the protective effect on dopaminergic neurons against glutamate toxicity *in vitro* (86,181). Previously, we have established several therapies targeting neuroinflammation and neurodegeneration in an animal model of PD and these therapies include D-morphinan-related compounds (120), anti-

inflammatory cytokines such as TGF-β (transforming growth factor-beta) (115) and IL-10 (111,182), IKK (inhibitor of kappa B (IκB) kinase) inhibitors (124), NADPH (nicotinamide adenine dinucleotide phosphate) oxidase inhibitors (126), and β2-AR (beta 2-adrenergic receptor) agonists (20,23). We have conducted a number of experiments using different classes of anti-inflammatory compounds to determine their efficacy in preventing dopaminergic neurotoxicity by activated microglial cells both *in vitro* and *in vivo*. First, it was found that morphinan compounds and their stereoisomers (L-morphine and its D stereo enantiomers) can inhibit microglial activation and LPS- or MPP+-induced neurotoxicity in rat primary mesencephalic cultures. We and others observed that several dextrorotatory isomers of morphine compounds, including D-morphine, dextromethorphan, and sinomenine, showed neuroprotective effects against LPS and MPP+ (1-methyl-4-phenylpyridinium) which were mediated through the inhibition of microglial PHOX (NADPH-oxidase) activity (119,120,183). Furthermore, these studies also suggest that these morphinan compounds bind to the catalytic subunit of PHOX, inhibit its activity, and reduce the production of superoxide and other pro- inflammatory cytokines (119). In another set of studies using a different anti-inflammatory approach, a specific inhibitor of IKK-β (IkappaB kinase-beta) protects dopaminergic neurons against LPS-induced neurotoxicity both *in vitro* and *in vivo* through inhibition of NF-κB activation, resulting in the decreased production of ROS and inflammatory cytokines (124). We have also developed therapies targeting neuroinflammation in PD models by using anti-inflammatory cytokines such as IL-10 and TGF-β1, and found that treatment with IL-10 on rat mesencephalic neuron-glia culture protects against LPS-induced neurotoxicity via suppression of pro-inflammatory mediators and superoxide production (111). Similarly, the neuroprotective effect of TGFβ1 is primarily due to its ability to inhibit ERK phosphorylation, the

serine phosphorylation on p47*phox*, and the production of ROS from microglia during activation by LPS (115).

2.6. Adrenergic Receptors

One of the most potent and successful therapeutic treatments for inflammation mediated dopaminergic neurotoxicity is the use of long-acting agonists to the β2-AR. Adrenergic receptors (AR) are seven-transmembrane proteins that serve as adrenoreceptors for catecholamines such as norepinephrine and epinephrine on multiple cell types, and cells within the CNS that express AR include neurons, immune cells, and astrocytes. Pharmacological classification of the adrenergic receptor was first introduced in 1948 and broadly classified as α and β adrenergic receptors (184) by Ahlquist. The classification was based on the order of potency and specificity of natural and synthetic agonist and blocking agents. The α-AR response corresponds to mainly excitatory response, while β-AR responses were correlated mainly with the inhibitory response. The α-AR response showed the order of potency: norepinephrine > epinephrine > isoproterenol and β-AR-mediated response exhibited order of potency: isoproterenol > epinephrine > norepinephrine (185,186). After the discovery of new drugs which have a high affinity to adrenergic receptors, these receptors were sub-classified. α-AR were subdivided into α1 and α2 adrenergic receptors (187). Further studies subdivided β-AR into β1 and β2 which are normally present on immune cells, cardiac muscles, and airway smooth muscles, respectively (188). A third β-AR, now called as β3-AR was identified on adipose tissues (189). Tissue distribution, physiological effects, mechanism of action, and the major agonists/antagonists of ARs are summarized in **Table 2.1**. Pharmacological compounds that serve as short, long, and ultra-long-acting agonists for these receptors have now been developed, and they are normally thought to stimulate adrenergic receptors by four different mechanisms: (i) by direct receptor binding, the most common

mechanism where drugs activate peripheral adrenergic receptors via direct binding to receptor and mimic the actions of endogenous agonists (NE, epinephrine), (ii) by regulating NE release, where drugs act on sympathetic nerve terminals and results into NE release, (iii) by inhibition of NE reuptake, where these drugs can cause NE to accumulate within synaptic gaps at sympathetic nerve terminals, (iv) by blockade of NE inactivation where drugs inhibit the activity of monoamine oxidase (MAO) which inhibits the activity of monoamines such as NE and dopamine (190).

Table 2. 1: Characteristics of adrenergic receptors.

Receptor Type	Tissue Distribution	Mechanism of Action	Agonist Potency	Physiological Effects	Agonist	Antagonist
α1	Vascular Smooth Muscles, Visceral smooth Muscles	Gq-protein coupled activates Phospholipase C, IP3+DAG	Epi ≥ NE >> Iso	Smooth muscle contractions, Gluconeogenesis, Vasoconstriction	Norepinephrine, Phenylephrine, Methoxamine	Doxazosin, Phentolamine, Prazosin
α2	Pre-synaptic terminals, pancreas, platelets, Ciliary epithelium, Salivary Glands	Gi-protein coupled inhibits Adenyl cyclase	Epi ≥ NE >>Iso	Inhibits release of Neurotransmitter	Clonidine, Monoxidine	Yohimbine, Idazoxan, Tolazoline
β1	Heart, Kidney, some pre-synaptic terminals	Gs-protein coupled activates Adenyl cyclase +PKA	Iso > Epi ≥ NE	Increase heart rate and Renin secretion	Isoproterenol, Norepinephrine, Dobutamine	Propranolol, Metoprolol, Atenolol
β2	Visceral smooth muscles, Bronchioles, Liver, Skeletal Muscles	Gs-protein coupled activates Adenyl cyclase +PKA, Ca-channels	Iso > Epi >> NE	Vasodilation, Bronchodilation, Inhibits insulin secretion	Isoproterenol, Salbutamol, Salmeterol, Albuterol, Formoterol, Terbutaline, Levalbuterol	Propranolol, ICI-118,551, Nadolol, Butoxamine
β3	Adipose Tissue	Gs-protein coupled activates Adenyl cyclase +PKA	Iso = NE > Epi	Increase lipolysis	Isoproterenol, Amibegron, Solabegron	SR59230A

NE: Norepinephrine, **Epi:** Epinephrine and **Iso:** Isoproterenol

2.7. General properties of β2-adrenergic receptors: a G-protein coupled receptor

2.7.1. Structure

The β2-ARs belong to a diverse superfamily of human cell surface seven transmembrane receptors for hormones and neurotransmitters called G-protein coupled receptors (GPCRs). GPCRs are divided into six classes on basis of sequence homology: class A (Rhodopsin-like), class B (Secretin receptor family) class C (Metabotropic glutamate), class D (Fungal mating pheromone receptor), class E (Cyclic AMP receptor) and class F (Frizzled/smoothened) (191). GPCRs are one of the most extensively studied proteins for the development of pharmaceutical drugs and target for approximately 50% of the marketed pharmaceutical drugs (192). The adrenergic receptor family belongs to the rhodopsin-like subfamily, the largest class of the GPCR. The β2-AR, an intron-less gene, is present on the long arm of chromosome 5 (5q31) and encodes for 413 amino acid polypeptide of 46kD (193). Similar to all GPCRs, β2-AR is composed of seven transmembrane spanning α-helices with an intracellular C-terminus and an extracellular N-terminus. The β2-AR was the first GPCR to be cloned (194) and the first GPCR structure to be solved (195). The β2-AR has been studied extensively and also serves as a model system for investigating the regulation and signal transduction of GPCRs. The study of the 3-D protein structure of this family of GPCRs took a giant leap forward when rhodopsin was first crystallized in 2000 and this crystalline structure has been used as an important template for modeling other GPCRs in this family (196). The crystalline structure of human β2-AR wasn't solved until 2007, when a non-active structure of β2-AR was identified (195). Posttranslational modifications such as glycosylation, palmitoylation, disulfide bond formation and phosphorylation have now been found to affect receptor functions. Interestingly, β2-AR is glycosylated at amino acid 6, 15 and 187 which is important for the trafficking of the β2-AR from the endoplasmic reticulum to the plasma

membrane (197). Mutation in these sites also results in reduced expression of receptor on the cell membrane, suggesting a role for glycosylation in cell surface expression (198). Conversely, the cysteine amino acid in the cytoplasmic tail at position 341 is palmitoylated, and is now found to be an important residue for the adequate coupling of the receptor to the G_s-protein (199). Finally, β2-AR have disulfide bonds which are essential for agonist binding and also for maintaining their tertiary structure (200).

2.7.2. Localization

Adrenergic receptors are widely distributed on human body organs and regulate physiologic functions such as bronchodilation (201,202), vasodilation, glycogenolysis in the liver, and relaxation of uterine and bladder muscles (203). The human β2-AR are widely expressed not only on airway smooth muscles but also on the wide variety of cells such as epithelial cells, endothelial cells, brain cells and immune cells including mast cells, macrophages, adaptive immune cells and eosinophils (202). The expression of β1- and β2-AR have also been found on microglial cells, suggesting that microglia, the brain's resident immune cell, is predominantly regulated by NE since NE is the predominant catecholamine in the CNS. Conversely, peripheral immune cells such as macrophages and T cells, which also express high levels of β1 and β2 AR, are thought to be regulated primarily by epinephrine (204).

2.7.3. β2-adrenergic receptors activation and signaling pathways in inflammation

Activation of adrenergic receptors could result into both pro- and anti-inflammatory actions, depending on certain parameters such as the type of cell, duration of ligand exposure to the receptor, and type of the adrenergic receptor (205). It is the diversity of the β2-AR that leads to the complexity of signaling mechanisms and to this duality of function. Activation of β2-AR by

receptor agonists initiate intracellular signaling pathways that function either via G-proteins or through β-arrestins. Like other GPCR, β2-AR can activate either canonical (traditional) or non-canonical (non-traditional) signal transduction pathways. In the canonical pathway, similar to a typical GPCR the β2-AR signals via a heterotrimeric G-protein complex, and when the receptor is coupled to inactive GDP-bound G-protein, it appears to have high affinity to the agonist or ligand. After ligand binding, the transmembrane domains of the receptor undergo conformational change with the exchange of GDP to GTP. Further, this conformational change reduces the affinity of the ligand to its receptor, increasing the possibility of retraction of ligand from the receptor, thereby preventing the over-activation of G-protein. This provides evidence that β2-AR appear to oscillate between an active and inactive form under normal conditions. After the exchange of GDP to GTP, the G_{α}-subunit dissociates from $G_{\beta\gamma}$-subunit which remains associated with plasma membrane and the G_{α}-subunit activates effector proteins. The downstream signaling of this process normally results in the production of intracellular second messengers which further activates the cAMP-PKA-mediated intracellular signaling pathway. The activated β2-AR binds with the α-subunit of the G-protein together with a guanosine triphosphate (GTP) molecule. Further, the receptor coupled with adenylate cyclase (AC) which catalyzes the conversion of ATP into cAMP (cyclic adenosine monophosphate; a second messenger for β2-AR) by hydrolysis of GTP into GDP. The cAMP activates and regulates protein kinase A (PKA) which further mediates the transcription of genes and degradation of cAMP by phosphodiesterase (PDE) leading to termination of signaling (206).

Earlier it was determined that β2-AR exhibit their inhibitory signals in immune cells via the canonical (PKA) signaling pathway. It has now been found that GPCR can also signal through a non-canonical pathway in addition to their classical signaling pathway (207). Activation through

the non-canonical signaling pathway is cell type dependent and G-protein independent, but rather the G-protein coupled receptor kinases (GRKs) and β-arrestins are involved in activation of this non-canonical signaling pathway. Various types of GRKs phosphorylate specifically serine and threonine at C-terminal of the β2-AR which further determines whether receptors undergo desensitization or initiate non-canonical signaling (208). For example, phosphorylation of receptor by GRK5/6 initiate β-arrestin-mediated non-canonical signaling while phosphorylation by GRK2 leads to β-arrestin-mediated desensitization of the receptor (209). During non-canonical signaling, β-arrestin2 couples β2-AR to MAPK signaling pathways which induces activation of transcription factors and allows their nuclear translocation. Activation of β2-AR with high agonist concentration can lead to sustained activation of ERK1/2 via β-arrestin2. This explains why β2-AR activation can either enhance or suppress the proliferation of immune cells and cytokine production particularly at a high concentration of agonists (146,205). Studies suggest that during inflammatory conditions immune cells can switch from canonical to the non-canonical pathway (205,206). Engagement of β2-AR receptors by agonists can result in immunomodulatory actions. Depending on the type of immune stimuli and timing of β2-AR activation relative to immune activation, β2-AR stimulation can positively or negatively regulate the response of immune activator (205,210). The initial data obtained in animal models of dopaminergic neurotoxicity suggests that the primary immunomodulatory mechanism of β2-AR activation that regulates CNS inflammation in microglial cells occurs through the non-canonical β-arrestin2 pathway of activation.

2.7. β2-agonists

β-agonists are a group of pharmaceutical compounds or sympathomimetic drugs that mimic the effects of endogenous catecholamines such as epinephrine, norepinephrine, and dopamine. These

drugs do not comprise a similar structure to catecholamines but still directly or indirectly activate the β2-adrenergic receptor. The first β-agonist was used around 5000 years ago in Chinese medicine where an ephedrine containing plant, ma huang, was used to treat respiratory problems (211). Further research in the 20th century has led to increased use of β-agonists for the treatment of respiratory diseases. The first β2-AR selective agonist, Salbutamol was synthesized by Glaxo in 1968 (212,213). Later, the same team at Glaxo modified Salbutamol into Salmeterol with long-lasting effects and reduced side effects. Recently, they have synthesized β2-agonists with ultra long-lasting effects such as Indacaterol (213). After successful trials, these β2-agonists were approved by the US Food and Drug Administration (FDA) for the treatment of respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). Since 1968, a number of companies have labored to develop β2-AR agonists, and some have now been commercialized for use in the treatment of COPD. A list of some of these agonists is given below and in Table 1.

2.8.1. Classification of β2-agonists: A pharmacogenetic study of β2-agonists has summarized the relationship between polymorphisms in the β2-adrenoreceptor (*ADRB2*) gene and the effects of select β2-agonists (214). Two hypotheses aim to account for the differences in functioning and *in vivo* half-lives of these compounds: exosite/exoreceptor (specific binding site) or plasmalemma diffusion micro-kinetics. Briefly, the exosite hypothesis focuses on the ability of the side-chain of these compounds to interact with a distinct site on the receptor such that it allows the active component to 'swing back-and-forth' to activate the receptor. The plasmalemma diffusion micro-kinetic hypothesis suggests that high concentrations of agonists are achieved in close proximity to the receptor and allows for a longer duration of action (215). Both of these hypotheses require further investigation and need to be studied within the CNS. Depending upon their mechanism and

duration of action, all β2-agonists are grouped into three major classes: short-acting, long-acting and ultra-long-acting β2-agonists.

2.8.1.1. Short-Acting β-agonist (SABA): These drugs are mostly hydrophilic in nature and access the active site of β-AR directly from the aqueous extracellular area and show the fast onset of action (216). These SABAs bind to the receptor for short time, therefore, their duration of action is short. Salbutamol (Ventolin), Albuterol (AccuNeb), Pirbuterol (Maxair) and Levalbuterol (Xopenex) are examples of SABAs.

2.8.1.2. Long-Acting β-agonist (LABA): These drugs are a frontline treatment for COPD and usually prescribed alone or in combination with inhaled corticosteroids. LABAs are lipophilic in nature and taken up by cell membrane as a reservoir, progressively seep out and interact with the active site of the receptor (216). They diffuse in the plasma membrane where they interact with the active site of the β2-AR which allows for the close proximity with the receptor and longer duration of action. The onset of action of these drugs is slower as compared to SABAs but the duration of action is prolonged thereby, referred to as LABAs. The duration of action is also dependent on the concentration of the agonist. Salmeterol (Serevent), Salmeterol with an inhaled corticosteroid (Advair), Formoterol (Foradil), and Formoterol with an inhaled corticosteroid (Symbicort) are commercially available LABAs and used in medication for asthma and COPD (217).

2.8.1.3. Ultra-long Acting β-agonists: These agonists are also lipophilic in nature and onset of action is similar to LABAs but the duration of action last longer than LABAs. Vilanterol with an inhaled corticosteroid (Breo) and Indacaterol (Arcapta) are ultra-LABAs, approved by FDA for the treatment of COPD (218).

2.9. β2-adrenergic receptors agonists in neuroprotection

The majority of adrenergic neurons are present in brainstem locus coeruleus (LC) nuclei which is a predominant site for the production of norepinephrine (NE) in the brain. LC neurons play a key role in the regulation of cognitive behavior such as mood, and arousal (219). These neurons also play role in the development of the brain, mainly the neocortex (220). The degeneration of LC-neurons has been identified in patients with PD and AD (221). Also, the classical 'monoamine hypothesis of depression' says that the deficiency of NE is a culprit for the cognitive impairment (222). NE/noradrenaline, the primary neurotransmitter released by the LC neurons targets the adrenergic receptors present on the microglia and astrocytes in the brain (223). NE-activated ARs on glial cells stimulate the second messenger system and maintain the homeostasis in the brain. Activation of AR on glial cells elicit anti-inflammatory actions, inhibit neuroinflammation, and thereby limit the degeneration of neurons (224). Moreover, drugs that stimulate the release of NE/NA have potential to reduced inflammation and amyloid pathology in a mouse model of AD (225). According to Braak's hypothesis, early stage of progression starts in LC before it spreads to SN (226). Overall, these and many other studies suggest the role of the adrenergic signaling in neurodegeneration. Therefore, enhancing NE/NA signaling, transplanting noradrenergic neurons or use of drugs that mimic the activity of NA/NE on glial cells have great potential to reverse or halt the progressive degeneration of neurons (227). The endogenous agonist/ligand for β2-AR is norepinephrine which acts as a neurotrophic factor and can influence protein/DNA synthesis in developing adult brain (228,229). NE protects cholinergic and dopaminergic cultured neurons against oxidative stress and catechol moiety of NE plays role in neuroprotection (230,231). It suggests that a compound containing catechol moiety such as β-agonists can mimic the neuroprotective effects of NE. Treatment with NE stimulates the synthesis of BDNF in astrocytes

and neuro *in vitro* and *in vivo* (232,233) and these neuroprotective effects were reversed by the antagonist of α1, β1 and β2-AR (234).

The use of β2-agonists as an adjunct therapy to L-DOPA in PD was first described in 1994 (235). Chai et al. showed that the β2-AR activation enhances hippocampal neurogenesis, ameliorates memory deficits, increased dendritic branching and spine density in a mouse model of Alzheimer's disease (236). Recently, *Mittal et al.* have found that β2-AR activation regulates the gene expression of α-synuclein in various animal and *in vitro* models of PD. Salbutamol, a blood-brain-barrier-permeable β2-agonist, reduces expression of SNCA gene via histone-3-lysine-27 acetylation of its promoter and enhancer. They also analyzed the pharmacological history of 4 million Norwegians over 11 years and found that Salbutamol was also associated with reduced risk of developing PD (237). In a mouse model of Down syndrome, Formoterol, a long-acting β2-AR agonist, causes significant improvement in synaptic density and cognitive functions (238). Salmeterol (Sal) is an inhaled long-acting highly selective β2-AR agonist which is currently being used as the active ingredient in Advair$^{@}$ as a bronchodilator. Our previous studies and others have shown that Salmeterol has anti-inflammatory and DA-neuroprotective activities, even at very low doses. Pre-treatment with Salmeterol protects DA neurons against LPS and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity in both *in vitro* and *in vivo* animal models of PD (20,239). Collectively, these studies suggest that β2-AR agonists not only protect neurons against degeneration but also have anti-inflammatory effects, and therefore, hold significant promise for the treatments of a wide variety of neurodegenerative conditions including PD (23). The clinical efficacy of β2-AR agonists have been examined in various neurological disorders and a few of them are summarized in **Table 2.2.**

Table 2. 2: Clinical trials using β2-agonist in neurological conditions.

Disease condition	Design	Dose	Drug	Ref.
Spinal cord Injury	Randomized controlled	4mg twice/day for 1st week then 8mg twice/day for 15 weeks	Albuterol	(240)
Alzheimer's disease	Randomized controlled	20mg/2ml for 12 months	Formoterol	(241)
Multiple sclerosis	Blinded controlled	4mg/day	Albuterol	(242)
Memory and cognition	Randomized controlled	4mg, single oral administration	Salbutamol	(243)
Neuropathic Pain	Controlled, double blinded	5mg twice/day for 28 days	Terbutaline	(244)
ALS	Uncontrolled	60ug/day for 6 months	Clenbuterol	(245)
SMA	Uncontrolled	3-8mg/day for 6 months	Albuterol	(246)
SBMA	Uncontrolled	20ug/day for 2days, then 40ug/day	Clenbuterol	(247)

SMA: Spinal Muscular Atrophy, **SBMA:** Spinal and Bulbar Muscular Atrophy, **ALS:** Amyotrophic Lateral Sclerosis.

2.10. β2-adrenergic receptors and neuroinflammation

Extensive previous investigations into the etiology of PD demonstrate a central role for the inflammatory microglial cell in the progression of PD. Thus, targeting neuroinflammation mediated by microglia may serve as a potential therapeutic benefit in the treatment of PD. Since traditional treatment for PD is aimed only at controlling the disease symptoms, the search for more effective neuroprotective therapies which target the cause of the disease is now receiving significant attention. Studies targeting neuroinflammation are aimed to promote the development of a novel therapeutic approach and aid in the drug discovery for neurodegenerative conditions such as PD. One such anti-inflammatory approach that has been found to be effective in protection

against dopaminergic neurodegeneration is accomplished by natural and therapeutic compounds that activate the β2-AR. Brain cells including neurons, microglia, and astrocytes as well as immune cells express a high density of β2-AR on their surface (204,248). Catecholamines such as epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine are the most abundant catecholamines found in the nervous system. As evidenced by many unrelated studies, catecholamines can modulate the immune response (224,249).

Further studies have found that the endogenous agonist of β2-AR, NE, controls microglial motility and functions during pathogenic conditions (250). NE also protects cortical neurons against microglia-mediated inflammation while decreased levels of NE enhance microglial activation (251). One study showed that β2-AR negatively regulates NF-κB activation and stabilizes the NF-κB/IκBα complex via β-arrestin2 in LPS activated murine macrophages (252). Interestingly, activation of β2-AR in astrocytes modulates TNF-α induced inflammatory gene expression *in vitro* and *in vivo*. In addition, an *in vivo* study demonstrated increased expression of β2-AR in glial cells in response to neuronal injury. This suggests that β2-AR may provide a therapeutic target for regulation of glial cell functioning and the inflammatory response in the brain (253). Activation of β2-AR on astrocytes stimulates the release of trophic factors such as BDNF, bFGF, NGF-1 and TGF-β1 via canonical signaling, showing anti-apoptotic and neuroprotective effects in animal models of cerebral ischemia and excitotoxicity (254,255). It has also been shown that noradrenaline acting on β2-AR enhances the expression of anti-inflammatory and neurotrophic cytokine IL-10 in the brain. This suggests an endogenous ligand of β2-AR is neuroprotective during inflammatory conditions in CNS disease pathology (253,256). Both canonical and non-canonical signaling of β2-AR can selectively regulate the adaptive immune response (205), since β2-AR are expressed by naïve CD4+ T (T-helper (Th0)) and Th1 cells but absent on Th2 cells

(257,258). Naïve CD4+ T-cell treated with a β2-AR agonist or NE suppress the production of interferon (IFN)-γ and IL-2 and affect their differentiation (259). Collectively, these studies and several others suggest the role of β2-AR in the regulation of immune response.

2.11. Molecular mechanism of inflammation in PD or molecular mediators of inflammation in PD

2.11.1. Effect of β2-AR agonists on NF-κB pathway: We have characterized and examined the effects of β2-AR agonists including Salbutamol, Salmeterol, Indacaterol, and Vilanterol on neuroinflammation in models of PD *in vitro* and *in vivo*. However, the short-acting agonists were neuroprotective and able to reduce inflammation *in vitro* at higher doses but the long-acting agonist showed beneficial effects at low concentration (10^{-9} M) in neurotoxicity and inflammatory models of PD (20). Salmeterol, a β2-AR agonist, can effectively serve as a therapeutic treatment for PD by inhibiting microglia-mediated inflammatory responses *in vivo*. We have found that Salmeterol functions to inhibit innate pro-inflammatory response in both murine macrophages and microglia through its inhibition of the NF-κB signaling pathways (20). We have also investigated whether Salmeterol is specific to neuroinflammation in PD or if it can be used as a universal anti-inflammatory drug against other chronic inflammatory diseases. To test this, we used murine macrophages stimulated with LPS from *Porphyromonas gingivalis* (*Pg*LPS), an oral pathogen as an *in vitro* model for the periodontal disease. We have found that Salmeterol shows similar anti-inflammatory effects on *Pg*LPS stimulated macrophages (260). Additionally, Feng et al. have also shown neuroprotective effects of β-arrestin2 via endogenous opioid arrest in inflammatory microglial cells (261).

2.11.2. Effect of β2-AR agonists on MAPK pathway: The agonist-activated β2-AR stimulate MAPK signaling pathway via non-canonical and G-protein independent pathway. Agonist-

activated β2-AR reduces phosphorylation of ERK1/2 and p38 MAPK in macrophages stimulated with LPS. In contrast, β2-AR activation stimulates MAPK signaling and TNF-α, IL-12 and NO production in murine macrophages treated with PMA (phorbol 12-myristate-13-acetate) (210). Similarly, our previous studies have shown that activation of β2-AR with the high concentration of agonist (up to 10^{-5}M) leads to sustained phosphorylation of ERK1/2 and enhanced production inflammatory mediators in murine microglia and macrophages (262). High-dose treatment of β2-AR agonists on mixed neuro-glia culture enhances neurotoxicity via NADPH oxidase activity in the ERK-dependent manner (18). Like others, we have found that the low-doses of the β2-AR agonist Salmeterol reduces the MAPK activity, NF-κB activation and production of TNF-α in LPS-activated primary microglia (20). We have also found that low-dose Salmeterol inhibits the phosphorylation of TAK1 (TGF-β-activated kinase1) which is an upstream regulator of NF-κB signaling in LPS-stimulated microglia. We have also found that Salmeterol increases the expression of β-arrestin2 and enhances the interaction between β-arrestin2 and TAB1 (TAK1-binding protein), reduced TAK1/TAB1 mediated activation of NF-κB and expression of pro-inflammatory genes. Furthermore, silencing of β-arrestin2 abrogates the anti-inflammatory effects of Salmeterol in LPS-stimulated BV2 cells (263). These studies suggest that the anti-inflammatory effects of Salmeterol work through the inhibition of pro-inflammatory pathways in microglial cells.

2.11.3 The β-arrestin-mediated biased effects of β2-AR agonist: Previous findings show that high dose Salmeterol enhances the expression of IL-1β and IL-6 mRNA and protein in unstimulated human monocytes and murine macrophages. These effects were β-arrestin2-dependent but PKA and NF-κB independent, while treatment with ERK1/2 and p38 MAPK inhibitor could reverse this effect (262). This finding and several others suggest Salmeterol or other long-acting agonist have

β-arrestin "biased" signaling of β2-AR. These agonists activate receptors via β-arrestin signaling with a much greater extent than their effect on G-protein-dependent signaling (264). Our studies suggest that a very low concentration of Salmeterol does not enhance cAMP signaling and its downstream mediators while it activates the β-arrestin2-mediated signaling events (20). β-arrestin2 has been shown as a novel regulator of IκB stability via the direct interaction of β-arrestin2 and IκB in HEK293 cells (265). In addition, β-arrestin2 negatively regulates the activation of NF-κB via direct binding with IκBα (266). One study showed that overexpression of β-arrestin2 significantly reduces L-DOPA-induced dyskinesia in animal models of PD (267). Collectively, these studies suggest that β2-AR agonists can be used therapeutically not only to inhibit chronic inflammation and progressive degeneration of neurons but also to treat some of the most debilitating neurologic symptoms in PD.

2.11.4. cAMP/PKA/CREB pathway induced by β2-AR: After binding with an agonist or endogenous ligand, β2-AR normally activate the classical cAMP-dependent signaling pathway. The downstream effect of the cAMP/PKA pathway is the phosphorylation and nuclear translocation of the CREB transcription factor which further enhances the expression of cAMP-inducible genes (216). Activation of CREB via this pathway regulates the synthesis of proteins which are mandatory for neuronal homeostasis (268). The classical signaling of β2-AR also increases the activity of PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) which is a key regulator of mitochondrial biogenesis and ROS metabolism (269). Activation of β2-AR also elevated the release of neurotrophic factors via cAMP/PKA/CREB pathway and provides neuroprotective benefits against degeneration (270). An endogenous agonist of β2-AR (NE) affects immune cell functions, production of cytokines and antibody secretion (257). β2-AR agonists have anti-inflammatory activity and inhibit release of pro-inflammatory

mediators via cAMP/PKA/CREB pathway and also by alternate cAMP-dependent pathway (cAMP/Epac1/2) (20,271,272). We have also found that pro-inflammatory effects of high-dose of Salmeterol are through cAMP/Epac pathway while the anti-inflammatory effects of low-dose of Salmeterol are independent on cAMP and Epac activation (18,20).

THESIS STATEMENT

Inflammation plays an important role in the etiology of a number of different forms of PD, and anti-inflammatory drugs hold promise as a therapeutic treatment for patients with PD. One such promising drug is Salmeterol and we have found that Salmeterol treatment is neuroprotective in PD models. The mechanism by which β2-AR agonists such as Salmeterol inhibits pro-inflammatory cytokine release and up-regulates anti-inflammatory cytokine release in the microglial cell population is still unclear and has never been tested before in a PD model. Therefore, to continue our previous work, we have tested the efficacy of Salmeterol in generating long-term protection against neurodegeneration in an inflammatory model of PD. The overall goal of this study was to determine the mechanisms by which β2-AR agonists exhibit anti-inflammatory effects and subsequently examine the underlying mechanism of immunomodulatory effect, on microglial cells. Also, we sought to test whether or not Salmeterol with its anti-inflammatory properties can be used to target other chronic inflammatory diseases such as periodontitis (an oral-inflammatory disease). We hypothesized that treatment with Salmeterol has anti-inflammatory effects and leads to phenotypic conversion of microglial cells by enhancing the production of anti-inflammatory mediators such as IL-10. We proposed that the use of β2-AR agonists would be a novel therapeutic approach to inhibit inflammation and death of DA neurons. To corroborate our hypothesis, we have addressed the following aims:

Project Objectives:

1) Determine the mechanism by which long-acting β2-AR agonist Salmeterol can inhibit microglial-mediated inflammatory response induced by LPS stimulation.
2) Examine if and how the β2-adrenergic receptor agonist Salmeterol mediate anti-inflammatory conversion in BV2 microglial cells.
3) Examine the effect of Salmeterol on global gene expression in LPS-activated microglia using RNA-sequencing.
4) Determine if the β2-adrenergic receptor agonist Salmeterol can exhibit anti-inflammatory effects on macrophages and monocytes stimulated with LPS from *Porphyromonas gingivalis* and/or can be used as a universal anti-inflammatory drug against other chronic inflammatory diseases.

The aim of chapters 1 and 2 is to provide the background information relevant to my thesis. Chapter 1 details the role of inflammation in PD and how it has been linked with disease progression. I have explained various anti-inflammatory therapies that have been used in PD to target neuroinflammation. Chapter 2 describes the general properties of adrenergic receptors, mechanism of action of β2-AR agonist and their role in neuro protection and inflammation. Chapter 3 describes the materials and methodology used in the study. Chapter 4, 5, 6 and 7 detail the results from objective 1, 2, 3 and 4 respectively. Chapter 8 explains the overall summary of important findings and future directions.

CHAPTER 3

MATERIALS & METHODS

3.1. Cell culture and treatments:

The human monocyte cell line THP-1, murine macrophage cell line RAW264.7 and murine microglial cell line BV2 (a kind gift from Prof. Christopher Power, University of Alberta) were maintained in culture media RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution at 37ºC and 5% CO_2 in humidified incubator. RAW-Blue cells (a kind gift from Prof. Maria Febbraio, School of Dentistry, University of Alberta) containing NF-κB secretory alkaline phosphatase (SEAP) reporter construct were cultured in same conditions.

The human monocyte cell line THP-1, murine macrophage cell line RAW264.7 were pre-treated with Salmeterol (10^{-9}M) for 45min and then stimulated with *Pg*LPS (500ng/ml) for 6h or 24h. BV2 microglial cells were also pre-treated with Salmeterol (10^{-9}M) for 45min and then stimulated with *E. coli* LPS (1µg/ml) for 6h. BV2 cells were also treated with ultra-long-acting β2-AR agonists, Vilanterol and Indacaterol (listed in **Table 3.1**). In other experiments BV2 cells were incubated with pharmacological inhibitors and β2-AR antagonist (listed in **Table 3.2**) before treatment with Salmeterol.

Table 3. 1: List of reagents used for cell culture treatment.

Reagent	Concentration Used	Purchased From
Lipopolysaccharide from *E.coli*	1 µg/ml	Sigma-Aldrich
Lipopolysaccharide from *P. gingivalis*	500 ng/ml	InvivoGen
Salmeterol	10^{-9} M	Tocris
Vilanterol	10^{-9} M	MedChemExpress
Indacaterol	10^{-9} M	Selleckchem
2′,7′-Dichlorofluorescin diacetate	1 µM	Sigma-Aldrich
Brefeldin A (BFA)	10 µg/ml	Thermo Fisher

Table 3. 2: List of inhibitors used for cell culture treatment.

Inhibitor	Effector	Specificity	Concentration Used	Purchased From
U0126	Extracellular signal-regulated kinase 1/2	MEK1/2	10 μM	Tocris
SP600125	c-Jun N-terminal kinase	JNK Kinase activity	10 μM	Selleckchem
SB203580	p38 MAPK	p38 MAPK	5 μM	Tocris
KT5720	PKA	PKA	1 μM	Tocris
Wortmannin	PI3K	PI3K	1 μM	Tocris
ICI 115 855 hydrochloride	β2-adrenergic receptor	β2-adrenergic receptor	1 nM	Tocris

3.2. RNA Assay

3.2.1. RNA extraction and cDNA synthesis:

After treatment, cells were washed with sterile 1X PBS and homogenized with Trizol reagent (Ambion, Thermo Fisher Scientific). Total cellular RNA was extracted from RAW264.7, THP-1 and BV2 cells by phenol-chloroform method (Trizol reagent) according to manufacturer's protocol. The quality of RNA was assessed by Nanodrop (Thermo Scientific) for purity (OD260/OD280) and by Agarose gel electrophoresis for RNA integrity and potential contamination. cDNA was synthesized from 1μg of total RNA by using QuantiTect Rev. Transcription Kit (Qiagen) as recommended by the manufacturer. After reaction, cDNA was diluted with nuclease-free water and quality and quantity of cDNA was assessed by Nanodrop.

3.2.2. Semi-quantitative RT-PCR:

The semi-quantitative RT-PCR was performed using 500ng of cDNA template. RT-PCR was performed by using 500ng of cDNA template in 10X Ex Taq buffer, 20mM $MgCl_2$, 2.5mM dNTPs

mix, Taq polymerase (TaKaRa ExTaq) and volume of the reaction was made up to 20µl by nuclease free water. The PCR reaction condition were as follows: denaturation at 95ºC for 30s, primer annealing at 55ºC or 58ºC for 30s followed by the final extension at 72ºC for 60s. The amplified products were loaded at equal volume and resolved on a 2% agarose gel by electrophoresis in 1X Tris-Acetate-EDTA (TAE) buffer. The PCR products were visualized with ethidium bromide and then viewed with UV transillumination by ChemiDoc gel Imaging system (BioRad) and intensity of bands was quantitatively determined by Image J densitometry analysis software (NIH). Primer pairs used for amplification are listed in **Table 3.3 and 3.4**.

3.2.3. Quantitative real-time PCR (qPCR):

The qPCR was performed by using 300-500ng of cDNA template with SYBR Green (BioRad) and the volume of reaction was made up to 20µl by nucleases free water. The PCR reaction condition were as follows: denaturation at 95ºC for 30s, primer annealing at 55ºC or 52ºC for 30s followed by the final extension at 72ºC for 60s. Results were analyzed using cycle threshold (ΔC_T) and relative gene expression was calculated with $2^{-\Delta\Delta CT}$ after normalizing the target gene ΔC_T value with the housekeeping gene (GAPDH) ΔC_T value. Data expressed as fold-change compared to control. Primer pairs used for amplification are listed in **Table 3.4**.

Table 3. 3: List of human primers used in gene expression analysis.

Target Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Accession Number
TNFA	GGA GAA GGG TGA CCG ACT	CTG CCC AGA CTC GGC AA	NC_000006.12
IL1B	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	NM_000576.2
IL6	AGCCACTCACCTCTTCAGAACGAA	AGTGCCTCTTTGCTGCTTTCACAC	NM_000600.5
IL8	CTT TGT CCA TTC CCA CTT CTG A	TCC CTA ACG GTT GCC TTT GTA T	NC_000004.12
ACTB	ATTGCCGACAGGATGCAGAA	GCTGATCCACATC TGCTGGAA	NC_000002.12

Table 3. 4: List of mouse primers used in gene expression analysis.

Target Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Accession Number
TNFA	ACCATGAGCACTGAAAGCAT	AGATGAGGTACAGGCCCTCT	NM_013693.3
IL1B	ATCCGTGTCTTCCTAAAGTATG	CCTGAGCGACCTGTCTTG	NM_008361.4
IL6	GCTGGAGTCACAGAAGGAG	GAGAACAACATAAGTCAGATACC	NM_031168.2
IL18	TCCAACTGCAGACTGGCAC	GTCTGGTCTGGGGTTCACTG	NM_001357221.1
NOS2	ACATCAGGTGGTCGGCCATCACT	CGTACCGGATGAGCTGTGAATT	NM_010927.4
CCL2	GATGCAGTTAACGCCCCACT	ACCCATTCCTTCTTGGGGTC	NM_011333.3
CCL3	GCAACCAAGTCTTCTCAGCG	AGCAAAGGCTGCTGGTTTCA	NM_011337.2
CCL4	TGTGCAAACCTAACCCCGAG	TGGAGCAAAGACTGCTGGTC	NM_013652.2
CXCL10	TCCCCATCAGCACCATGAAC	GGACCATGGCTTGACCATCA	NM_021274.2
ARG1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC	NM_007482.3
IL10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG	NM_010548.2
CXCL14	GAGTCACCGAGTGGTTCTGCAT	CTTCGTAGACCCTGCGCTTC	NM_019568.2
ACTB	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC	NM_000077.6
GAPDH	GCCTTCCGTGTTCCTACC	CTTCACCACCTTCTTGATGTC	NM_008084.3

3.3. Protein Assay:

3.3.1. Preparation of cell lysates and quantification:

Protein samples were prepared either whole-cell lysate or nuclear and cytoplasmic extract. For whole cell protein extraction cells were lysed in NP-40 lysis buffer (50mM Tris-pH8.0, 150mM NaCl, 1% NP-40 and freshly added 10^{-6} M Protease inhibitor). Cells were lysed with the cytosolic extraction buffer (comprised of 0.2% TritonX-100, 1.5mM magnesium chloride, 10mM potassium chloride, 10mM HEPES buffer (pH 7.6), 10mM sodium butyrate, 20mM β-glycerol phosphate, and protease inhibitor) and then centrifuge. Supernatant was collected and pellet was lysed with nuclear extraction buffer that contain 50mM Tris (pH 7.5), 150mM NaCl, 50mM NaF, 1mM EDTA, 1mM $NaVO_3$, 2% SDS, and protease inhibitor. Protein concentration was determined by the standard procedure of Pierce BCA protein assay kit (Thermo Scientific).

3.3.2. Western blotting:

Protein from each sample (30-50 µg) was mixed with 1X SDS loading buffer and heated at 95^{o}C for 5min. Cell lysates were separated on 10% or 12% polyacrylamide gels and blotted onto a nitrocellulose membrane by electrophoretic transfer. The membranes were blocked with 5% non-fat skimmed milk (in Tris-buffer saline containing 0.1% Tween-20; TBST). After blocking, blots were probed with following primary antibodies (indicated in **Table 3.5**) in 5% milk overnight at 4^{o}C. After 3 washes with TBST, blots were probed with respective HRP conjugated secondary antibodies (indicated in Table 3.5). Later blots were washed and developed with enhanced chemiluminescence reagent (Amersham, GE healthcare). Blots were further visualized using Bio-Rad ChemiDoc gel Imaging system and band intensities were quantitatively determined by Image J Software (NIH). Relative protein expression levels were calculated by obtaining a ratio of the target protein to housekeeping protein (β-actin or histone H3) band intensities via densitometry analysis. Data expressed as fold-change compared to respective control group.

Table 3. 5: List of antibodies used in the study.

Antibody	Purchased From
Anti-phospho-ERK1/2 (1:1000)	Cell Signaling
Anti-ERK1/2 (1:1000)	Cell Signaling
Anti-phospho-JNK (1:1000)	Cell Signaling
Anti-JNK (1:1000)	Cell Signaling
Anti-phospho-p38 (1:1000)	Cell Signaling
Anti-p38 (1:1000)	Cell Signaling
Anti-phospho-IκBα (1:1000)	Cell Signaling
Anti- IκBα (1:1000)	Cell Signaling
Anti-β-actin (1:3000)	Cell Signaling
Anti-histone H3 (1:1000)	Cell Signaling
Anti-Rel A/ NF-κB-p65 (1:1000)	Cell Signaling
Anti-phospho-TAK1 (1:1000)	Cell Signaling
Anti-TAK1(1:1000)	Cell Signaling
Anti-TAB1(1:1000)	Santa Cruz
Anti-β-arrestin2 (1:1000)	Cell Signaling
Anti-CREB (1:1000)	Cell Signaling
Anti-rabbit-IgG-HRP-linked (1:4000)	Cell Signaling
Anti-mouse-IgG-HRP-linked (1:4000)	Cell Signaling
Anti-goat-IgG-HRP-linked (1:4000)	Santa Cruz
Anti-rabbit-Alexa Fluor 488 (1:500)	Thermo Fisher Scientific
Anti-goat-Alexa Fluor Cy3 (1:500)	Thermo Fisher Scientific
Anti-CD11b (PE-Cy7;Y780)	BD Bioscience
Anti-TNF-α (APC;R670)	BD Bioscience
Anti-IL-10 (PE;Y586)	BD Bioscience

3.4. Enzyme-linked immunosorbent assay (ELISA):

RAW264.7 and THP-1 cells were seeded in 24-well tissue culture plates (5 x10^4cells per well). Cells were pre-incubated for 45min with Salmeterol (10^{-9}M). This concentration of Salmeterol was chosen because it has previously been shown to be very effective in blocking the inflammatory

responses in microglial cells induced by TLR-4 agonist (20). After 45min culture media replaced with fresh media and cells were stimulated with *Pg*LPS (500ng/ml) and supernatant was collected after 24h. The concentration of cytokines TNFα, IL-6, IL-1β, IL-10 and IL-8 (THP-1) in culture medium was measured by commercially available ELISA kits according to manufacturer's protocol. Also, BV2 cells were seeded in 24-well tissue culture plates (5 $x10^4$ cells per well). Cells were pre-incubated for 45min with Salmeterol (10^{-9}M). After 45min culture media replaced with fresh media and cells were stimulated with LPS (1μg/ml) and supernatant was collected after 6h. The concentration of cytokines TNFα, IL-1β and IL-10 in culture medium was measured by commercially available ELISA kits (R&D systems, Minneapolis, MN) according to manufacturer's protocol.

3.5. Secretory alkaline phosphatase (SEAP) promotor activity assay:

In this assay, we have used RAW (RAW-Blue) cells stably expressing SEAP gene inducible by NF-κB transcription factor **(Figure 3.1)**. RAW-Blue cells were seeded in 24 well plate (1 $x10^5$ cells/well) and treated with Salmeterol (10^{-9}M) and incubated for 45min. After treatment, media is replaced with fresh medium and cells were stimulated with *Pg*LPS (500ng/ml) for 24h. The SEAP detection medium (QUANTI-Blue solution) is prepared as per manufacturer's instructions. Supernatant from RAW-Blue cells collected and incubated with the quanti-blue solution at 37ºC for 1h. The NF-κB activation was detected by measuring pNF-κB-SEAP promotor activity spectrophotometrically at 635 nm by Infinite200 PRO plate reader (Tecan) and Magellan software (Tecan).

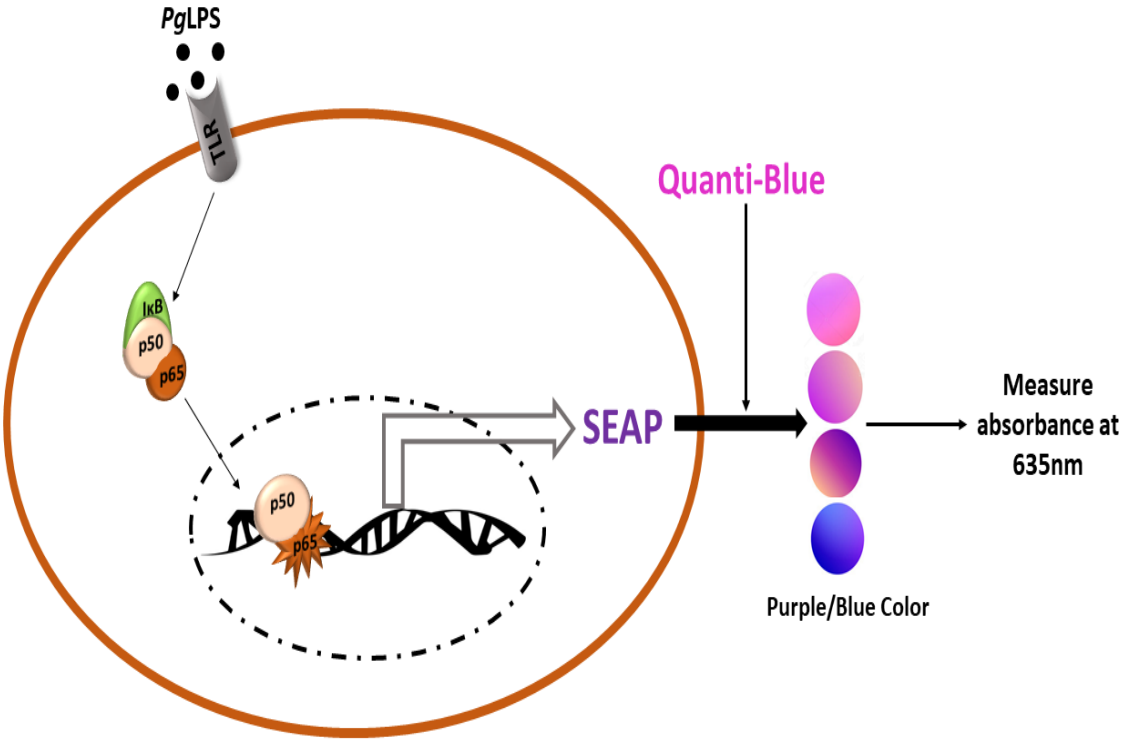


Figure 3. 1: SEAP-assay.

Raw-Blue cells co-expressing NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Once stimulated with *Pg*LPS, cells activate downstream NF-κB pathway. Using quanti-blue detection medium activation of NF-κB measured by spectrophotometer at 635nm.

3.6. Nitric oxide measurement:

The nitric oxide (NO) is oxidized to nitrite and nitrate and measurement of concentrations of these anions used as a quantitative measure of NO production (273). This assay involves nitric oxide based enzymatic conversion of nitrate to nitrite by nitrate reductase and the concentration of nitrite was determined by mixing supernatant from treated cells with Griess reagent, as described below. Murine macrophages RAW264.7, human-monocyte THP-1 cells and murine microglia BV2 cells (1×10^5) were seeded in 24 well culture plates. Cells were pre-incubated with Salmeterol for 45min and then stimulated with either *P*gLPS (500ng/ml) or *E.coli* LPS (1µg/ml) for 24h. A total of 50µl of culture supernatant was incubated with equal amount of Griess reagent on 96-well plate and incubated for 30min at room temperature. After incubation, absorbance ($A_{540\ nm}$) of samples was measured by Infinite200 PRO plate reader and Magellan software (Tecan). A standard curve was generated using 2-fold serial dilution of 50-200µM of sodium nitrite solution. The concentration of NO is indirectly measured by calculating the amount of NO_2 in the culture supernatant using standard curve of $NaNO_2$.

3.7. Reactive oxygen species (ROS) detection assay:

The oxidation of 2'-7' dichlorofluorescin diacetate (H_2DCFDA) to 2'-7'dichlorofluorescein (DCF) has been used extensively for the detection of the reactive oxygen species (274). The diacetate form, H_2DCFDA is taken up by cells where cellular esterases convert it into non-fluorescent H_2DCF inside the cell. Further, intracellular ROS oxidized non-fluorescent H_2DCF molecule to a fluorescent DCF molecule and the measurement of fluorescence are 485nm for excitation and 535nm for emission. The quantification of ROS in BV2 cells was measured as described below.

BV2 cells were plated in 96-well clear bottom black well plates. Cells were incubated with the non-fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma Aldrich) and after 30min incubation cells were washed with 1X PBS. Next, cells were treated with Salmeterol for 45min, and then stimulated with LPS. Cells were also incubated with hydrogen peroxide (H_2O_2) as control. After 1h incubation fluorescence intensity was measured using microplate reader at 485nm for excitation and 535nm for emission.

3.8. Immunocytochemistry:

RAW264.7 cells were seeded on coverslip (in 24-well plate) at density of 40,000 cells per well. After treatment cells were fixed for 20min with 4% paraformaldehyde at room temperature and washed three times with TBS. Cells were further blocked and permeabilized using 4% bovine serum albumin (BSA) containing 0.5% Triton X-100 for 1h. Cells were incubated with primary antibody (NF-κB-p65; 1:500 dilution) for overnight at 4^{o}C. After incubation, cells were washed thrice and incubated with appropriate secondary antibody tagged with Alexa Fluor 488. After washing, coverslips were mounted with mounting medium containing DAPI. For each group, 5-7 images were captured from random fields. Images were acquired using AxioImager.Z1 microscope.

BV2 cells were seeded on coverslip (in 24-well plate) at density of 40,000 cells per well. After treatment cells were fixed for 20min with 4% paraformaldehyde at room temperature and washed three times with TBS. Cells were further blocked and permeabilized using 4% bovine serum albumin (BSA) containing 0.1% Triton X-100 for 1h. Cells were incubated with primary antibody (NF-κB-p65; 1:500, anti-β-arrestin2 and anti-TAB1) for overnight at 4^{o}C. After incubation, cells were washed thrice and incubated with appropriate secondary antibody tagged with Alexa Fluor 488 and Alexa Fluor 594. After washing, coverslips were mounted with mounting medium

containing DAPI. For each group, 5-7 images were captured from random fields. Images were acquired using AxioImager.Z1 microscope (Carl Zeiss, Germany). The quantitative analysis of captured images were performed using Fiji (Fiji Is Just Image J) (NIH; https://imagej.nih.gov/ij/) as described by Noursadeghi et al (277). Also, the cytoplasmic and nuclear Rel A fluorescence intensities were used to calculate the nuclear: cytoplasmic ratio as a relative measure of nuclear translocation of Rel A (p65).

3.9. Co-immunoprecipitation:

BV2 cells were pretreated with Salmeterol (10^{-9}M) for 45min followed by stimulation with LPS for 2h. Whole cell lysate from BV2 microglial cells were prepared as described above. Lysates form each experimental group (300µg) were incubated overnight with anti-TAB1 (2µg) at 4ºC with gentle agitation. The antigen-antibody complexes were subjected to immunoprecipitation by mixing with protein A/G plus agarose beads (50µl per sample) for 2h at 4ºC with agitation. The tubes containing immuno-complexes were centrifuged at 5,000 rpm for 5min and beads were further washed three times with 1X PBS. Elution step was performed by mixing the beads with 2X sample buffer and incubating for 5min at 100 ºC. The eluted samples were further analysed by immunoblotting for anti-β-arrestin2 and anti-TAK1.

3.10. RNA interference:

BV2 cells were transfected with 20pmoles of *Arrb2* and *CREB* siRNA using Lipofectamine RNAiMAX (Invitrogen). In control experimental groups 20pmoles of scrambled (Scr) siRNA was transfected. Both *CREB* and *Arrb2* siRNA were transfected in BV2 for 24h and subsequently subjected to treatments as described in the respective figure legends. Silencing of target gene expression was confirmed with immunoblotting.

3.11. Flow Cytometry:

Fluorophore conjugated antibodies specific to surface maker and cytokines were purchased from BD Bioscience. For surface marker staining anti-CD11b (PE-Cy7;Y780), and for intracellular cytokine staining anti-TNF-α (APC;R670) and anti-IL-10 (PE;Y586) antibodies were used. BV2 cells were first treated with Salmeterol (10^{-9}M) for 45min. Next, cells were incubated with protein transport inhibitor; Brefeldin A (BFA; 10 μg/ml, ThermoFisher) prior to stimulation with *E. coli* LPS (1μg/ml) for 6h. Cells were then initially stained with surface marker CD11b. Next, cells were washed with Perm/wash buffer (BD Bioscience), and for intracellular cytokine staining cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Bioscience), then stained with antibodies to TNF-α and IL-10. Paraformaldehyde-fixed cells were acquired using BD LSR Fortessa flow cytometer (BD Bioscience) and analyzed by FlowJo (version 10) software.

3.12. Statistical Analysis:

Data collected from at least 3-5 independent experiments with three replicates per experiments and presented as mean ± SD and median (for box-plots) of repeated measures. The statistical significance of comparison between control and treated groups determined by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-tests. The statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA, USA). The p-values of <0.05 was considered statistically significant.

3.13. Methods for RNA Sequencing:

3.13.1. Total RNA extraction:

Total cellular RNA was extracted from BV2 cells by Trizol reagent (Invitrogen) according to manufacturer's protocol. The quality of RNA was assessed by Nanodrop (Thermo Scientific) for

purity (OD260/OD280), by Agarose gel electrophoresis for RNA integrity and potential contamination. The purity and integrity were also checked by Agilent 2100 bio analyzer **(Table 3.6).**

Table 3. 6: Quantification of RNA samples.

Sample Name	Conc. (ng/µl)	Amount (µg)	260/280	260/230	RIN	Result
Control (1)	725.60	30.480	1.93	2.19	9.8	Pass
LPS (1)	808.46	38	1.94	2.14	9.8	Pass
Sal (1)	1670.12	27.891	1.95	2.25	9.6	Pass
Sal+LPS (1)	1843.40	38.711	1.9	2.13	9.6	Pass
Control (2)	1541.90	29.604	1.96	2.19	9.6	Pass
LPS (2)	1672.46	35.122	1.92	2.15	9.6	Pass
Sal (2)	1430.09	27.315	1.93	2.21	9.6	Pass
Sal+LPS (2)	1451.38	30.479	1.91	2.16	9.5	Pass

RIN: RNA integrity number, **Pass:** Library preparation can be proceeded.

3.13.2. cDNA library construction and quality assessment for RNA-sequencing:

Total RNA was isolated, purified and quantified. The RNA samples were pass through the quality assessment before library preparation. cDNA libraries were prepared by using NEBNext Ultra library preparation kit from Illumina (Illumina, San Diego, USA). mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads and then fragmented randomly by addition of fragmentation buffer. The first strand of cDNA was synthesized using random hexamer primer and M-MuLV reverse Transcriptase (RNase-H), followed by second strand synthesis using DNA polymerase I and RNase-H. The double-stranded cDNA was purified and converted into blunt end

via exonuclease/polymerase activities (end-repair reaction). After adenylation at 3'end, DNA fragments were ligated with NEBNext adaptor sequences. In order to select cDNA fragments preferentially 150-200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly USA). Later, libraries were amplified by PCR and purified by using AMPure XP beads. Prepared libraries were quantified by Qubit2.0 and library size was detected by Agilent 2100 bio analyzer. To accurately quantify the library concentration and to ensure the library quality, qPCR was performed. Finally, the libraries were sequenced using Illumina HiSeq 2500 platform with paired-end 150bp sequencing strategy. Sequencing was performed by NovoGene Corporation Inc. (Sacramento, USA). Biological replicates (n=2) RNA sequencing was performed on each treatment condition of BV2 microglia cells: control (2 samples), LPS-stimulated (2 samples), Salmeterol-treated (2 samples) and Salmeterol-treated and LPS-stimulated (2 samples). This step of sequencing was performed by Novogene Inc. Workflow of library construction and sequencing is represented in **Figure 3.2**.

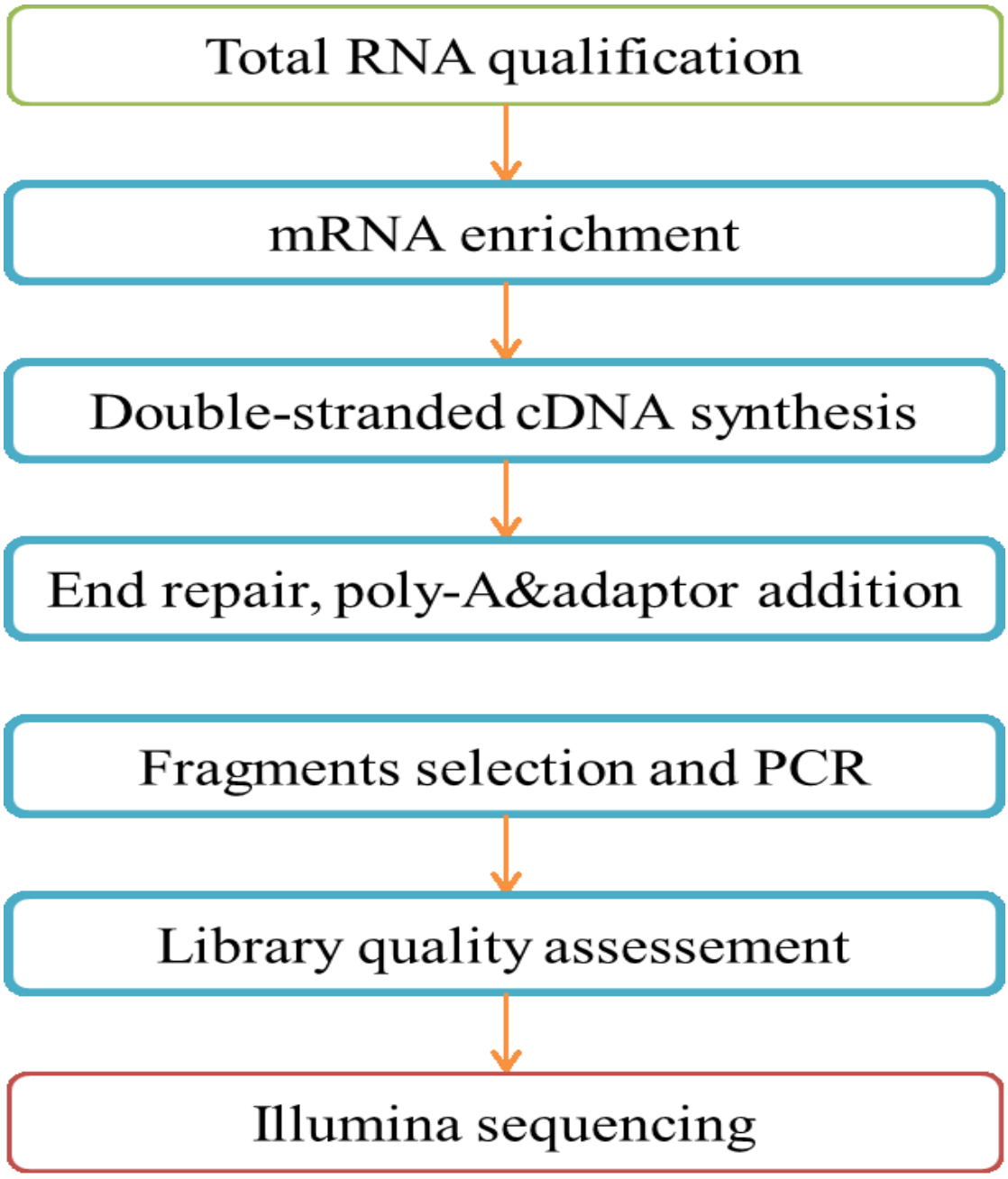


Figure 3. 2: The workflow of library construction and sequencing.

3.13.3. Raw data processing and quality control analysis:

Raw image data files from Illumina were transformed to sequencing reads by CASAVA base recognition (base calling) and stored in FASTQ file format containing read sequence and corresponding base quality. FASTQ files of RNA-sequencing experiments pass through the quality control analysis. The adaptor sequences were trimmed from raw reads and low quality reads were removed. Reads containing N>10% were also removed in order to filter raw reads to get the clean reads. RNA-Seq data was mapped to the reference genome (mouse assembly GRCm38/mm10) and assembled by Tophat2 alignment and Cufflinks pipeline from Illumina. Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5). STAR used the method of Maximal Mappable Prefix (MMP) which can generate a precise mapping result for junction reads. HTSeq v0.6.1 was then applied to count the read numbers mapped of each gene. The bioinformatics analysis was performed by NovoGene Inc. The workflow of bio-informatics analysis is shown in **Figure 3.3.**

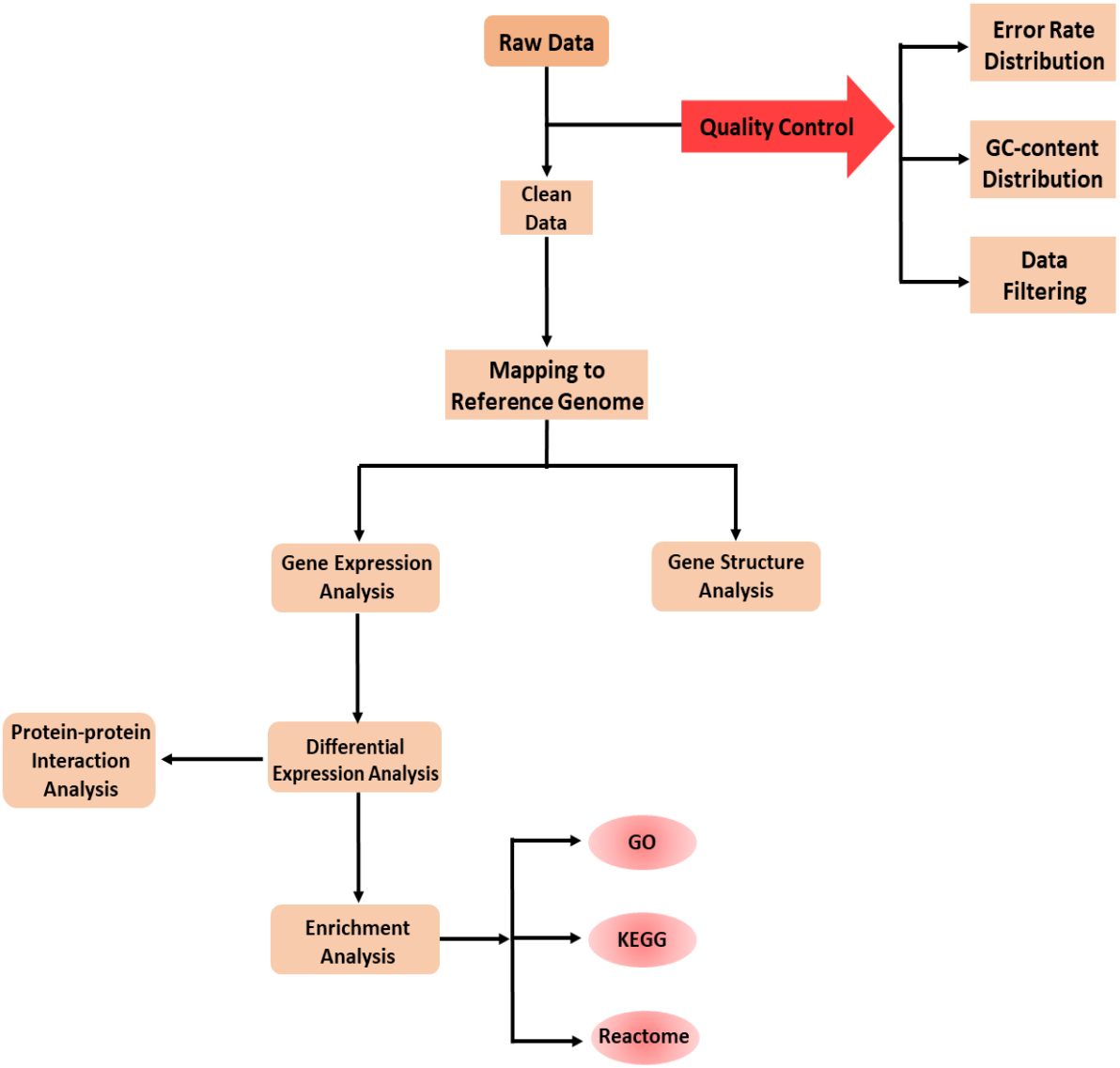


Figure 3. 3: The workflow of Bioinformatics analysis.

Raw image data file from high-throughput sequencing was transformed to Sequenced Reads (called Raw Data or Raw Reads). Quality control analysis was performed on raw data by determining the sequencing error rate distribution, GC content (used to detect potential AT/GC separation) and data filtering to get the clean reads. Next, the clean data was mapped to reference genome and proceed to gene expression analysis.

3.13.4. Differential gene expression analysis:

Differential expression analysis (for biological replicates) between two groups or treatment conditions were performed using DESeq2 R package (2_1.6.3) (275,276). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. The Venn diagrams were prepared using the function 'vennDiagram' in R on basis of the gene list for different experimental groups.

3.13.5. Functional analysis of differentially expressed genes:

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster profiler (v2.4.3) R package in which gene length bias were corrected. GO terms with corrected P-value <0.05 were considered significantly enriched by differential expressed genes. The cluster Profiler R package (277) was also used to test the statistical enrichment of differential expression genes in KEGG pathways (278). All software packages used for analysis are listed in **Table 3.7.**

Table 3. 7: List of software used for sequencing analysis.

ANALYSIS	SOFTWARE	VERSION	PARAMETER	USED FOR
Mapping	STAR	V2.5	Mismatch=2	Mapping to reference genome
Quantification	HTSeq	V0.6.1	-m union	-
Differential Analysis	DESeq2	V2_1.6.3	Padj<0.05	Differential gene expression between samples with biological replicates.
	EdgeR	V3.16.5	Padj<0.05 and log2(fold change)>1	
Enrichment Analysis	Cluster Profiler	V2.4.3	Padj<0.05	For GO, KEGG and Reactome Enrichment Analysis

Table 3. 8: List of buffers.

Buffer	Composition
10X PBS	1.5M NaCl, 27mM KCl, 80mM Na_2HPO_4, 20mM KH_2PO_4, bring up to 1L; pH: 7.2-7.4
10X TBS	0.2M Tris, 1.5M NaCl bring up to 1L; pH: 7.6
10X Western Transfer Buffer	250mM Tris, 1.92M Glycine, bring up to 1L H_2O
10X TAE	0.4M Tris, 10mM EDTA, 17.4M Glacial Acetic Acid, bring up to 1L; pH:8.0
SDS-PAGE Running Buffer	250mM Tris, 1.92M Glycine, 35mM SDS, bring up to 1L; pH: 8.3
TBST	100 ml 10X TBS, 900 ml H_2O, 1 ml Tween-20
5X loading buffer	1M Tris, 50% Glycerol, 1% Bromophenol Blue, 5% Beta mercaptoethanol
NP-40 Lysis Buffer	50mM Tris, 150mM NaCl, 1% NP-40, add fresh protease inhibitor.
HEPES-Lysis Buffer	50mM Tris-pH: 7.5, 150mM NaCl, 50mM NaF, 1mM EDTA, 1mM $NaVO_3$, 2%SDS, add fresh protease inhibitor.
SDS-Lysis Buffer	0.2% Triton-X100, 10mM HEPES (pH: 7.6), 1.5mM $MgCl_2$, 10mM KCl, 10mM Sodium butyrate, 20mM Beta glycerol phosphate, add fresh protease inhibitor
Stripping Buffer	62.5mM Tris-HCl (pH: 6.8), 2% SDS, 100mM beta-mercaptoethanol, bring up to 25ml H_2O.
1X Western Transfer Buffer	100 ml 10X Transfer buffer, 200 ml Methanol, 700 ml H_2O
Flow Buffer	2% FBS in 1X PBS
Reagent Diluent for Elisa	1% BSA in 1X PBS
Elisa Wash Buffer	0.05% Tween20 in 1X PBS

CHAPTER 4

β-arrestin2 regulates the anti-inflammatory effects of Salmeterol in lipopolysaccharide-stimulated BV2 cells

This chapter has been modified from the published article:

Sharma, M., Flood, P.M., 2018. **β-arrestin2 regulates the anti-inflammatory effects of Salmeterol in lipopolysaccharide-stimulated BV2 cells.** *J. Neuroimmunol*. 325, 10–19.

4.1. ABSTRACT

Microglial activation contributes to chronic inflammation and neuronal loss in progressive neurodegenerative disorders such as PD. Thus, treatments suppressing microglial activation may have therapeutic benefits to prevent neuronal loss in neurodegenerative diseases. Our previous findings show that Salmeterol, a long-acting β2-adrenergic receptor (β2-AR) agonist, is neuroprotective in two distinct animal models of PD, including where LPS from *E. coli* was used to initiate chronic neurodegeneration. Salmeterol was found to be a potent inhibitor of dopaminergic neurodegeneration by regulating the production of pro-inflammatory mediators from activated microglial cells. In the present study, we investigated the molecular basis of the anti-inflammatory effects of Salmeterol on LPS-activated murine microglial BV2 cells. BV2 cells were pretreated with Salmeterol and followed by stimulation with LPS. Salmeterol inhibited LPS-induced release of the pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and nitric oxide from BV2 cells. Additionally, Salmeterol suppressed nuclear translocation of nuclear factor kappa-B (NF-κB) p65 by inhibiting the IκB-α degradation and TAK1 (transforming growth factor-beta-activated kinase1) phosphorylation. We have also found that Salmeterol increases the expression of β-arrestin2 and enhances the interaction between β-arrestin2 and TAB1 (TAK1-binding protein), reduced TAK1/TAB1 mediated activation of NFκB and expression of pro-inflammatory genes. Furthermore, silencing of β-arrestin2 abrogates the anti-inflammatory effects of Salmeterol in LPS-stimulated BV2 cells. Our findings suggest that the anti-inflammatory properties of Salmeterol is β-arrestin2 dependent and also offers novel therapeutics targeting inflammatory pathways to prevent microglial cell activation and neuronal loss in neuroinflammatory diseases like PD.

4.2. INTRODUCTION

Parkinson's disease, the second most prevalent neurodegenerative disorder, is characterized by loss of dopaminergic (DA) neurons in the substantia nigra pars compacta in the CNS. PD is not only characterized as loss of DA-neurons and motor impairment but also recognized to have an inflammatory component which plays a crucial role in the progression of the disease. Research studies of post-mortem brain from patients with PD and related parkinsonian syndromes suggest the presence of activated microglia around degenerating DA-neurons in SN (17). These reactive microglia result in prolong inflammation and induce highly detrimental neurotoxic effects by producing a large amount of inflammatory mediators such NO (280), super oxide (281) and TNF-α) (282). Production of these inflammatory mediators is a pivotal process in the progression of PD, and therapeutic intervention inhibiting the production of these mediators by microglia may serve as a potential treatment for PD. Brain cells including neurons, microglia, and astrocytes express a high density of β2-AR which belong to the G-protein coupled seven transmembrane receptor (GPCR) superfamily, and engagement of these receptors by β2-AR agonists can result in immunomodulatory actions (283). Activation of β2-AR by these agonists initiates signaling either through classical G protein coupled activation or through β-arrestin-dependent activation pathways (284,285). Several studies show the agonist stimulated activation of β2-AR in brain inhibited LPS-induced inflammatory response by suppressing pro-inflammatory mediators (IL-1β, TNF-α, and NO) in NF-κB dependent manner (286,287). In addition, β2-AR agonists have been proposed as a therapeutic to treat chronic inflammatory diseases such as sepsis (288) as well as neurodegenerative disorders (23,286). Interestingly, an endogenous agonist of β2-AR, norepinephrine (NE) inhibited LPS-induced inflammatory responses by reducing the expression of nitric oxide synthase (NOS2) and IL-1β production in primary rat microglial cells (291), and

furthermore depletion of NE has been shown to cause microglia mediated neuroinflammation (290). Collectively, these findings implicate the role of agonist-activated β2-AR signaling in microglial mediated inflammatory response and neuronal death.

Salmeterol is an FDA-approved long-acting selective β2-AR agonist which is currently being used as a bronchodilator for the treatment of severe persistent asthma and COPD (291–293). In our previous studies, we have shown that Salmeterol has potent anti-inflammatory and dopaminergic neuroprotective effects against both LPS and 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity *in vivo* (20), and pre-treatment of mesencephalic cultures or primary microglial cells with Salmeterol protects DA neurons from neurodegeneration induced by either LPS or MPTP in vitro. These studies showed that Salmeterol can inhibit dopaminergic neurodegeneration by suppressing the production of inflammatory mediators as well as inhibition of TAK1 (transforming growth factor-beta activated kinase 1) mediated phosphorylation of MAPK and NF-κB (22). In our current studies, we tested the anti-inflammatory effects of Salmeterol on the murine microglial cell line BV2, in order to determine the underlying cellular and molecular mechanisms that regulate microglial activity. Here, we reveal that Salmeterol suppresses production of inflammatory mediators in LPS-induced BV2 microglial cells and β-arrestin2 is a critical anti-inflammatory signal mediator in this response. Sal/β2-AR activation in BV2 cells enhances β-arrestin2 expression and prevents the formation of the TAB1 (transforming growth factor-beta-activated kinase-1 (TAK1) binding protein 1)/TAK1 molecular complex, thereby arresting the downstream NF-κB inflammatory pathway. In contrast, knockdown of β-arrestin2 using siRNA (si*Arrb2*) attenuates the anti-inflammatory effects of Sal/β2-AR activation. These results suggest that β2-AR agonists can be developed as anti-inflammatory therapy to subside neuro-inflammation in PD and other inflammation-dependent neurodegenerative disorders.

4.3. RESULTS

4.3.1. Agonist activated BV2 microglial β2-AR attenuates LPS-stimulated pro-inflammatory cytokines production

Microglia release variety of cytokine and chemokines in response to inflammatory stimuli. We first investigated the effect of Salmeterol on the production of inflammatory mediators by BV2 cells in response to the endotoxin LPS derived from *E. coli*. We used a 10^{-9}M dose of Salmeterol because we have found in our previous studies this as the most effective anti- inflammatory dose in vitro on primary microglial cells (20,260). **Figure 4.1** indicates that LPS stimulation leads to significant elevation in mRNA (A and C) and protein expression (B and D) of the inflammatory mediators, IL-1β and TNF-α, respectively in BV2 cells. The expression of these inflammatory mediators was significantly decreased by pre-treatment with Salmeterol. Moreover, pre-treatment with Salmeterol inhibited the LPS-induced production of nitric oxide by 3-fold (Figure4.1E). Collectively, this data suggests that Salmeterol exerts anti-inflammatory properties by suppressing endotoxin stimulated release of inflammatory mediators in BV2 cells.

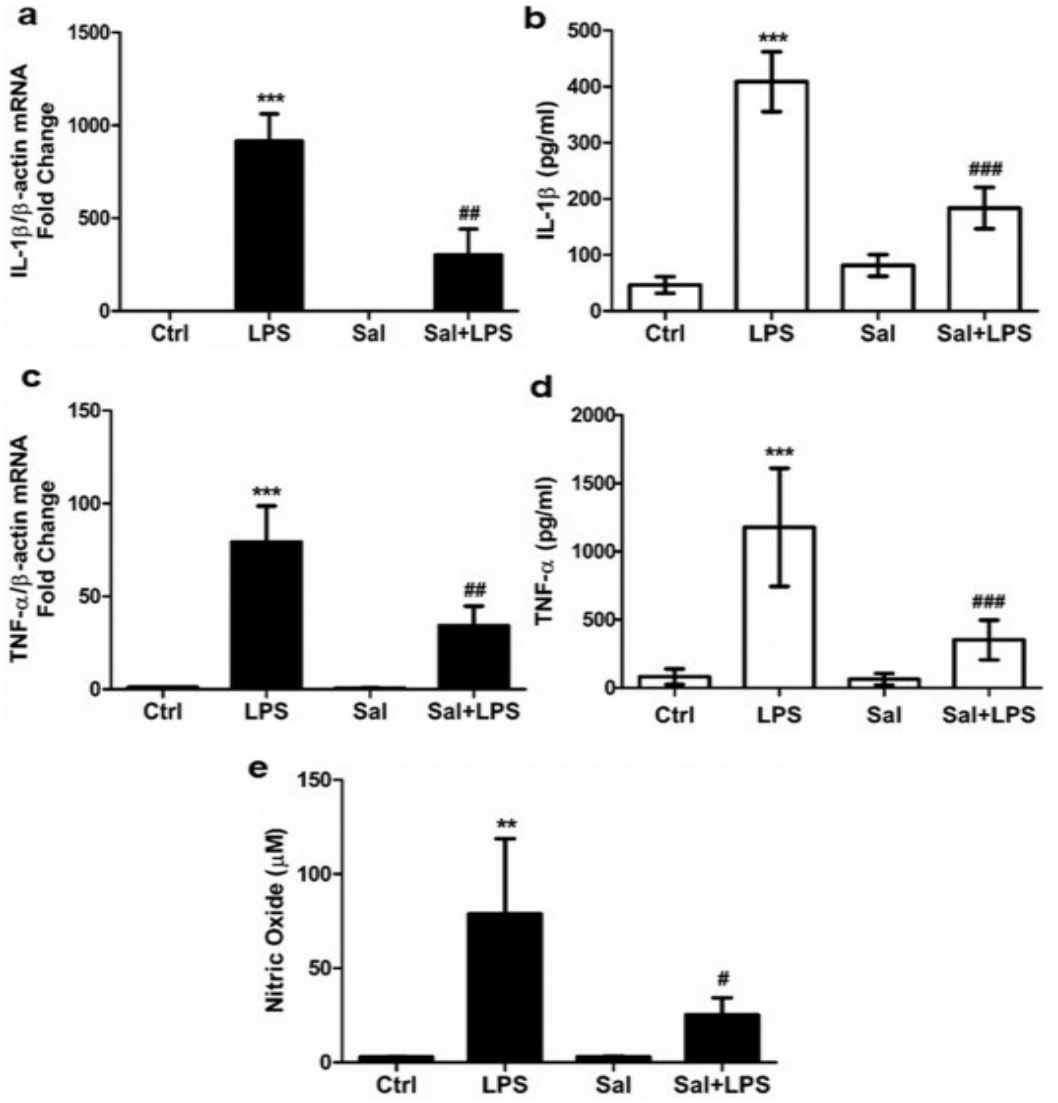


Figure 4.1: Effect of β2-AR agonist, Salmeterol on production of pro-inflammatory mediators.

BV2 cells were pre-treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with LPS (1 μg/ml) and the mRNA and protein levels of IL-1β (A and B) and TNF-α (C and D) in BV2 cell culture were measured. For mRNA analysis cells were stimulated with LPS for 6 h and the extracted RNA of cells was subjected to qRT-PCR analysis. The relative mRNA levels of TNF-α and IL-1β were normalized to β-actin. mRNA data expressed as fold change compared to control (A and C). For protein quantification, BV2 cells were pretreated with Salmeterol for 45 min, then stimulated with LPS for 24 h, and supernatant were collected and measured by ELISA kits. The level of nitric oxide (E) was measured by Griess reaction assay. Data represented as mean ± SD from independent experiments ($n = 4$). ***$p < .001$ and **$p < .01$ represents significant difference from control group and ###$p < .001$, ##$p < .01$ and #$p < .05$ represents significant difference from LPS-treated group.

4.3.2. Salmeterol inhibits activation of NF-κB pathway and its nuclear translocation in LPS-stimulated BV2 microglial cells

The LPS-induced expression of inflammatory mediators depends on the activation of TLRs and the initiation of the NF-κB signaling pathway (294). First, we sought to determine if Salmeterol inhibits the activation of the NF-κB pathway by performing immunofluorescence staining to image the nuclear translocation of NF-κB (p65) in BV2 cells treated with LPS and Salmeterol. **Figure 4.2A** demonstrates the immunofluorescence images captured by AxioImager.Z1 microscope. In Fig. 4.2B and C, the quantitative analysis of nuclear translocation of p65 shows increased nuclear fluorescence intensity (66%) and higher nuclear: cytoplasmic ratio in LPS-treated group compared to unstimulated BV2 cells. While pre-treatment with Salmeterol reduces the nuclear fluorescence intensity (43%) and nuclear: cytoplasmic ratio of p65 in response to LPS stimulation. Further, we have verified the inhibitory effects of Salmeterol on NF-κB pathway by immunoblotting analysis. **Figure 4.3** shows that Salmeterol significantly inhibited the activation of NF-κB signaling pathway by suppressing the degradation of IκBα (A), the phosphorylation of TAK1 (B), and the translocation of NF-κB (p65) from cytosol (C) to nucleus (D). The immunoblots showing this quantification are shown in Fig. 4.3E. These results demonstrate that Salmeterol exerts anti-inflammatory effects via inhibition of NF-κB signaling pathway and nuclear translocation of NF-κB in LPS-activated BV2 microglial cells.

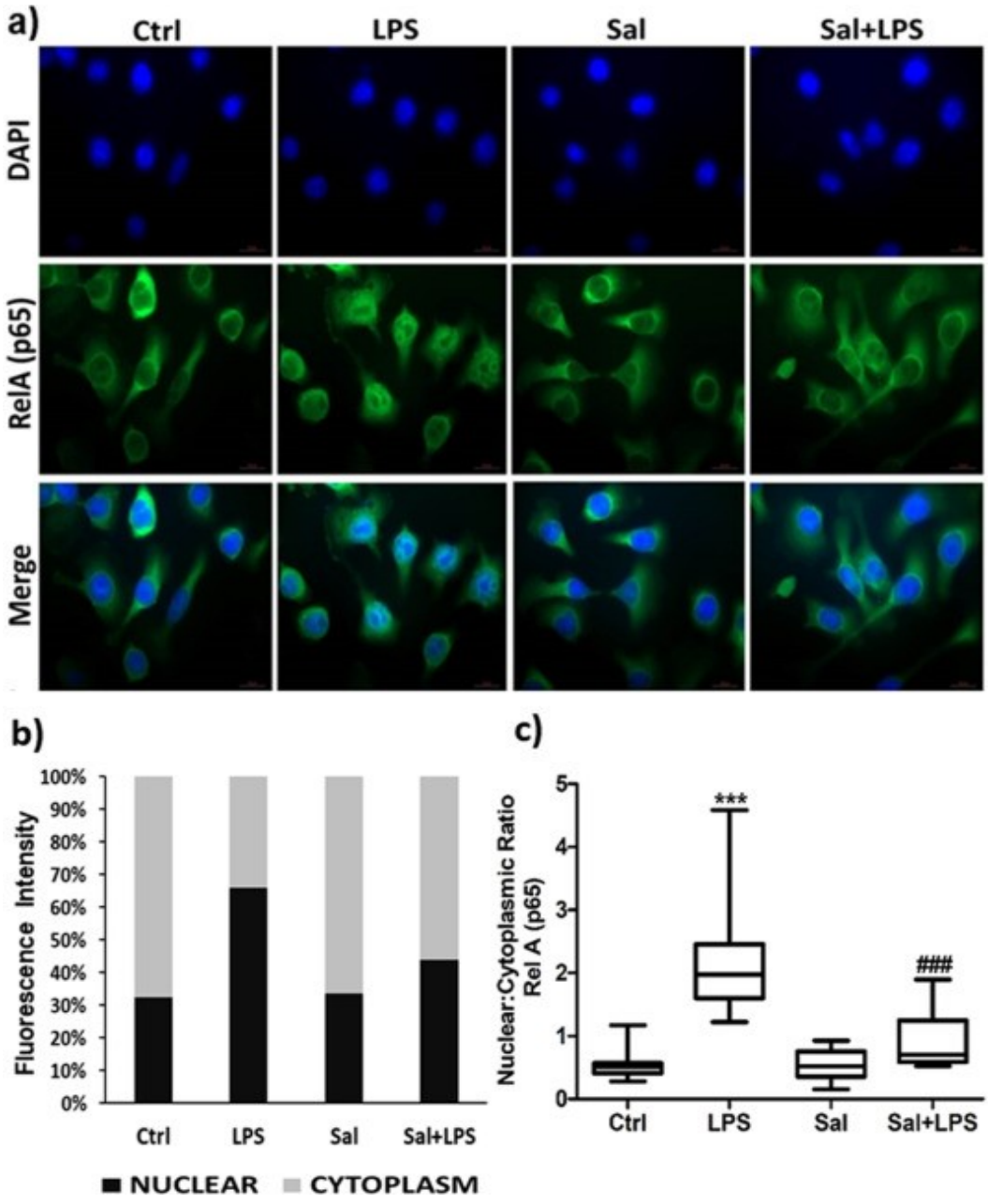


Figure 4. 2: Effect of Salmeterol on LPS-mediated nuclear translocation of NF-κB in BV2 cells.

(A) Immunofluorescence staining demonstrates Rel A (p65) nuclear translocation-Scale bar, 20 µm. Using ImageJ nuclear and cytoplasmic ROI (region of interest) were applied to measure the fluorescence intensities of Rel A (p65). (B) The stacked bar graph represent percentage distribution of average nuclear and cytoplasmic fluorescence intensities in each experimental group. (C) The box-plot depict the quantification of the nuclear: cytoplasmic ratios of p65 fluorescence intensities. Data points collected from analysis of four separate images of each experimental group.

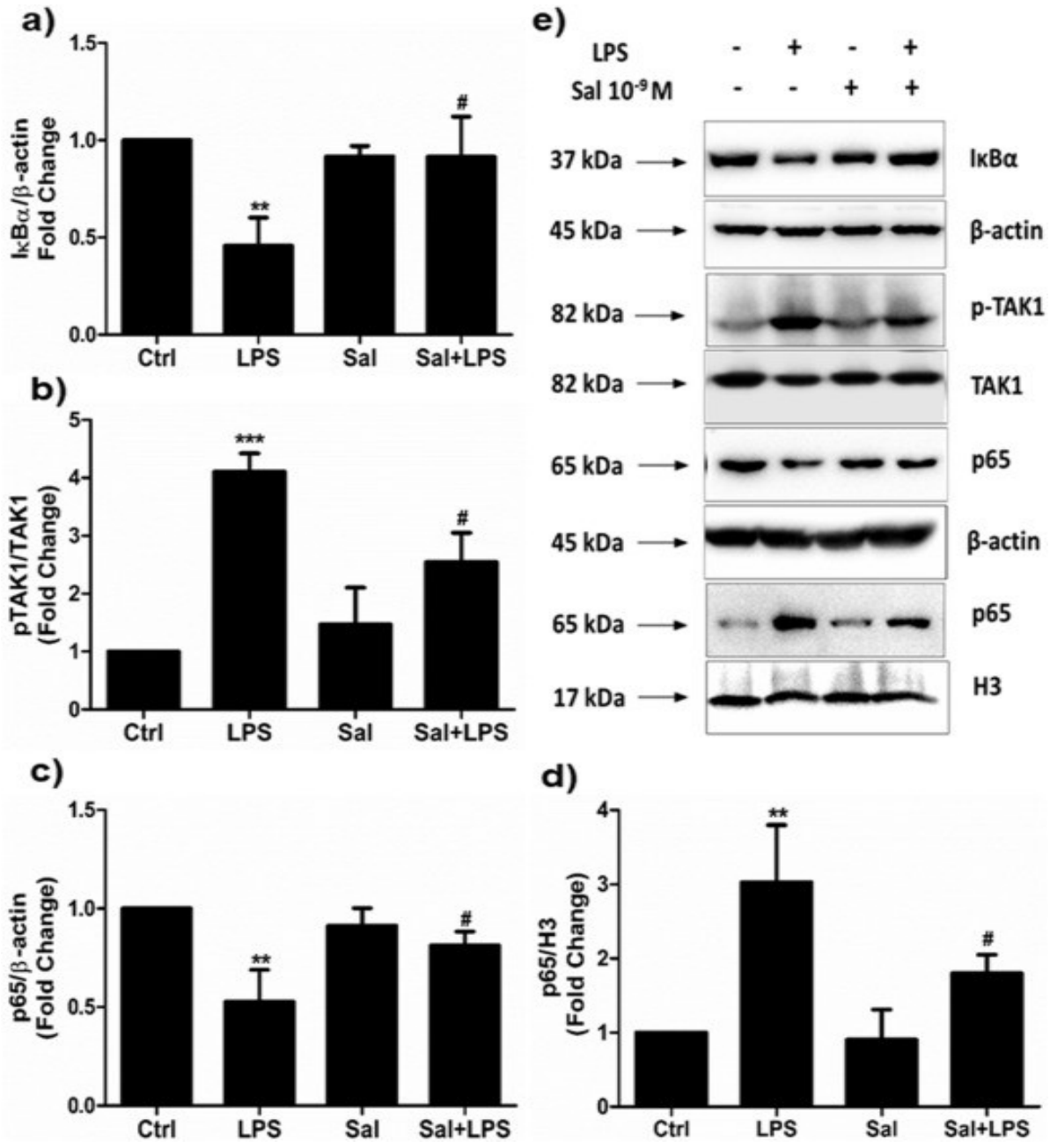


Figure 4. 3: Effect of Salmeterol on LPS-mediated NF-κB activation.

Cells were pre-incubated with Salmeterol (10^{-9} M) for 45 min and stimulated with LPS for 1 h. Representative western blots (E) and quantitative analysis show effect of Salmeterol on IκBα degradation (A), and TAK1 phosphorylation (B), and p65 NF-κB expression in cytosol (C) and nuclear (D) fractions of BV2 cells. Bar graphs represent fold change compared to control. Data represents mean ± SD of three independent experiments. ***p < .001 and **p < .01 indicates significant difference from control group and ##p < .01 and #p < .05 indicates significant difference from LPS-treated group.

4.3.3. Expression of β-arrestin2 is required for the anti-inflammatory effect of Salmeterol

β-arrestins are well described as critical regulators of inflammatory response and their expression alters in response to inflammatory stimuli (295). To examine the role of β-arrestin2 in regulating β2-AR/Sal mediated anti-inflammatory effects in LPS-stimulated BV2 cells, we first checked the effect of exposure to Salmeterol on the expression of β-arrestin2 in BV2 cells. Here, we have found **(Figure 4.4)** that β-arrestin2 expression was reduced in BV2 cells in response to LPS stimulation, and this reduction was significantly abrogated by Salmeterol pre-treatment. These results demonstrate that activation of β2-AR with Salmeterol prevents the inhibition of protein levels of β-arrestin2 when BV2 cell were stimulated by LPS, and suggests that this level of expression may be crucial for the anti-inflammatory effect of Salmeterol. In order to check the role of β-arrestin2 in regulating the anti-inflammatory response of Salmeterol in response to LPS, we next reduced the β-arrestin2 expression using siRNA against β-arrestin2 (Si-β-arrestin2).

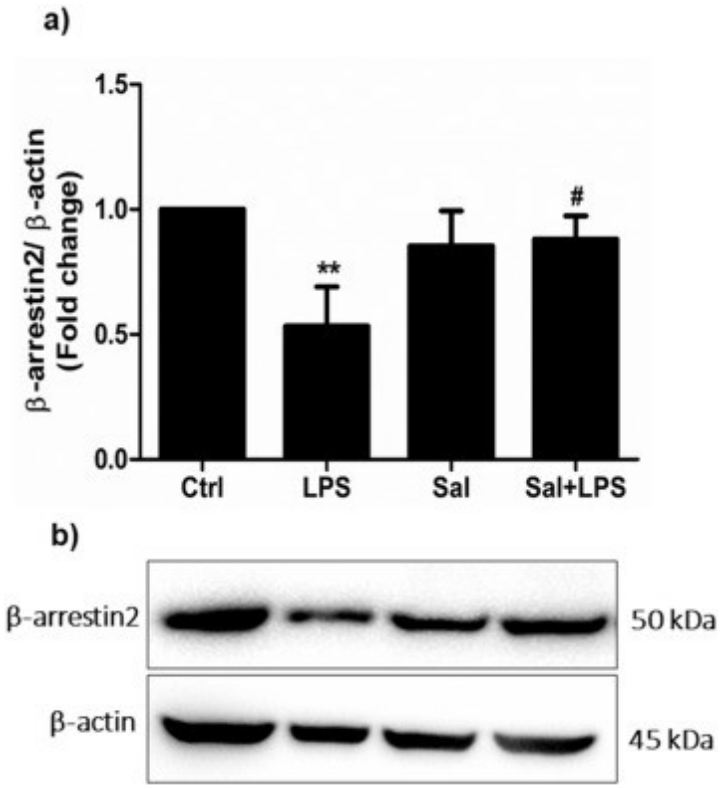


Figure 4. 4: Salmeterol prevents the inhibition of expression of β-arrestin2 by LPS in stimulated BV2 cells.

Cells were pre-treated with Salmeterol (10^{-9} M) for 45 min and stimulated with LPS for 24 h. Representative immunoblots (B) and quantitative analysis (A) of β-arrestin2 in BV2 cells. Data represents mean ± SD of independent experiments ($n = 3$) and **$p < .01$ represents the significant difference from control group and #$p < .05$ indicate the significant difference from LPS-treated group.

Figure 4.5A, shows that while the BV2 cells transfected with scrambled siRNA (Si control) had no effect on β-arrestin2 expression, BV2 cells transfected with siRNA to β-arrestin2 (Si-β-arrestin2) showed a 5-fold reduced expression of the β-arrestin2 protein.

Furthermore, the cells exposed to the scrambled siRNA (Si control) showed significant reduction in the production and release of the LPS-induced pro-inflammatory mediators NO (B), TNF-α protein (C) and mRNA (D), and IL-1β protein (E) and mRNA (F) upon treatment with Salmeterol. In contrast, the silencing of Arrb2 (Si-β-arrestin2) in BV2 cells significantly abrogates the anti-inflammatory effects of Salmeterol (Fig. 4.5B-F). These results suggest that silencing of Arrb2 gene abolished the inhibitory effects of Salmeterol on inflammatory mediator production and demonstrates that the anti-inflammatory effects of β2-AR activation in response to inflammatory stimuli depend on the expression of β-arrestin2.

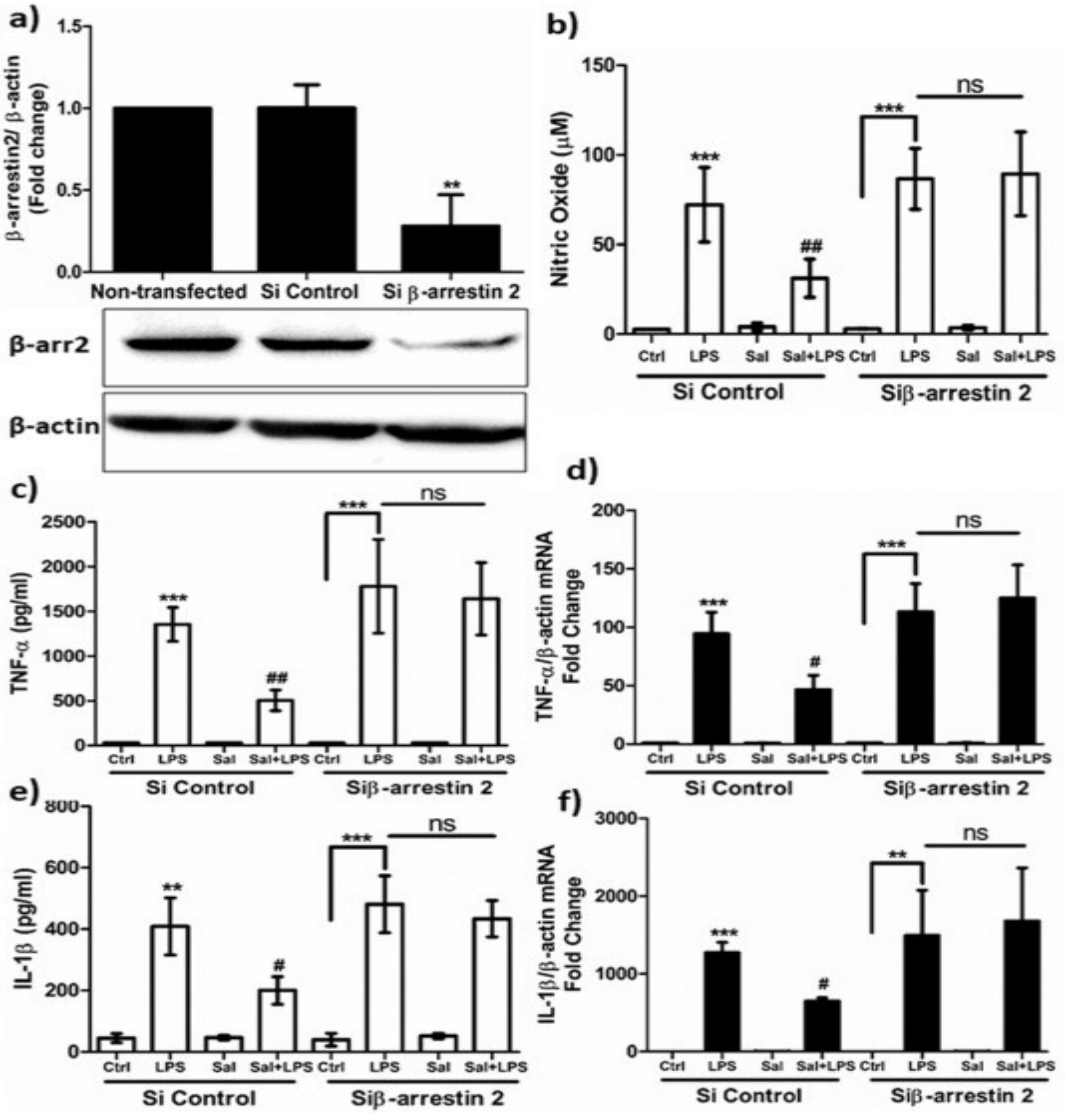


Figure 4. 5: Anti-inflammatory effects of Salmeterol is β-arrestin2 dependent.

To inhibit β-arrestin2 expression, BV2 cells were transfected with β-arrestin2 siRNA (Siβ-arrestin2) and scrambled siRNA (SiControl) for 24 h and then treated with Salmeterol and followed by stimulated with LPS for 24 h. The expression of β-arrestin2 proteins in SiControl and Siβ-arrestin2 transfected BV2 cells was measured by Western blot (A). The level of nitric oxide was measured by Griess reaction (B), while levels of protein and mRNA levels of TNF-α (C and D) IL-1β (E and F) in BV2 cell culture were measured by ELISA and qRT-PCR, respectively. For protein quantification, BV2 cells were pretreated with Salmeterol for 45 min, then stimulated with LPS for 24 h, and supernatant were collected. For mRNA analysis cells were stimulated with LPS for 6 h and the extracted RNA of cells was subjected to qRT-PCR analysis. The relative mRNA levels of TNF-α and IL-1β were normalized to β-actin. mRNA data expressed as fold change compared to control (A and C). Data represented as mean ± SD from independent experiments (n = 4). ***p < .001 and **p < .01 represents significant difference from control group and ##p < .01 and #p < .05 represents significant difference from LPS-treated group.

4.3.4. Inhibition of the TLR4–induced activation of the NF-κB signaling pathway by Salmeterol is β-arrestin2 dependent

We next examined if the inhibition of β-arrestin2 expression had any effect on the Salmeterol-dependent inhibition of the NF-κB signaling pathway activated by LPS. **Figure 4.6** shows that abrogation of β-arrestin2 expression by siRNA abolishes the inhibitory effects of Salmeterol and restores IκBα degradation (A), phosphorylation of TAK1 (B), and translocation of p65-NF-κB from cytosol (C) to nucleus (D) induced by LPS activation. Fig. 4.6E shows the corresponding immunoblots representing these results. Collectively, results from 4.3.3 and 4.3.4 sections suggest the expression of β-arrestin2 is required to elicit the anti-inflammatory effects of β2-AR activation with Salmeterol.

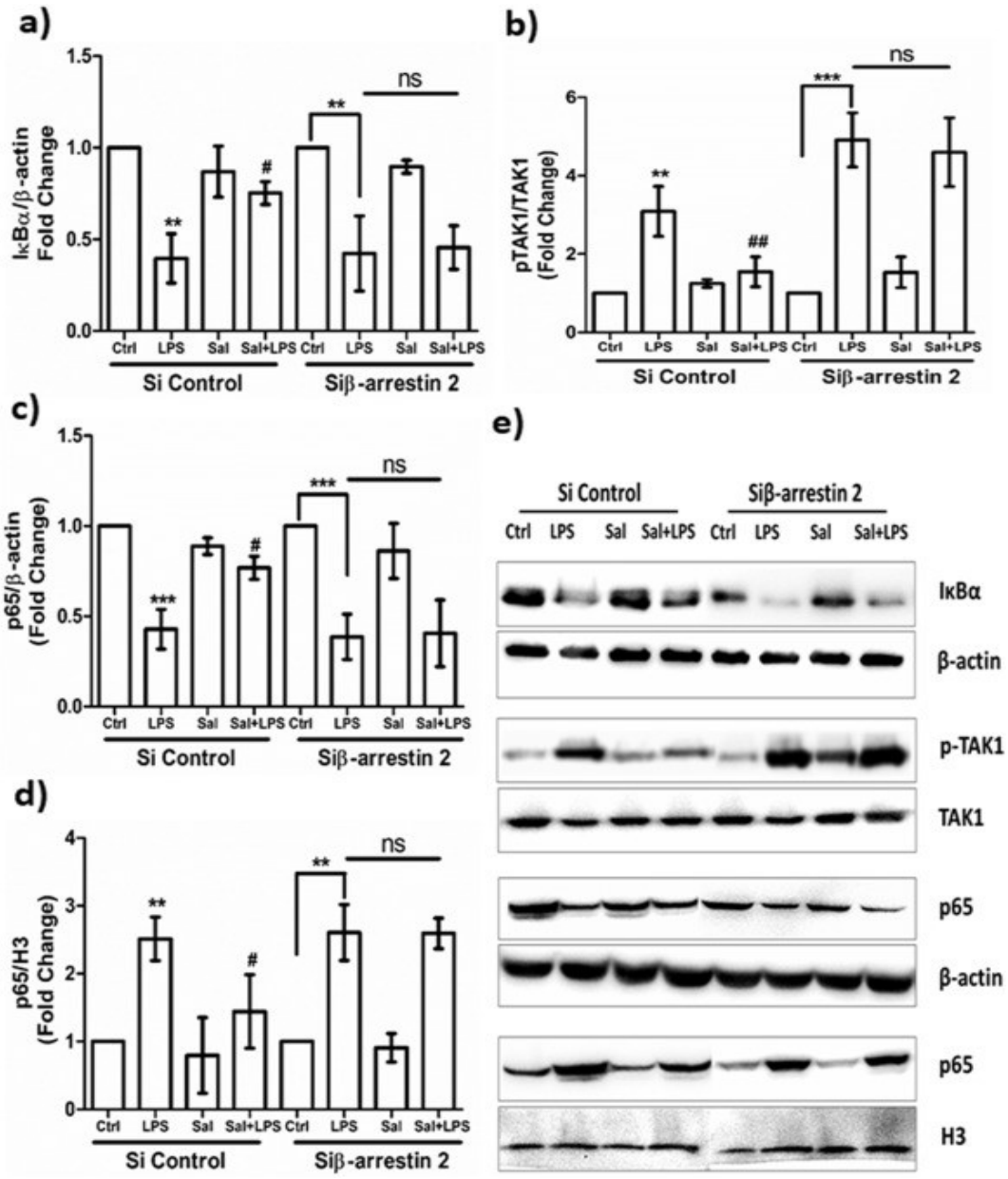


Figure 4. 6: Inhibition of NF-κB signaling pathway by Salmeterol is dependent on β-arrestin2.

Cells were transfected with β-arrestin2 siRNA (Siβ-arrestin2) and scrambled siRNA (SiControl) for 24 h and then treated with Salmeterol and followed by stimulated with LPS for 1 h. Cells were pre-incubated with Salmeterol (10^{-9} M) for 45 min and stimulated with LPS for 1 h. Representative western blots (E) and quantitative analysis show effect of Salmeterol on IκBα degradation (A), and TAK1 phosphorylation (B), and p65 NF-κB expression in cytosol (C) and nuclear (D) fractions of BV2 cells. Bar graphs represent fold change compared to control. Data represents mean ± SD of three independent experiments. ***p < .001 and **p < .01 indicates significant difference from control group and ##p < .01 and #p < .05 indicates significant difference from LPS-treated group.

4.3.5. Salmeterol suppresses LPS-induced NF-κB activation and TAK1 phosphorylation by enhancing the TAB1-β-arrestin2 interaction

Since the downstream activation of LPS-induced NF-κB activation requires the formation of a molecular complex of signaling molecules TAK1 and TAB1, we first examined if β-arrestin2 co-localizes with TAB1 in BV2 cells by immunocytochemistry. We found that β-arrestin2 shows co-localization with TAB1 **(Figure 4.7A)** within the cytoplasm of unstimulated BV2 cells. We next performed a co-immunoprecipitation assay to examine the association of TAK1 and TAB1 with β-arrestin2. In Fig. 7B and C, the immunoprecipitation assay showed association of TAB1 with β-arrestin2 in unstimulated BV2 cells which was reduced in response to LPS. Pre-treatment with Salmeterol restored the binding of TAB1 with β-arrestin2 in LPS-stimulated BV2 cells (Fig. 4.7C). On the other hand, LPS stimulation increased level of TAK1/TAB1 binding, which was then significantly reduced by Salmeterol treatment (Fig. 4.7B and D). Taken together, these results indicate the anti-inflammatory and protective effects of agonist activated β2-AR stimulation function via the conversion of a pro-inflammatory TAK1/TAB1 association to an anti-inflammatory β-arr2/TAB1 association.

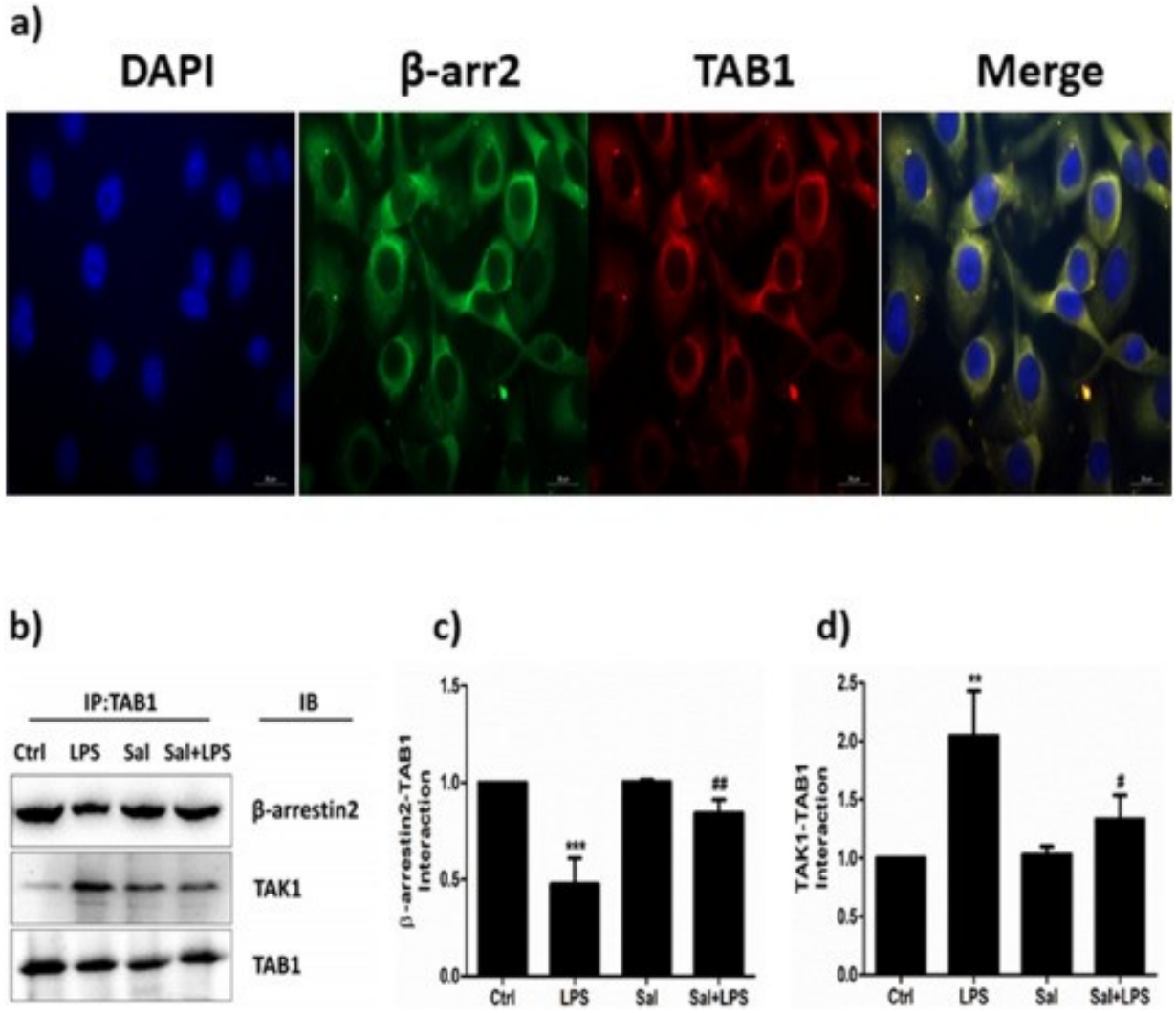


Figure 4. 7: β-arrestin2 binds to TAB1 and inhibits TAB1/TAK1 binding.

Immunocytochemistry analysis represents co-localization of β-arrestin2 and TAB1 in the cytoplasm of BV2 cells (A). Interaction of TAB1 with β-arrestin2 and TAK1 was determined by co-immunoprecipitation assay in BV2 cells pre-treated with Salmeterol and stimulated with LPS for 2 h (B). Graphs represent quantitative analysis of β-arrestin2-TAB1 interaction (C) and TAK1-TAB1 interaction (D). Data represents mean ± SD of three independent experiments. ***$p < .001$ and **$p < .01$ indicates significant difference from control group and ##$p < .01$ and #$p < .05$ indicates significant difference from LPS-treated group.

4.4. DISCUSSION

Neuroinflammation associated with PD pathology is one of the major hallmarks of neurodegeneration and disease progression (155,296,297). Microglia are the key regulators of immune responses within the SN and activated microglia contribute to DA-neuronal damage. Observations which demonstrated that inflammation in the SN plays a role in the progression of PD have led many investigators to initially consider the potential use of anti-inflammatory drugs for the treatment of PD. Our previous studies have shown that the neuropathological phenotype observed in PD brain is influenced by neuroinflammation and that Salmeterol, a long-acting β2-AR agonist, can effectively serve as a therapeutic treatment for PD by inhibiting microglia-mediated inflammatory responses. We have shown that Salmeterol has anti-inflammatory and DA-neuroprotective activities, even at low doses (10^{-9} M), and pre-treatment with Salmeterol protects DA neurons against LPS and 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) induced toxicity in both *in vitro* and *in vivo* models of PD (20). One of the key findings of our current study is that Salmeterol exhibits its β-arrestin2-dependent anti-inflammatory effects on LPS-activated BV2 microglial cells via inhibition of NF-κB pathway. Our results show that treatment with Salmeterol suppress the production of pro-inflammatory mediators TNF-α, IL-1β and NO, and these anti-inflammatory effects were abolished after silencing of β-arrestin2 (Si-β-arrestin2) using siRNA. Further analysis shows that Salmeterol functions by enhancing the expression of β-arrestin2 leading to the binding of β-arrestin2 to TAB1, inhibition of the TAK1/TAB1 interaction, and thereby suppression of NF-κB signaling pathway **(Figure 4.8)**.

Previously, several other investigators have shown that β2-AR agonists can play a significant role in neuroprotection in neurodegenerative conditions. Chai et al. showed that the β2-AR activation enhances hippocampal neurogenesis, ameliorates memory deficits, increased dendritic branching

and spine density in mouse model of Alzheimer's disease (236). Mittal et al. have found that a short-acting β2-AR agonist, Salbutamol, reduces the expression of α-synuclein (a pathological hallmark of PD) in different experimental models of PD, and patients regularly using this asthma drug have a reduced risk of developing PD (237). In a mouse model of Down syndrome, Formoterol, a long-acting β2-AR agonist, causes significant improvement in synaptic density and cognitive functions (238). Our current results show that β2-AR agonists can also have strong neuroprotective effects through inhibition of inflammatory signaling in microglial cells responsible for the neuroinflammation leading to dopaminergic cell death. Collectively, these studies suggest that β2-AR agonists not only protect neurons against degeneration but also have anti-inflammatory effects, and therefore, hold significant promise for the treatments of a wide variety of neurodegenerative conditions including PD (239). Consistent with our findings, catecholamines such as epinephrine and norepinephrine (NE), the endogenous ligands for β2-AR, have also been found to have neuroprotective properties against inflammatory damage in CNS disease pathology (224,231,249,298,299). Gyoneva et al. have found that NE controls microglial motility partially through its interactions with β2-AR during pathogenic conditions (250). In a different study, it was found that NE protects cortical neurons against microglia mediated inflammation while decreased levels of NE enhance microglial activation (251).

Interestingly, activation of β2-AR in astrocytes modulates TNF-α induced inflammatory gene expression in vitro and in vivo. This study also demonstrated increased expression of β2-AR in glial cells in response to neuronal injury (253). Taken together, these studies suggest that β2-AR may provide a therapeutic target for regulation of glial cell function and the inflammatory response in the brain. Additionally, it has also been shown that NE acting on β2-AR enhances the expression of the anti-inflammatory and neurotrophic cytokine IL-10 in the brain (256), and previous results

by ourselves and others have shown that IL-10 is a potent neuroprotective cytokine in PD (111,112,182,300,301). Collectively, these studies suggest a role of β2-AR in the regulation of CNS inflammation and neuroprotection against neurodegenerative conditions. Microglia serve as the first line of defense against pathogen and injury in brain, and an important contributor to neurodegeneration (176). During neuroinflammatory conditions microglia produces wide range of inflammatory cytokines including TNF-α, IL-1β, IL-6 and NO (302). It is proposed that degenerating DA-neurons release toxic factors that activate microglia, which in turn attract additional activated microglia into the SN. Collectively, these activated microglia and damaged neurons form a vicious cycle that leads to chronic inflammation and extensive DA neurodegeneration over time leading to the progression of PD (33,173). Microglial activation and production of pro-inflammatory cytokines is regulated by NF-κB signaling and inhibition of NF-κB activity is neuroprotective during chronic inflammatory conditions in PD (124,168,303). Therefore, targeting NF-κB activation in microglial cell (microglia-mediated neuroinflammation) may serve as a promising therapy for neurodegenerative diseases (304).

We have also found that Salmeterol suppress pro-inflammatory mediators in LPS-stimulated murine macrophages via inhibition of the NF-κB pathway (260), suggesting that β2-AR agonists may have a more universal role in controlling chronic inflammatory conditions through their inhibition of NF-κB in innate immune cell responses. Activation of β2-AR by receptor agonists initiate intracellular signaling pathways that function either via G proteins or through β-arrestin 1 or 2 (305). β-arrestin2 has been previously reported as a negative regulator of inflammation and to also play an important role in internalization and desensitization of GPCR (306,307). Our results suggest that the β-arrestin2 pathway is the major pathway of inhibition for inflammatory responses in microglial cells. In a study similar to ours, it has been shown that β2-AR negatively regulates

NF-κB activation and stabilizes NF-κB/IκB complex via β-arrestin2 in LPS-activated murine macrophages (252). β-arrestin2 has been shown as a novel regulator of IκB stability via the direct interaction of β-arrestin2 and IκB in HEK293 cells (265). In addition, Fluoxetine, a small pharmacological molecule used as an anti-depressant, has been shown to exert anti-inflammatory effects on LPS-stimulated microglial cells via the β-arrestin2 regulated pathway through the inhibition of TAK1 phosphorylation and NF-κB activation. Fluoxetine also enhances the expression of β-arrestin2 and stabilizes its association with TAB1 (308). Similarly, Feng et al. have also shown neuroprotective effects of β-arrestin2 via endogenous opioid arrest in inflammatory microglial cells (261). Taken together, it appears that increased expression and activation of β-arrestin2 in microglial cells leads to enhancement of β-arrestin2/TAB1 binding and destruction of the TAK1/TAB1 binding complex required for activation of the NF-κB pathway and pro-inflammatory mediator production.

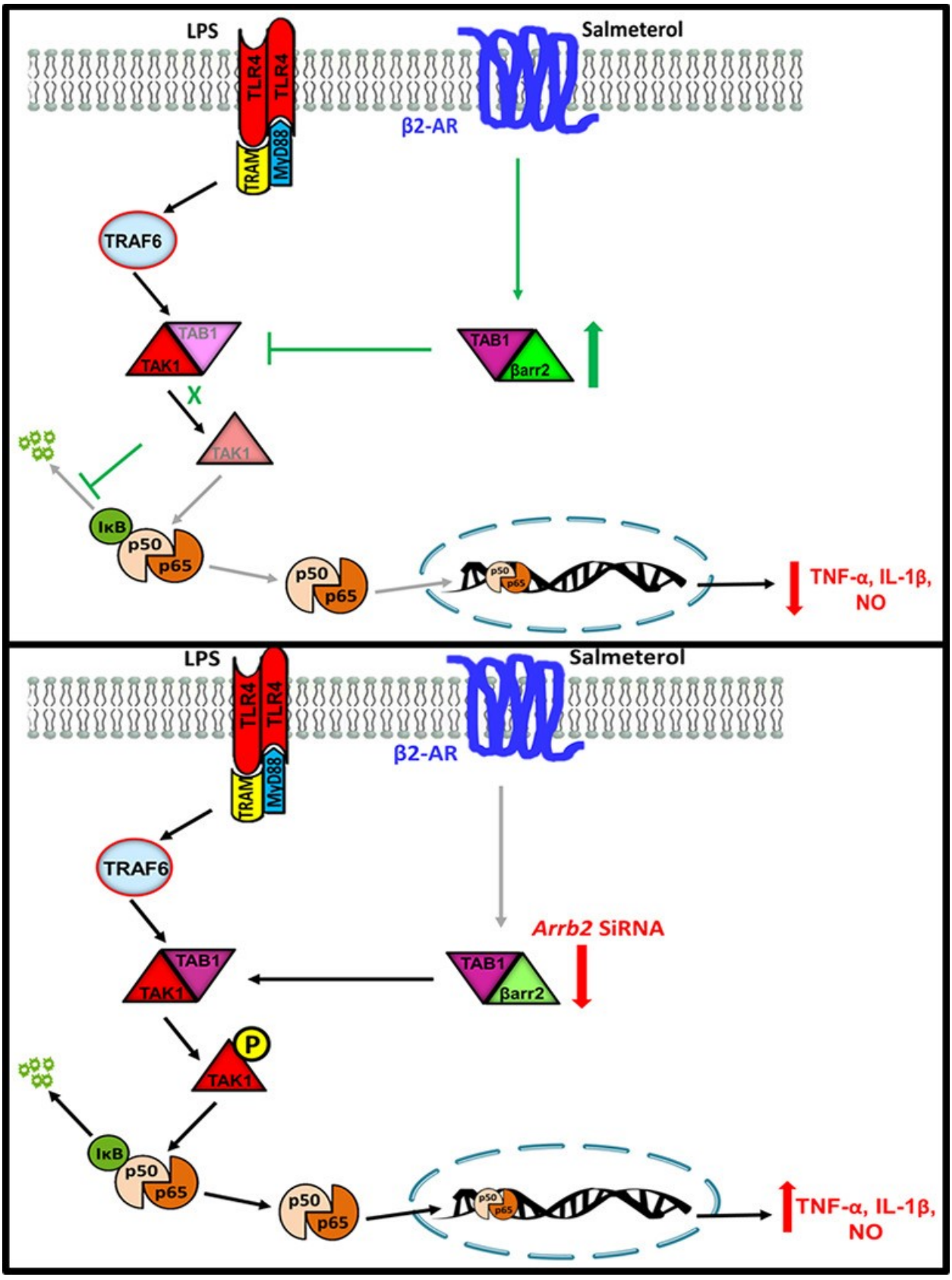

LPS
TLR4
TLR4
TRAM
MyD88
Salmeterol
β2-AR
TRAF6
TAB1
TAK1
TAB1
βarr2
X
TAK1
IκB
p50
p65
p50
p65
p50
p65
TNF-α, IL-1β,
NO
LPS
TLR4
TLR4
TRAM
MyD88
Salmeterol
β2-AR
TRAF6
Arrb2 SiRNA
TAB1
TAK1
TAB1
βarr2
P
TAK1
IκB
p50
p65
p50
p65
p50
p65
TNF-α, IL-1β,
NO

Figure 4. 8: Schematic diagram depicting anti-inflammatory effects of Salmeterol via β2-AR/βarrestin2 signaling in BV2 cells.

Stimulation of BV2 with LPS triggers TLR4 signaling pathway through TRAF6 and TAK1, leading to the activation of NF-κB and expression of pro-inflammatory genes (upper panel). However, activation of microglial β2-AR with Salmeterol enhances the expression of β-arrestin2 and leads to its increased interaction with TAB1, *preventing* formation of the TAB1/TAK1 complex, and *inhibiting* the activation of NF-κB. The lower panel shows lack of β-arrestin2 (knockdown using siRNA against *Arrb2*) in BV2 cells abrogates the anti-inflammatory effects of the Sal/β2-AR/β-arrestin2 pathway and leads to normal activation of BV2 microglial cells in response to inflammatory stimuli.

Currently, there are several synthetic β2-AR agonists available and several are currently being used as first-line medications in the treatment of chronic obstructive pulmonary disease (COPD) and bronchial asthma (291,309). Our current and previous studies suggest that the neuropathological phenotype observed in the PD brain is influenced by neuroinflammation and that Salmeterol, a β2-AR agonist, can effectively serve as a therapeutic treatment for PD by inhibiting microglia-mediated inflammatory responses (20,23). We also have found that Salmeterol functions to inhibit innate pro-inflammatory response in both murine macrophages and microglia through its inhibition of the NF-κB signaling pathways via a β-arrestin2/TAK1 dependent mechanism (260). These studies show that the mechanism by which agonist activated β2-AR inhibits pro-inflammatory responses is by enhancing β-arrestin2 expression, leading to increased β-arrestin2/ TAB1 binding, inhibiting the formation of the TAB1/TAK1 complex and the subsequent phosphorylation of TAK1, which in turn attenuates phosphorylation of the IκB/NF-κB complex, preventing the release of the NF-κB p50/p65 complex needed for pro-inflammatory gene activation. However, how the activation of β2-AR enhances expression of β-arrestin2 leading to its association with TAB1 is not well explained, and still remains to be determined. The recent evidence and results from our study suggest that β-arrestin2 expression is required for anti-inflammatory effects of β2-AR/Sal signaling by disrupting the formation of the TAK1-TAB1 molecular complex by inhibiting TAK1/TAB1 binding **(Figure 4.8)**. Collectively, these studies suggest Salmeterol could serve as a novel therapeutic for targeting microglia and macrophage-mediated chronic inflammatory conditions including PD.

CHAPTER 5

Mechanism underlying β2-AR agonist-mediated phenotypic conversion of LPS-activated microglial cells

This chapter has been modified from the published article:

Sharma, M., Arbabzada, N., Flood, P.M. **Mechanism underlying β2-AR agonist-mediated phenotypic conversion of LPS-activated microglial cells**. (*J. Neuroimmunol*. 332:37-48).

5.1. ABSTRACT

Fundamentally, microglia have two activation states, a pro-inflammatory neurotoxic (M1) and an anti-inflammatory neuroprotective (M2) phenotype, and their conversion from M1-like to M2-like microglia may provide therapeutic benefits to prevent neuronal loss in neurodegenerative diseases such as PD. Previously, we showed that Salmeterol, a long-acting β2-adrenergic receptor (β2-AR) agonist, has neuroprotective effects in PD models *in vitro* and *in vivo* through the β-arrestin2-dependent inhibition of pro-inflammatory M1-type mediator production. In the present study, we explored whether Salmeterol can mediate phenotypic conversion in LPS-activated murine microglial BV2 cells from the neurotoxic M1-like to a neuroprotective M2-like phenotype. Salmeterol inhibited the production of LPS-induced mediators of the pro-inflammatory M1 phenotype such as tumor necrosis factor-α (TNF-α), IL-(interleukin) 18, IL-6, chemokines (CCL2, CCL3, CCL4) and reactive oxygen species from BV2 cells. Conversely, treatment with Salmeterol and other β2-AR agonists robustly enhanced the production of the M2 cytokine IL-10 from LPS-activated microglia. In addition, Salmeterol upregulates the expression of arginase-1 and CXCL14. Furthermore, using siRNA approach we found that silencing of the transcription factor *Creb* abrogates the Salmeterol-mediated production of IL-10 in LPS-activated BV2 cells, but silencing of β-arrestin2 with *Arrb2* siRNA did not. Therefore, our data shows conversion from an M1- to M2-like phenotype in LPS-activated microglia by β2-AR agonists involves activation of the classical cAMP/PKA/CREB as well as the PI3K and p38 MAPK signaling pathways, and provides a novel therapeutic approach targeting microglial cell activation and inducing their phenotypic conversion in the treatment of neuroinflammatory diseases such as PD.

5.2. INTRODUCTION

Inflammation is a highly regulated process which involves multiple steps including the migration of immune cells, production of inflammatory mediators, a destructive phase that results in clearance of infection/debris and eventually the conversion into a regenerative stage that results in tissue repair. In chronic inflammation, this conversion to a regenerative stage is often delayed or absent, resulting in continuous tissue destruction and pathology. Similar to peripheral macrophages, microglial cells respond to endogenous stimuli in both a protective and pathogenic manner and functionally serve as the resident macrophages of the CNS. Activation of microglia and their subsequent production of inflammatory mediators have been shown in pathological studies of many chronic neurodegenerative diseases including Alzheimer's disease (310), PD (35), multiple sclerosis (311), stroke (312), neuropathic pain (313) and several others (314), with little to no evidence of conversion to a tissue regenerative state (315).

PD is a neurological movement disorder caused by degeneration of dopaminergic neurons which results into impaired motor basal ganglia circuitry in the mid brain region (316). The pathological hallmarks of PD are the presence of Lewy bodies containing insoluble α-synuclein protein aggregates (317), as well as neuroinflammation and activated glial cells (168). Dopaminergic neurodegeneration and the accumulation of protein aggregates convert microglia into an activated state, and this process results in microglial priming (176). These primed or activated microglia are then more susceptible to a secondary inflammatory stimulus which further leads to a pathological inflammatory response, characterized by the production of several inflammatory mediators which contribute to neuronal damage (176). It has been suggested that the inhibition of the pro-inflammatory microglial response and an enhanced anti-inflammatory response during PD progression is required for halting and potentially reversing dopaminergic cell loss, and this

immunologic conversion leads to resolved inflammation within the substantia nigra (SN) (318,319). An increased number of activated microglia in SN region is a hallmark of neuroinflammation and in the pathogenesis of PD (17). Activated microglia in CNS milieu can perform a variety of functions with diverse phenotypes. These phenotypes broadly divided into two main states: a classically activated M1-like phenotype with cytotoxic/neurotoxic properties and an alternate activated M2-like phenotype with regenerative and neuroprotective properties (318). M1 or classically activated microglia produce pro-inflammatory mediators such as TNF-α (tumor necrosis factor-α), IL (interleukin)-6, IL-1β, and IL-18, CXCL10/IP-10 (C-X-C motif chemokine ligand 10/interferon gamma-induced protein 10), MCP-1/CCL2 (monocyte chemoattractant protein1/C-C motif chemokine ligand 2), NO (nitric oxide) and ROS (reactive oxygen species). In contrast, the protective phenotype of microglia characterized by the expression of an anti-inflammatory phenotype, including the expression of arginase-1, IL-10 and CXCL14 (320).

Studies targeting neuroinflammation represent a novel therapeutic approach for neurodegenerative conditions such as PD (319,321,322), and previously we have established several therapies targeting neuroinflammation and neurodegeneration in an animal model of PD. These therapies include D-morphinan-related compounds (119), anti-inflammatory cytokines such as TGF-β (transforming growth factor-beta) (116) and IL-10 (111,300), and small molecule inhibitors targeting IKK (inhibitor of kappa B (IκB) kinase) (124) and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (126). One such highly effective anti-inflammatory approach is the use of therapeutic compounds that activate the β2-AR (20,260,263,323). A number of different types of brain cells, including microglia, astroglia, and neurons express the β2-ARs (283), which is one of the main targets of the regulatory effects for noradrenaline or norepinephrine (NE) in PD (324). Previously, we have found that the long-acting β2-AR agonist Salmeterol showed

neuroprotective properties in both neurotoxin-based MPTP and inflammatory LPS model of PD (20). Salmeterol is a long-acting β2-AR and an active ingredient in Advair[@] which is a FDA-approved drug and prescribed as a bronchodilator for the treatment of asthma and chronic obstructive pulmonary disorder (COPD) (291). Further, we found that Salmeterol shows anti-inflammatory effects by enhancing the binding between β-arrestin2 and TAB1 (TAK1 (transforming growth factor-beta-activated-kinase1) binding protein) and reducing TAK1/TAB1 interaction, thereby suppressing the activation of NF-κB (263).

Previous results show that treatment with the anti-inflammatory cytokine IL-10 on neuron-glia culture suppressed LPS-induced degeneration of DA neurons which shows the role of IL-10 in regulating neuro-inflammation in PD (111,300). In addition, both the endogenous agonist (NE) and pharmacological agonist of β2-AR have been found to alter the LPS-activated M1-like phenotype of macrophages by enhancing the expression of Arg-1 and IL-10 (325–327). Here, in the current study we reveal that the long-acting β2-AR agonist Salmeterol has potent anti-inflammatory effects and suppresses the production of pro-inflammatory cytokines and chemokines which are the characteristic markers of classically activated M1 microglia, while concurrently induces the production of anti-inflammatory cytokine IL-10 in LPS-activated BV2 cells. In addition, we have found that β2-AR stimulation by Salmeterol enhances expression of arginase-1 (Arg-1) and CXCL14, thereby converting the inflammatory M1-like microglia to an M2-like microglial phenotype. Inhibition of classical signaling pathway of β2-AR via silencing *CREB* by siRNA and inhibiting protein kinase A (PKA) abrogates Salmeterol-mediated production of IL-10. In contrast, inhibition of β-arrestin2 via siRNA did not affect the Salmeterol-mediated production of IL-10. These immunomodulatory effects of Salmeterol may serve as a potential therapeutic avenue for neuroinflammatory and neurodegenerative diseases including PD.

5.3. RESULTS

5.3.1. β2-AR agonist stimulation suppresses TLR-induced M1 markers of microglial activation. Many studies have shown that pharmacological and endogenous β2-AR agonists can suppress the LPS-induced production of TNF-α (328,329). Previously, we have shown that β2-AR agonist Salmeterol inhibited the activation of the LPS-induced NF-κB signaling pathway, which is a key transcription factor for the production of a number of pro-inflammatory mediators (20,263). Here, we wished to determine the effect of Salmeterol on the expression of microglial activation markers. BV2 cells were treated with long-acting β2-AR agonist Salmeterol and then activated with inflammatory stimulus LPS. The intracellular production and release of TNF-α was measured by flow cytometry, qPCR and ELISA. The intracellular staining of TNF-α was enhanced with LPS stimulation and suppressed by Salmeterol treatment **(Figure 5.1A)**. The quantitative analysis shown increased number of TNF-α^+ cells in LPS-stimulated group while the number of TNF-α^+ cells was significantly suppressed (by ~ 3-fold; *$p<0.001$*) in LPS+Salmeterol group (Figure 5.1B). Similarly, the production of TNF-α in culture supernatant was also significantly suppressed (by ~4-fold; *$p<0.001$*) in the LPS+Salmeterol group when compared to LPS-alone group (Figure 5.1C). Furthermore, upon stimulation with endotoxin, microglia secrete a variety of cytokines and chemokines other than TNF-α that are unique to the M1 inflammatory phenotype. We examined the effect of Salmeterol on mRNA expression of TNF-α and these other M1 inflammatory phenotype makers on LPS-activated BV2 microglial cells.

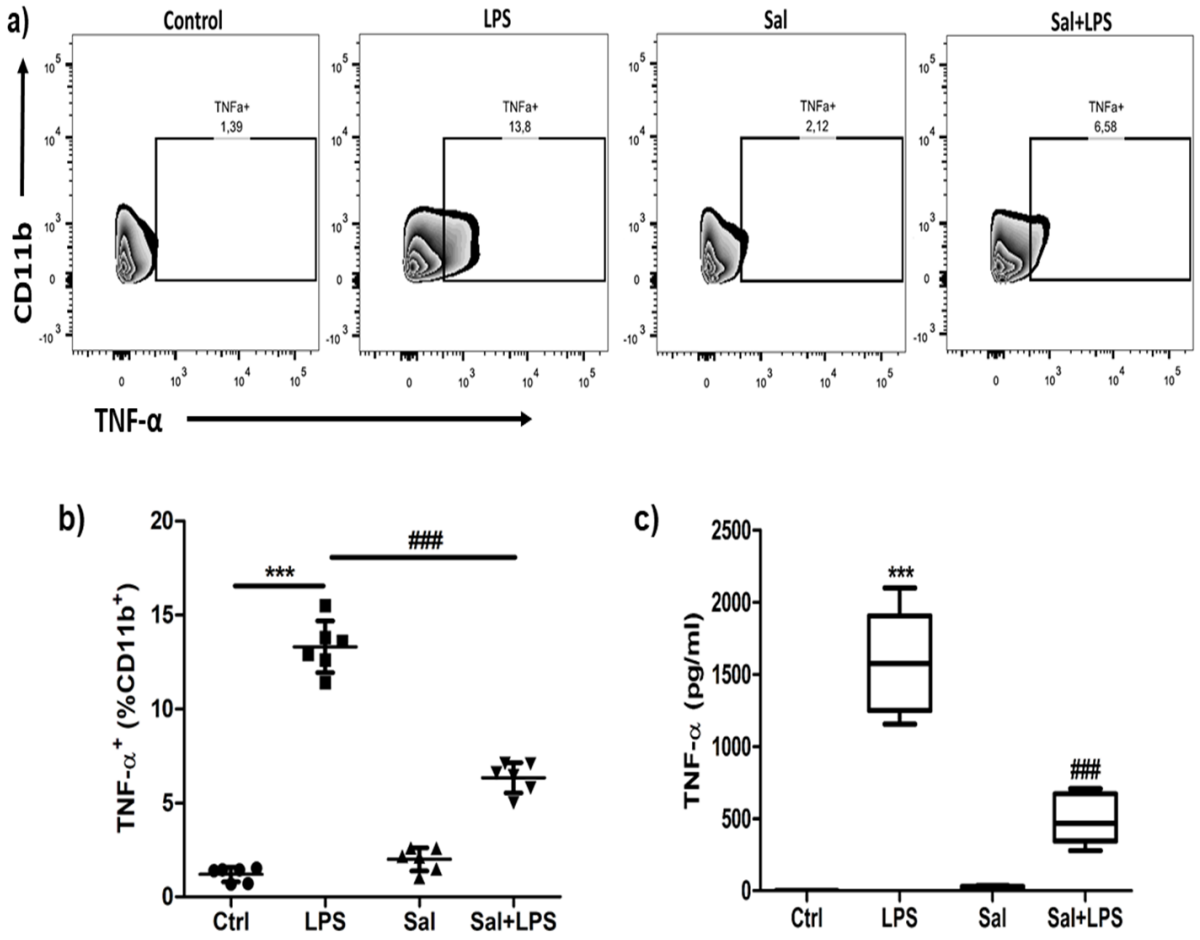


Figure 5. 1: β2-AR agonist Salmeterol suppresses LPS-induced TNF-α production in BV2 cells.

BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min then stimulated with *E. coli* LPS (1µg/ml) and Brefeldin A (10 µg/ml) (A and B). After 4h cells were harvested and intracellular TNF-α expression was measured by flow cytometry. (A) Represents the expression level of TNF-α in control (untreated), LPS-stimulated, Salmeterol-treated and Sal+LPS-treated experimental group. (B) Plot shows the percentage of TNF expressing cells. (C) BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min then stimulated with E. coli LPS (1µg/ml) and after 6h supernatants were collected. Box-plot represents the TNF-α production in culture supernatant which was measured by ELISA. Data represents mean±SD of 3-5 independent experiments. ***$p<0.001$ indicates significant difference from control group and ###$p<0.001$ indicates significant difference from LPS-treated group.

In **Figure 5.2**, we show that LPS exposure upregulates the expression of M1-specific cytokines TNF-α, IL-18, IL-6 which were significantly downregulated in LPS+Salmeterol group (*p<0.001 and p<0.01*; Figure 5.2A-C). In addition, stimulation of BV2 cells with LPS also upregulated the mRNA expression of pro-inflammatory M1-specific chemokines CCL2 (MCP-1), CCL3, CCL4 and CXCL10 (IP-10), and pre-treatment with Salmeterol significantly suppresses the expression of these chemokines (*p<0.001*; Figure 5.2D-G). As reported previously, Salmeterol also suppresses the LPS-activated nitric oxide (NO) secretion from microglia cells (263) and here we observe that Salmeterol also significantly downregulates the mRNA expression of LPS-activated iNOS (by ~6-fold; *p<0.001*, Figure 5.2H). In addition, Salmeterol significantly suppresses the LPS-induced generation reactive oxygen species in BV2 cells (*p<0.001*; Figure 5.2I). Collectively, this data suggests that Salmeterol exerts anti-inflammatory effects by inhibiting the expression of M1-specific pro-inflammatory cytokine and chemokine mRNAs and the production reactive oxygen species in endotoxin-activated BV2 cells.

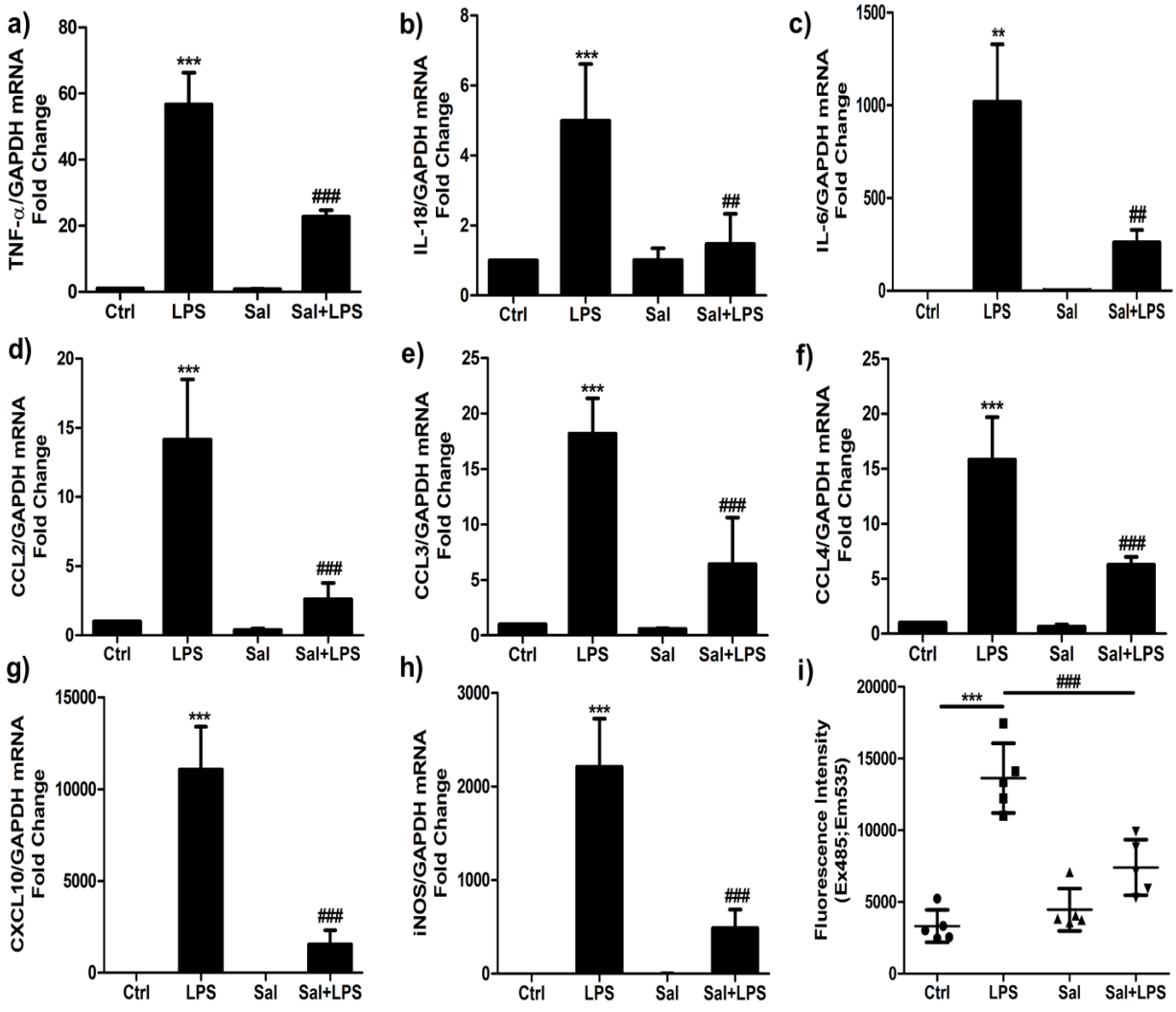


Figure 5. 2: Engagement of beta2 adrenergic receptor with agonist Salmeterol downregulates LPS-stimulated expression of M1 phenotypic markers of microglia.

BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with *E. coli* LPS (1µg/ml). After 4h cells were harvested, RNA was isolated from each experimental group and qRT-PCR analysis was performed. Bar graphs represent the expression level of (A) *TNFA*, (B) *IL-18*, (C) *IL-6*, (D) *CCL2*, (E) *CCL3*, (F) *CCL4*, (G) *CXCL10*, and (H) iNOS. The level of intracellular reactive oxygen species (ROS) was measured in culture supernatant 1h post-LPS stimulation (I). Data represents mean±SD of 3 independent experiments. ***$p<0.001$ and **$p<0.01$ represents significant difference from control group. ###$p<0.001$ and ##$p<0.01$ represents significant difference from LPS-treated group.

5.3.2. Salmeterol stimulates expression of M2 phenotypic markers in LPS-activated BV2 cells. In response to inflammatory stimuli such as LPS, microglia change their phenotype from resting to the activated pro-inflammatory type (M1) (320). Conversely, activation of TLRs with LPS can also weakly enhance the expression of the M2 phenotypic marker IL-10 in immune cells including microglia (330). Recent studies have shown that adrenergic receptor activation can robustly increase the expression of IL-10 in dendritic cells (331) and activated macrophages (326). Consequently, we tested if pre-treatment with Salmeterol can convert the pro-inflammatory M1-like phenotype to the M2-like or anti-inflammatory phenotype by increasing the expression of not only IL-10 but of other M2 markers as well. The intracellular production and release of IL-10 was measured by flow cytometry, qPCR and ELISA. While the intracellular staining of IL-10 was slightly enhanced in LPS-activated BV2 cells compared to unstimulated cells, IL-10 staining was robustly increased after Salmeterol treatment in these cells. Interestingly, no increased expression of IL-10 was observed in Salmeterol alone treated BV2 cells **(Figure 5.3A)**. The quantitative analysis shows a small increase in the number of IL-10^{+} cells in LPS-stimulated group, which was significantly enhanced by Salmeterol treatment (approximately by 3-fold; $p<0.001$) in LPS+Salmeterol group when compared to LPS alone group (Figure 5.3B). Also, pre-treatment with Salmeterol enhances the mRNA expression of IL-10 in LPS-activated BV2 cells ($p<0.01$; Figure 5.3C). Similarly, the production of IL-10 in culture supernatant was also significantly enhanced (by ~4-fold; $p<0.001$) in LPS+Salmeterol group when compared to LPS-alone group (Figure 5.3D). When we further examined the effect of Salmeterol on the expression of other M2-specific phenotype makers of activated microglia. We found that Salmeterol significantly enhances the expression of arginase-1 and CXCL14 in LPS-activated BV2 microglia ($p<0.001$; Figure 5.3E-

F). Consistent with IL-10 expression, there was no enhanced expression of arginase-1 and CXCL14 observed in Salmeterol alone treated group.

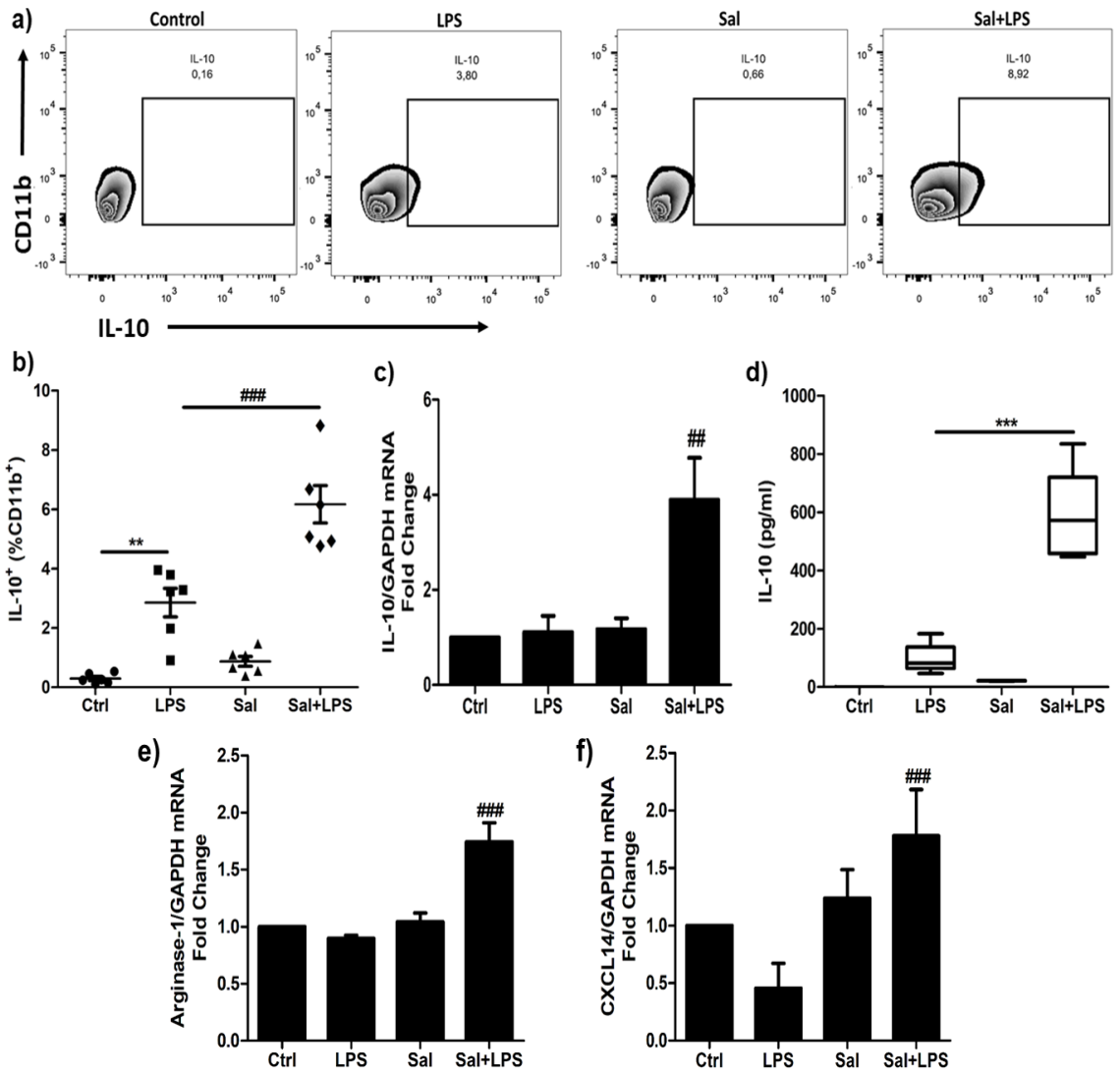


Figure 5. 3: Treatment with Salmeterol converts immunological phenotype of LPS-stimulated microglia.

BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with E. coli LPS (1µg/ml) and Brefeldin A (10 µg/ml). After 4h cells were harvested, and intracellular IL-10 expression was measured by flow cytometry. (A) Top panel represents the expression level of IL-10 in control (untreated), LPS-stimulated, Salmeterol-treated and Sal+LPS-treated experimental group. (B) Plot shows the percentage of IL-10 expressing cells. (C) Bar graph represents the mRNA level of IL-10 expression in experimental groups. (D) BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min then stimulated with *E. coli* LPS (1µg/ml) and after 6h supernatants were collected. Box-plot represents the IL-10 production in culture supernatant which was measured by ELISA. (E-F) Bar graphs depict the mRNA expression level of M2 phenotype markers *Arg-1* and *CXCL14* respectively. Data represents mean±SD of 3-4 independent experiments. **p<0.01 indicates significant difference from control group. ###p<0.001 and ##p<0.01 indicates significant difference from LPS-treated group.

5.3.3. Salmeterol-mediated anti-inflammatory effect and phenotypic conversion is specific to activation of β2-AR. Given the evidence that Salmeterol is a specific agonist of β2-AR, we sought to examine if other β2-AR agonists show similar effects to Salmeterol. For these experiments, we used the ultra-long-acting β2-AR agonists Vilanterol and Indacaterol. BV2 cells were pre-treated with Vilanterol and Indacaterol and then stimulated with LPS, and the production of inflammatory mediators TNF-α and IL-10 was measured in culture supernatant. Results **(Figure 5.4)** show both Vilanterol and Indacaterol significantly suppressed the LPS-induced production of TNF-α and enhanced the LPS-induced production of IL-10 in BV2 microglia ($p<0.001$; Figure 5.4A-B). Furthermore, the blockade of β2-AR with the specific antagonist ICI 118,551HCl reverses the immunoregulatory effects of Salmeterol in LPS-activated BV2 microglial cells (Figure 5.4E-F).

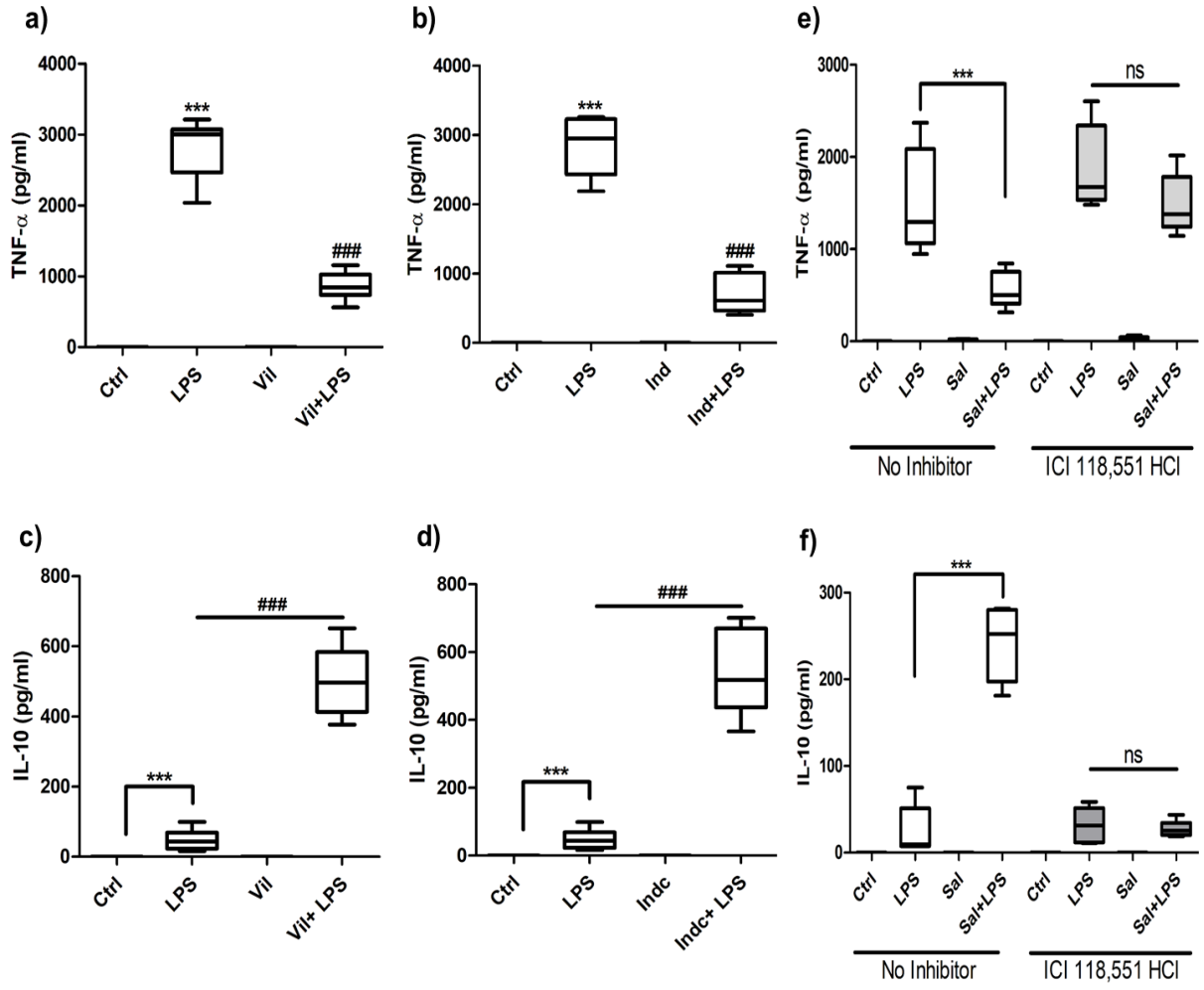


Figure 5. 4: Inflammatory conversion of microglia by is mediated by β2-AR activation and blocked by β2-AR antagonists.

BV2 cells were treated with the ultra-long acting β2-AR specific agonists Vilanterol and Indacaterol (10^{-9} M) for 45 min and further stimulated with *E. coli* LPS (1μg/ml). Production of IL-10 and TNF-α in culture supernatant was measured by ELISA after 6h post-LPS stimulation. Box plots represent the supressed TNF-α and enhanced IL-10 production by Vilanterol and Indacaterol. (A-D) In separate experimentBV2 cells were treated with β2-AR specific antagonists ICI 118,551 HCl prior to treatment with Salmeterol. Box plots depict the supressed TNF-α and enhanced IL-10 production and this effect was further blocked by β2-AR specific antagonist (E-F). Data represents mean±SD of independent experiments (n=4). ***p<0.001 indicates significant difference from control group. ###p<0.001 indicates significant difference from LPS-treated group. ns- not significant.

5.3.4. Salmeterol-mediated enhancement of IL-10 is β-arrestin2-independent. After binding with agonist, β2-AR activation leads to two different signaling pathways: the classical or G-protein-dependent pathway and the alternate β-arrestin-dependent or G-protein-independent pathway. Previously, we have found that anti-inflammatory effects of Salmeterol is β-arrestin2-dependent by demonstrating that following the silencing β-arrestin2 Salmeterol had no effect on the reduced production of TNF-α in LPS-activated BV2 cells (263). Consequently, we sought to examine whether the increased production of IL-10 is also regulated by β-arrestin2-mediated signaling by transfecting BV2 cells with β-arrestin2 siRNA and then treated these cells with Salmeterol, followed by stimulated with LPS. Results **(Figure 5.5)** indicate that cells exposed to the scrambled siRNA (Si control) showed significant increased production of the IL-10 in LPS+Salmeterol-treated group compared LPS alone ($p<0.001$), and the silencing of β-arrestin2 with siRNA to *Arrb2* had no effect on the enhancement of IL-10 production in LPS+Salmeterol activated BV2 cells ($p<0.01$; Figure 5.5A). The effect of Si-RNA on the inhibition of β-arrestin2 protein expression was verified by western blot (Figure 5.5C).

5.3.5. Salmeterol-mediated IL-10 enhancement in activated microglia requires CREB activation. It has been shown that the classical G-protein-dependent or cAMP/PKA/CREB signaling pathway is responsible for converting M2 polarization in microglia activated by GPCR such as cannabinoid CB2 receptors (332,333). We therefore first examined if the LPS-dependent Salmeterol-activated enhancement of the production of IL-10 is CREB dependent. To test this, BV2 cells were transfected with siRNA against *CREB* (Si-Creb) and then treated with Salmeterol, followed by stimulated with LPS. The release of IL-10 was measured by ELISA and results indicate that cells exposed to the scrambled siRNA (Si-Control) showed significantly increased production of the IL-10 in LPS+Salmeterol-treated group compared LPS alone ($p<0.001$). In

contrast, BV2 cells transfected with Si-Creb showed a much smaller but still slightly significant production of IL-10 in LPS+Salmeterol treated group ($p<0.05$, Figure 5.5B) when compared with the cells transfected with Si-Control. This shows IL-10 production was significantly reduced by Salmeterol in LPS-activated BV2 cells after the silencing of *CREB* ($p<0.001$; Figure 5.5B) when compared with Si-Control group. Effect of Si-RNA on protein expression of Creb was measured by western blot (Figure 5.5D).

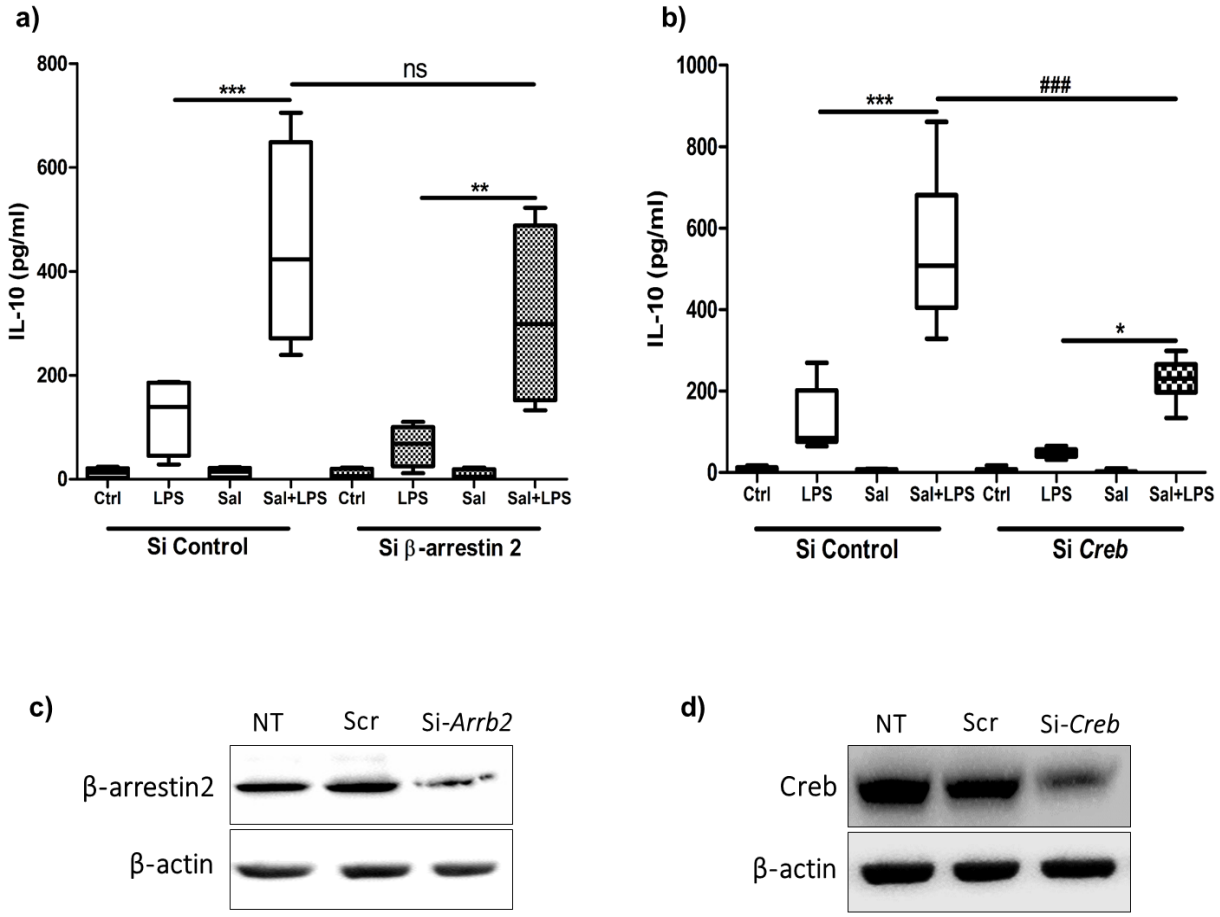


Figure 5. 5: Sal/β2-AR-mediated enhancement of IL-10 production is dependent on classical GPCR signaling pathway.

BV2 microglia were transfected with β-arrestin2 siRNA (Siβ-arrestin2) and scrambled siRNA (SiControl or Scr). In separate experiments BV2 cells were transfected with Creb siRNA (Si-Creb) and scrambled siRNA. After 24h of transfection cell were treated with Salmeterol and followed by stimulated with LPS. After 6h cell culture supernatants were collected and production of IL-10 was measured by ELISA. Box plots depict the effect of silencing of β-arrestin2 (A) and Creb (B) on IL-10 production in all experimental groups. Representative western blots (C) and (D) show reduced expression of β-arrestin2 and total Creb protein after transfection. Data represents mean±SD of four independent experiments (n=4). ***p<0.001, **p<0.01 and *p<0.05 indicates significant difference from LPS-treated group and ###p<0.001 indicates significant difference between SiControl and Siβ-arrestin2 and Si-Creb experimental groups. ns- not significant.

Activation of CREB has been shown to involve activation of the PKA signaling pathway, but can also be mediated by activation of MAPK (334,335) and PI3K (phosphoinositide 3-kinase) (336). Therefore, we examined whether LPS+Salmeterol-mediated enhancement of IL-10 production via CREB is mediated by these and/or other signaling molecules. BV2 cells were treated with the indicated pharmacological inhibitors targeting intracellular signaling molecules for 1h prior to treatment with Salmeterol and LPS. Next, production of IL-10 in culture supernatant was measured. Results **(Figure 5.6)** shows inhibition of ERK1/2 (extracellular signal-regulated kinase) (by 10μM of U0126) and JNK (c-Jun N-terminal kinase) (by 10μM of SP600125) was not capable to affecting the Salmeterol-mediated production of IL-10 (Figure 5.6A-B), while p38 MAPK inhibition (via 10μM of SB203580) significantly inhibited Salmeterol-mediated increased production of IL-10 ($p<0.001$; Figure 5.6C). Likewise, it has been previously shown that PI3K activation is required for M2 activation of macrophages (337), and we find that treatment with PI3K inhibitor (Wortmannin; 1uM and) also abrogates the Salmeterol-mediated IL-10 production ($p<0.001$; Figure 5.6D). As expected, protein kinase A inhibition (by KT5720; 1μM) also significantly inhibited IL-10 enhancement in LPS+Salmeterol-activated BV2 cells ($p<0.001$; Figure 5.6E), which further indicates the finding that Salmeterol-mediated IL-10 production is dependent on the classical cAMP/PKA/CREB signaling pathway.

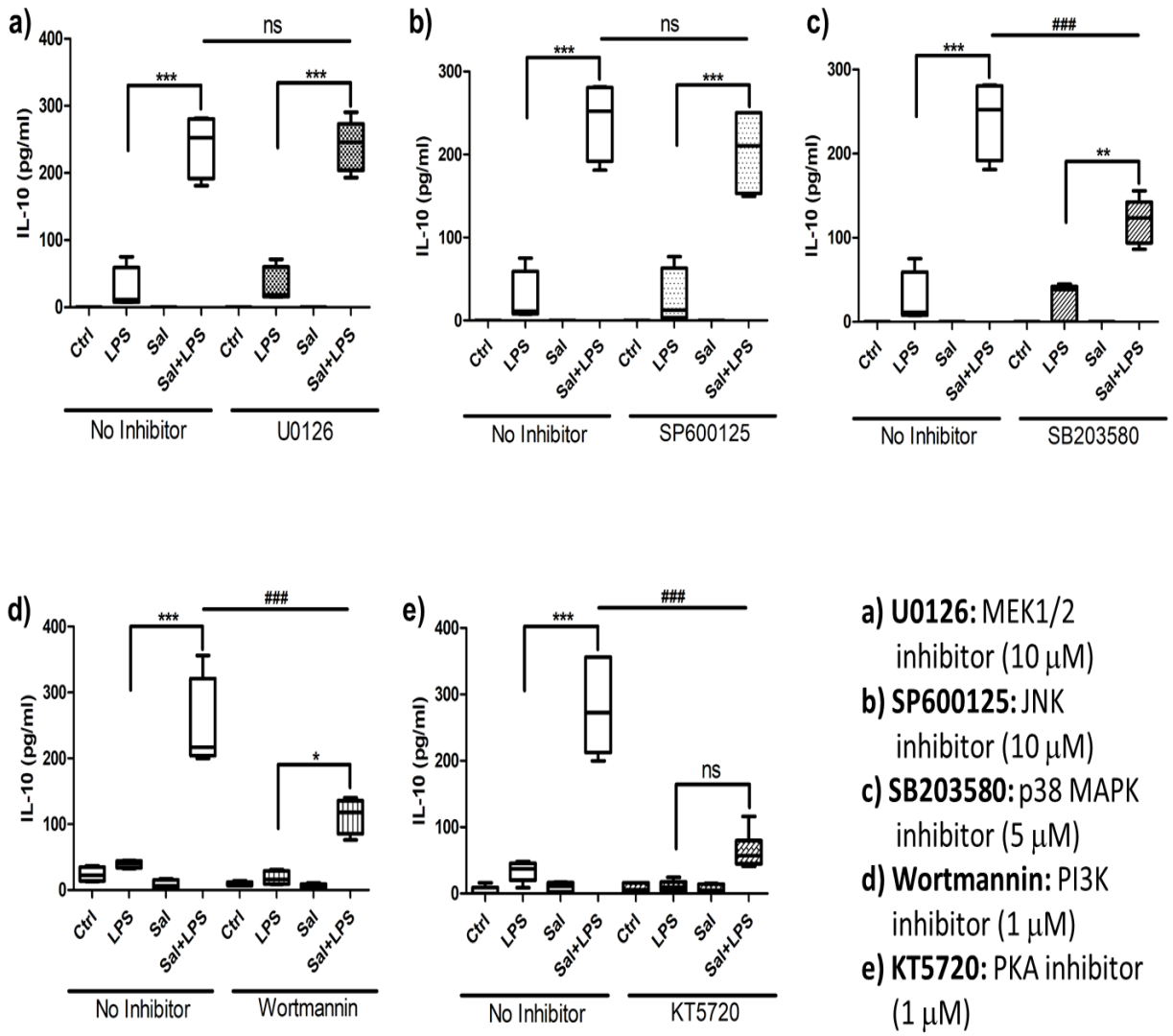


Figure 5. 6: Effects of blockade of various signaling pathways on Salmeterol-mediated production of IL-10.

BV2 cells were incubated with inhibitors indicated for 1h prior. Then, cells were treated with Salmeterol (10^{-9} M) for 45 min and further stimulated with *E. coli* LPS (1μg/ml). Production of IL-10 culture supernatant was measured by ELISA. (A-E) Box plots represent the IL-10 production by Salmeterol in presence and absence of pharmacological inhibitors of indicated signaling molecules. Data represents mean±SD of five independent experiments (n=5). ***p<0.001 and **p<0.01 indicates significant difference between LPS-treated and LPS+Salmeterol-treated group. ###p<0.001 indicates significant difference between experimental groups treated with inhibitors and no inhibitors. ns- not significant.

5.4. DISCUSSION

Inflammatory processes in the brain are mainly regulated by microglia and these microglial cells acquire different activation states in order to perform their molecular and cellular functions. Once activated, the M1 microglia responds to the toxic stimuli and promote inflammation. On the other hand, the M2 microglia secrete anti-inflammatory mediators and promote repair and tissue homeostasis (320,337). Our results indicate treatment of LPS-activated microglia with Salmeterol suppresses M1-type activation and promotes M2-type activation of microglia. Previously, we have studied the neuroprotective and anti-inflammatory properties of β2-AR in MPTP and LPS model of PD but the underlying mechanism is still not completely understood (20,263). Here, we find that the β2-AR agonist Salmeterol significantly inhibits the cytokine/chemokine expression such as TNF-α, IL-1β, IL-6, IL-18, IP-10 and MCP-1 (Figure 5.1 and 5.2), as well as other M1-phenotypic markers such as iNOS, reactive oxygen species, and even COX2 (cyclooxygenase-2, data not shown). These pro-inflammatory mediators have been shown to play crucial role in neuroinflammation and progression of PD and other neurodegenerative diseases (155,167,338,339). On the other hand, we found that Salmeterol significantly enhances the production of the anti-inflammatory cytokine IL-10 suggesting that its mode of action is not only to inhibit the pro-inflammatory phenotype, but to induce the production of anti-inflammatory cytokines that may help to control the continued destruction of dopaminergic neurons by other neighboring microglial cells.

The importance of inflammation in the destruction of dopaminergic neurons in PD has long been suggested (180,304,323). Elevated levels of pro-inflammatory mediators have been found in blood and cerebrospinal fluid of PD patients (340,341). The increased production of pro-inflammatory mediators have been associated with several neurodegenerative conditions including PD

(180,296,342). Similarly, the over-production of reactive oxygen or nitrogen species have been associated to the etiology of many diseases including neurodegenerative and neuropsychiatric diseases such as PD (343–346). A number of studies have shown an association between the use of NSAIDs and risk of Parkinson's disease (347–349). However, it has been suggested that non-specific shut-down of inflammation may not have sufficient beneficial effects on the disease pathogenesis (350). Therefore, suppressing neuroinflammation and toxicity is an important therapeutic approach but strengthening the neuroprotective and restorative properties of microglia may also be required to halt the disease progression. Results from this study also address this hypothesis.

One of the important features of our findings is that Salmeterol not only suppresses neurotoxic phenotype of microglia (M1) via inhibiting TNF-α, IL-6, IL-1β, IL-18, CCL2, CCL3, CCL4, CXCL10, nitric oxide and ROS production, but also elicits anti-inflammatory effects by inducing IL-10 production and promoting the microglial conversion from the M1- to the M2-like phenotype **(Figures 5.1, 5.2 and 5.7)**. It has been found that M1 cytokines and chemokines play a major role in neurodegenerative diseases. For example, in addition to the known neuropathic effects of TNF-α (22,351,352) and IL-1β (353) the higher expression of CCL2/MCP-1 exacerbates the chronic inflammation in many neurodegenerative conditions (354). Increased levels of chemokines including MCP-1, CCL3, CCL4 and CXCL10 have been associated with depressive symptoms and cognitive impairment in PD patients (355,356). Salmeterol also reduces the expression of IL-18 which contributes to dopaminergic neurodegeneration (357) and several other inflammation-related disorders (358,359). In addition, polymorphism in IL-18 gene promoter has been associated with idiopathic PD in two different population studies (360,361).

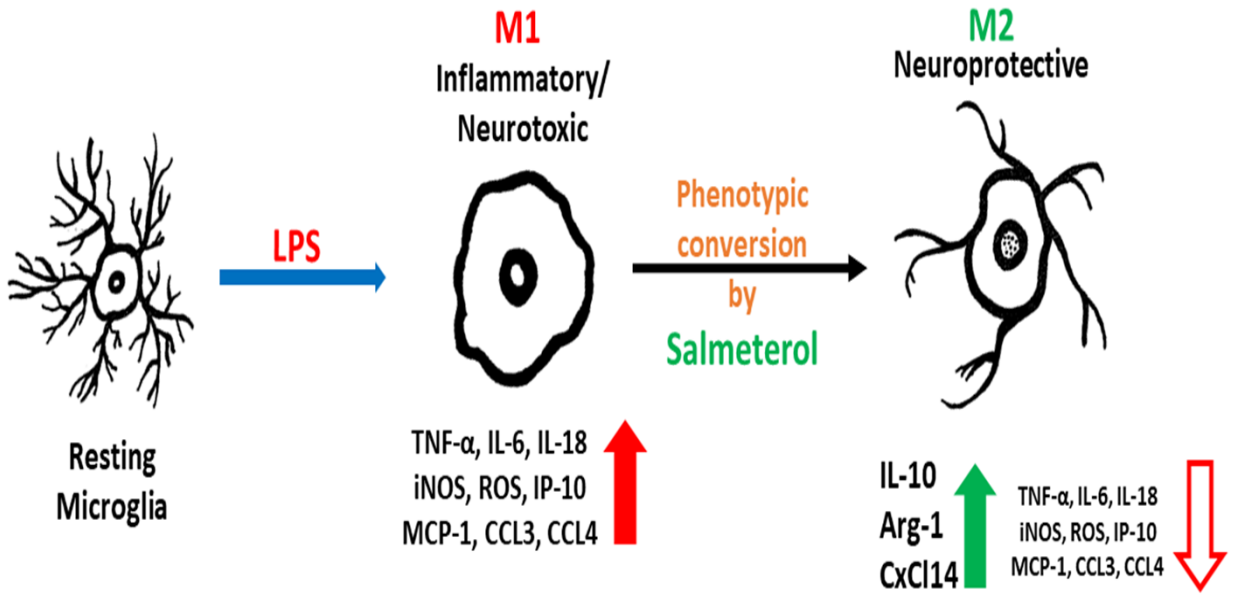


Figure 5. 7: Schematic diagram showing immunomodulatory effect of Salmeterol on inflammatory conversion of LPS-stimulated BV2 cells.

Resting microglia, once activated with endotoxin, expresses an M1-like phenotype which is neurotoxic. These M1-activated microglia robustly enhance production of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-18, MCP-1, CCL3, CCL4, ROS and CXCL10/IP-10. In contrast, treatment of LPS-activated microglia with Salmeterol changes their phenotype to an M2-like phenotype which is characterized by the inhibition of pro-inflammatory cytokines and the enhancement of anti-inflammatory cytokines and chemokines IL-10, arginase-1, and CXCL14.

Conversely, we find that β2-AR agonists Salmeterol enhances the expression and production of IL-10 in LPS-activated BV2 microglia and also enhances the expression of M2 markers arginase-1 and CXCL14 **(Figure 5.3)**. IL-10 has been found as an anti-inflammatory and neurotrophic factor which helps in tissue repair and homeostasis process (362,363). Previously, we have shown neuroprotective effects of anti-inflammatory cytokine (such as IL-10 and TGF-β) therapies in PD models (111,119,300). IL-10 is also known to protect LPS-induced dopaminergic neurodegeneration in SN and mesencephalic culture (301). Similarly, IL-10 also suppresses programmed-cell death in ventral mesencephalic neurons via JAK-STAT3 pathway (182). IL-10 has been shown to prevent glutamate-induced excitotoxicity in brain ischemia. Protective and immunoregulatory effects of IL-10 have been explained in gut inflammation and also in cancer (364), infection (365), autoimmune diseases (366), and neurodegenerative diseases (362,363). These findings give further insight to the idea that neuroprotective role of β2-AR agonists are not only due to the suppression of pro-inflammatory mediators but also due to the conversion of microglia from pro- to anti-inflammatory-like phenotype.

Similar to our findings, activation of β2-AR in murine macrophages promotes an M2-like phenotype and shows protection against endotoxemia and acute lung injury (326). Furthermore, β2-AR activation by NE in macrophages robustly enhances the IL-10 production *in vitro* and *in vivo*, and consequently, *ADRB2* knockout mice were more susceptible to infection and LPS challenge (328). Transcriptome analysis of β2-AR-activated macrophages showed transcriptome with up-regulation of M2-spectrum gene expression which is regulated by CREB, C/EBPβ (CCAAT-enhancer-binding protein-beta) and ATF (activating transcription factor) transcription factors (327). However, we are able to define β2-AR-stimulated microglia only as M2-like microglia because classically defined M2 macrophages are activated by IL-4 and the transcription

factors involved in the M2 phenotype are different than β2-AR-related transcription factors (327,367). Further studies need to be done to determine the exact nature of the conversion of activated microglia to the M2-like phenotype we see in BV-2 cells.

Early degeneration of locus coeruleus (LC) adrenergic neurons and their circuitry to substantia nigra have been found in PD patients, suggested a role for the noradrenergic system in the neuropathology of PD (227). Extensive dysfunction of the adrenergic neurons in LC has been demonstrated as the ubiquitous feature of PD and AD pathology (368,369). The pathological studies by Braak suggest LC as the first brain region to be affected in PD (226). In addition, pharmacological approaches to mimic the effects of noradrenaline exhibit neuroprotective effects (324,370). For example, it has been found that activation of β2-AR via NE leads to suppressed microglial activation and to the production of growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor-1 (NGF-1) by astrocytes (254,371), thereby providing neuroprotective and neuro-restorative effects and also limit the cytotoxicity of DA-neurons (255,372). In addition, since β2-ARs belong to the seven transmembrane G-protein-coupled receptor superfamily, the engagement of β2-ARs with their agonists initiates either the classical G-protein-dependent or the alternate β-arrestin-dependent signaling pathway, both of which can result in immunomodulatory actions (284,373).

Consequently, we have further investigated the molecular mechanism of the immunoregulatory effects of Salmeterol. In a recent study we have shown that the anti-inflammatory effects or regulation of production of pro-inflammatory mediators by Salmeterol is dependent on the alternative/β-arrestin-dependent pathway of G-protein coupled signaling. Silencing of β-arrestin2 by using siRNA against *Arrb2* gene reverses the inhibition of TNF-α and IL-1β production by Salmeterol (263). In this study we show that silencing of *Arrb2* gene did not alter the enhanced

IL-10 production by Salmeterol **(Figure 5.5A)**. Rather, in our results blockade of PKA by pharmacological inhibitor reduces the Salmeterol-mediated production of IL-10, so it appears that cAMP/PKA activation leading to CREB activation through the classical pathway of G-protein coupled activation leads to IL-10 enhancement. Similar mechanisms have been suggested by Ghosh et. al where they have found that treatment with cAMP in combination with Th2 cytokine (IL-4) promoted microglia polarization towards to the M2 phenotype (374) and this M2 conversion requires the activation of PKA. In addition to that, Salmeterol also suppress LPS-induced systemic inflammation via inhibition of NLRP2 inflammasome and this anti-inflammatory effect of Salmeterol is dependent on both classical GPCR/cAMP pathway as well as β-arrestin2 pathway (375). Collectively, data from our previous study (263) and this study suggest that the suppression of pro-inflammatory phenotype (M1) of microglia is dependent on β-arrestin pathway while enhancement of anti-inflammatory-like phenotype (M2) requires the activation of the classical cAMP/PKA/CREB pathway of GPCR signaling.

Another interesting finding of our study is Salmeterol alone did not enhance the IL-10 production in resting microglia and only induced IL-10 production in LPS-activated microglia. Activation of TLR pathway not only activates the NF-κB pathway but also activates MAPK pathway. Blockade of ERK1/2 and JNK MAPK did not affect the Salmeterol-mediated IL-10 production but blockade of p38 and PI3K reverse the enhanced production of IL-10 by Salmeterol. It is known that p38 and PI3K pathway play a role in the regulation of the production of IL-10 in macrophages (326,376). The PI3K pathway also regulates macrophage activation and their M1/M2 polarization. Therefore, it appears that IL-10 enhancement by Salmeterol involves both the PI3K/p38 pathway as well as the PKA pathway, both of which appear to be needed to get optimal activation of CREB leading to IL-10 production.

We have yet to determine if the immune-conversion effects are the major therapeutic benefits of β2-AR agonists in the treatment of dopaminergic neurodegeneration. Interestingly, β2-AR activation has also been linked with the inhibition of transcription of α-synuclein in animals and *in vitro* models of PD. They have shown that β2-AR agonists Clenbuterol and Salbutamol suppresses the expression of *SNCA* gene via histone-3-lysine-27 acetylation of its promoter and enhancer region. Interestingly, it was also found that patients on Salbutamol, had a reduced risk of developing PD (237). In contrast, patients using a β2-AR antagonist Propranolol were found to be at increased risk of developing PD (237). A similar study by Gronich et al. also shows that within a large cohort of the Israeli population, the use of various β2-agonists was associated with reduced risk of PD while patients treated with Propranolol appear to have higher risk of developing PD (377). Interestingly, in a mouse model of Alzheimer's disease, investigators showed that the β2-AR activation enhances hippocampal neurogenesis, ameliorates memory deficits, and increases dendritic branching and spine density (236). Taken together, we suggest that β2-AR agonist may have therapeutic benefits against neurodegeneration via: i) suppression of pro-inflammatory mediator production and conversion of microglia activation from neurotoxic/M1- phenotype to neuroprotective/M2-like phenotype, ii) promoting the release of neurotrophic factors from glial cells, iii) regulating the gene and protein expression of SNCA and iv) promoting neurogenesis and repopulation of neurons.

CHAPTER 6

RNA-seq transcriptome profiling of BV2 cells treated with Salmeterol and/or LPS

6.1. ABSTRACT

Microglial activation is a pivotal event for neuroinflammation and neurodegeneration in a number of neurodegenerative diseases, including Parkinson's disease. We have found that a β2-AR agonist Salmeterol can regulate this microglial activation and ultimately converts the immunologic phenotype of these cells from inflammatory to immunoregulatory. However, transcriptome profiling of these activated microglia following Salmeterol treatment has not yet been studied. Here, we have compared effect of Salmeterol on global gene expression in LPS-activated BV2 microglial cells. To study the immunomodulatory and neuroprotective effects of Salmeterol, RNA-Seq was performed with untreated (control), Salmeterol-treated, LPS-activated and LPS+Salmeterol treated BV2 cells. Bioinformatic analysis was performed, and DESeq analysis showed differentially increased or decreased expression of select genes in response to LPS-stimulation and Salmeterol treatment (cut off value $p<0.05$). Consistent with our earlier findings, annotation analysis showed that Salmeterol significantly downregulated the genes related to microglia activation and immune response such as pro-inflammatory cytokines and chemokines. Interesting, Salmeterol treatment significantly upregulates the expression of neurotrophic factors in LPS-activated and resting microglia. In addition, Salmeterol significantly modulates the expression of genes related to Parkinson's and other neurodegenerative diseases. The KEGG pathway analysis shows that Salmeterol modulates the expression of various pathways related to inflammatory response and pathogenesis of neurodegenerative disorders. Taken together, our results confirm the immunomodulatory effects of Salmeterol and suggest that Salmeterol may have therapeutic applications for inflammation-mediated neurodegenerative diseases.

6.2. INTRODUCTION

Microglial cells are one of the important inflammatory mediators of the CNS. Although microglia constitute only 5-12% of CNS, where they perform wide variety of functions. They act as the first line of defense and mediate the innate immune response against pathogens and damaged tissue within the brain and central nervous system. Other than the immunoregulatory functions, microglia also perform a "housekeeping" job to maintain CNS homeostasis. A number of studies have shown the various roles of microglia in mental health, aging, neuroinflammation and neurodegenerative conditions (297,378). Transcriptome analysis of microglial suggests the key functions of microglia are: sensing their environment, maintaining physiological homeostasis and protecting against toxic agents. Under normal conditions microglia stay in sessile state and constantly inspect their environment, but once activated by toxic stimulus microglia alter their phenotype, produce pro-inflammatory mediators including reactive oxygen species (ROS), nitric oxide (NO), cytokines and chemokines, and contribute to neuronal damage (319). Prolonged activation of primed microglia results in chronic neuroinflammation, which is an important contributing factor for initiation and progression of many neurodegenerative diseases such as Alzheimer's disease (310), Parkinson's disease (33), multiple sclerosis (311) and numerous others (314).

Microglia express a wide variety of pattern recognition receptors (PRRs) and respond to the presence of various PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). The most common and potent toxic stimuli is LPS produced by gram negative bacteria which is widely used to study activation of the innate immune response, including the activation of microglial cells. *In vitro* stimulation of microglia with LPS enhances the production of pro-inflammatory mediators (379), and leads to a strong neurotoxic response. Previously, we have therapeutically targeted microglia-mediated inflammation using several anti-

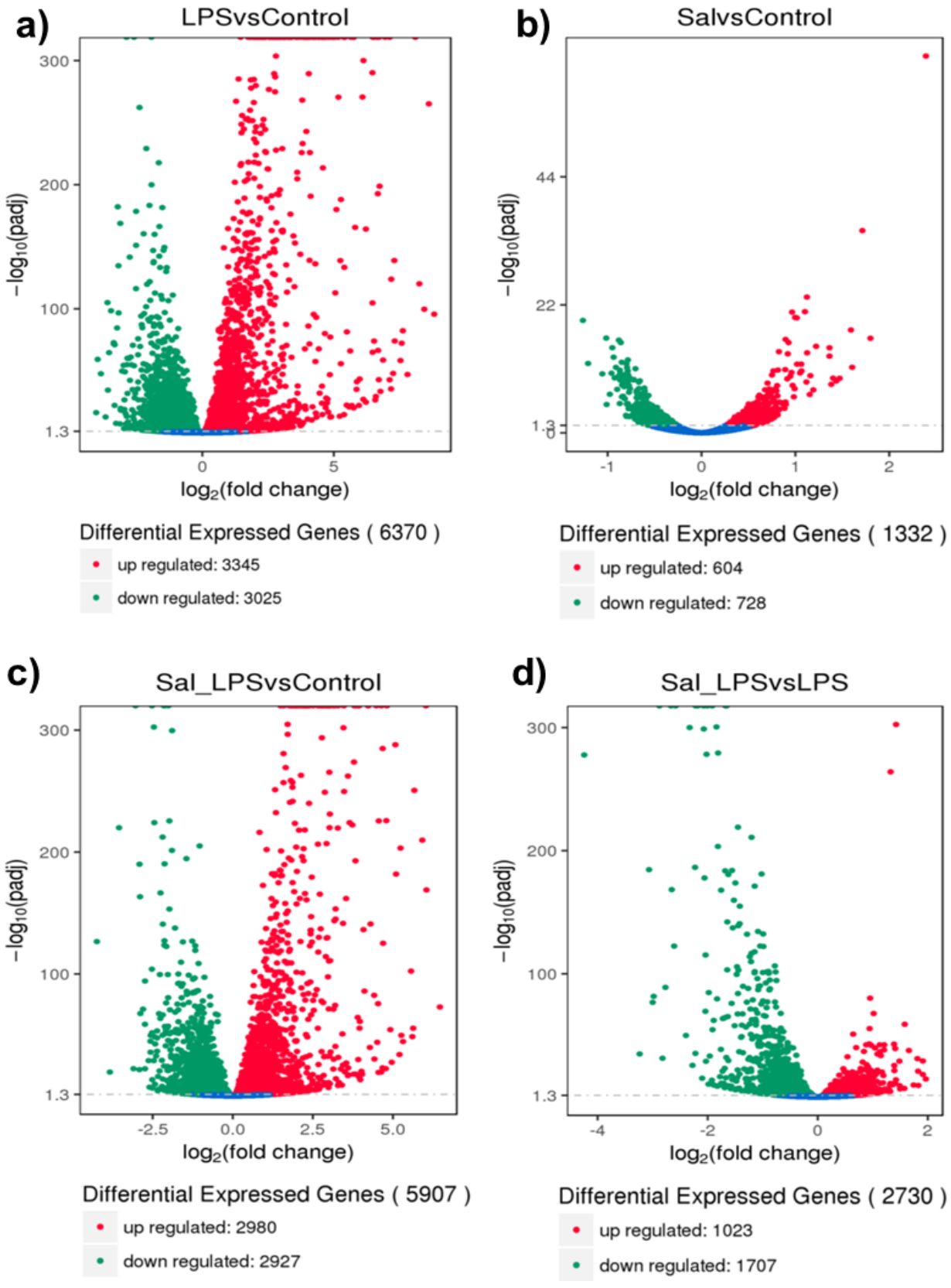
inflammatory approaches (111,115,120,124,126). One such potent therapy we have discovered is the use of β2-AR agonists targeting neuroinflammation and degeneration in PD models. The long-acting β2-AR agonist Salmeterol, which is an FDA-approved drug normally used as bronchodilator and prescribed for the treatment of asthma and chronic obstructive pulmonary diseases, has been found to be one of the most effective β2-AR agonists *in vitro* and *in vivo* to mediate microglial inflammatory activation.

Our previous and current observations clearly suggest a protective effect of the β2-AR agonist against neuroinflammation and dopaminergic neurodegeneration. We have also investigated the mechanism behind the anti-inflammatory and immunoregulatory effects of Salmeterol in LPS-activated microglia and macrophages, but a genome-wide analysis of Salmeterol's effect on LPS-activated microglial cells has not been performed. Therefore we sought to perform this analysis which, to best of our knowledge, this is the first genome-wide study of Salmeterol-mediated global gene expression changes in LPS-activated BV2 microglia. In this study, by using RNA-seq, we have found that treatment with Salmeterol regulates the expression of not only inflammatory genes but also of other genes related to the pathogenesis of neurodegenerative diseases. Our findings suggest that Salmeterol can be an effective multi-purpose therapeutic drug against inflammation-mediated neurological diseases.

6.3. RESULTS

6.3.1. Distinct genes were identified by RNA-Seq analysis in Salmeterol-treated and LPS-activated BV2 microglia. To identify the transcriptome in response to Salmeterol treatment and LPS stimulation, BV2 microglial cells were first treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with *E. coli* LPS (1μg/ml) for 6h. After sequencing, DESeq2 analysis was

performed and results revealed a total of 6370 differentially expressed genes (DEG) (increased and decreased expression) in LPS-stimulated BV2 cells when compared with unstimulated cells **(Figure 6.1)**. Among these 6370 differentially expressed genes, 3345 genes were upregulated and 3025 genes were downregulated (Figure 6.1A). In the group treated with Salmeterol-alone, 604 genes were increased in expression while 728 were decreased in expression compared to control group (Figure 6.1B). In cells treated with both Salmeterol and LPS 2980 genes were upregulated and 2927 genes were downregulated in comparison with control group (Figure 6.1C). Interestingly, 1023 genes with increased expression and 1707 genes with decreased expression were identified in cells treated with Salmeterol and then LPS when compared with group treated with LPS only (Figure 6.1D). Significant differences were identified by fold change ≥1.5 log2 with P≤ 0.05. Differentially expressed genes by Salmeterol and LPS+Salmeterol are also presented in Venn diagram analysis (Figure 6.1E-F). Furthermore, Cluster analysis of DEGs was performed to estimate the expression pattern of DEGs under experimental conditions. Hierarchical clustering was applied with the log10 (FPKM+1; Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) and a heat map was created **(Figure 6.2)**.


a)
LPSvsControl
300
200
100
1.3
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5
log2(fold change)
Differential Expressed Genes (6370)
up regulated: 3345
down regulated: 3025
b)
SalvsControl
44
22
1.3
0
−log10(padj)
-1
0
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log2(fold change)
Differential Expressed Genes (1332)
up regulated: 604
down regulated: 728
c)
Sal_LPSvsControl
300
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Differential Expressed Genes (5907)
up regulated: 2980
down regulated: 2927
d)
Sal_LPSvsLPS
300
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Differential Expressed Genes (2730)
up regulated: 1023
down regulated: 1707

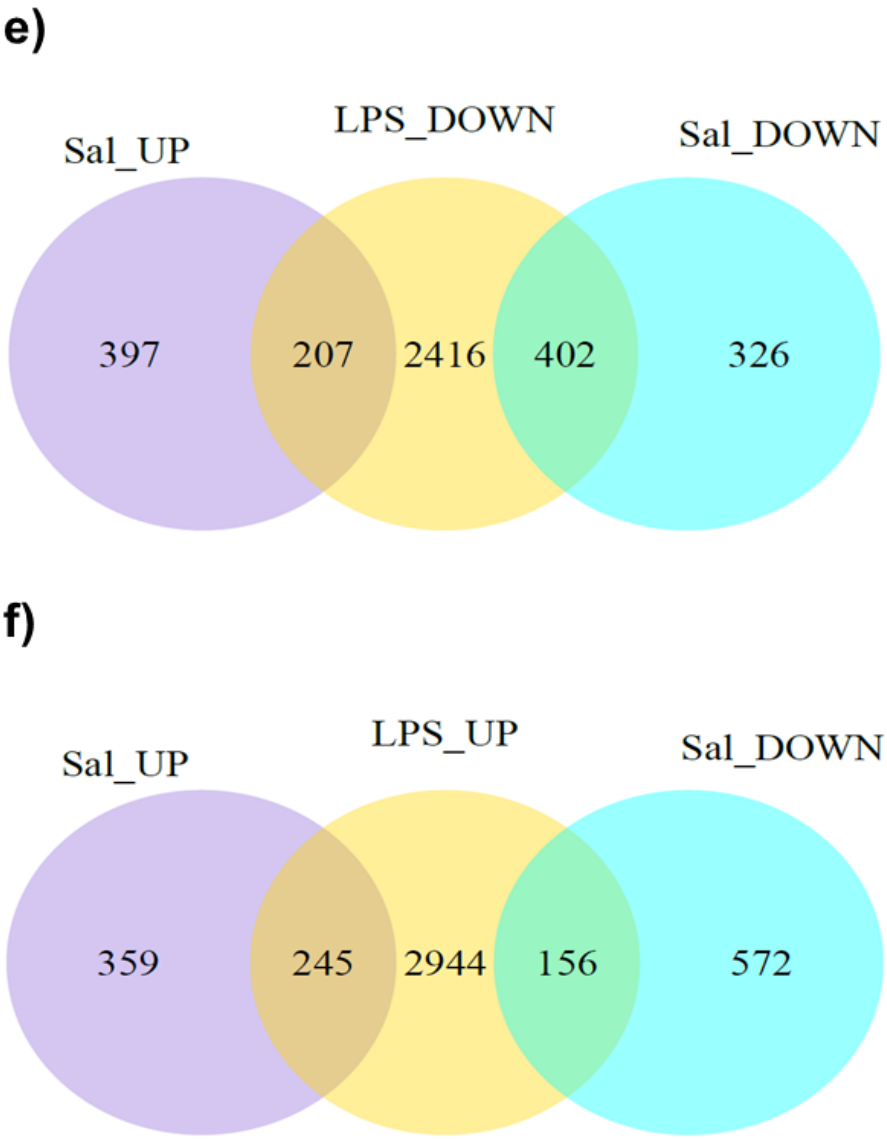


Figure 6. 1: RNA-sequencing analysis shows differentially expressed genes.

Volcano diagram showing the distribution of DEGs (A-D). Horizontal axis is representing the fold change of genes in different experimental groups and vertical axis is showing statistically significant degree of changes in gene expression level. Each point represents a gene, blue dots indicate no significant difference in genes. Red dots indicate upregulated DEGs and green dots indicate downregulated DEGs between experimental groups. (E-F) Data also represented as Venn diagram showing the number of LPS inducible genes that were suppressed or up regulated by Salmeterol treatment in activated BV2 cells. Data shown from biological replicates (n=2) and threshold of DEGs is padj <0.05.

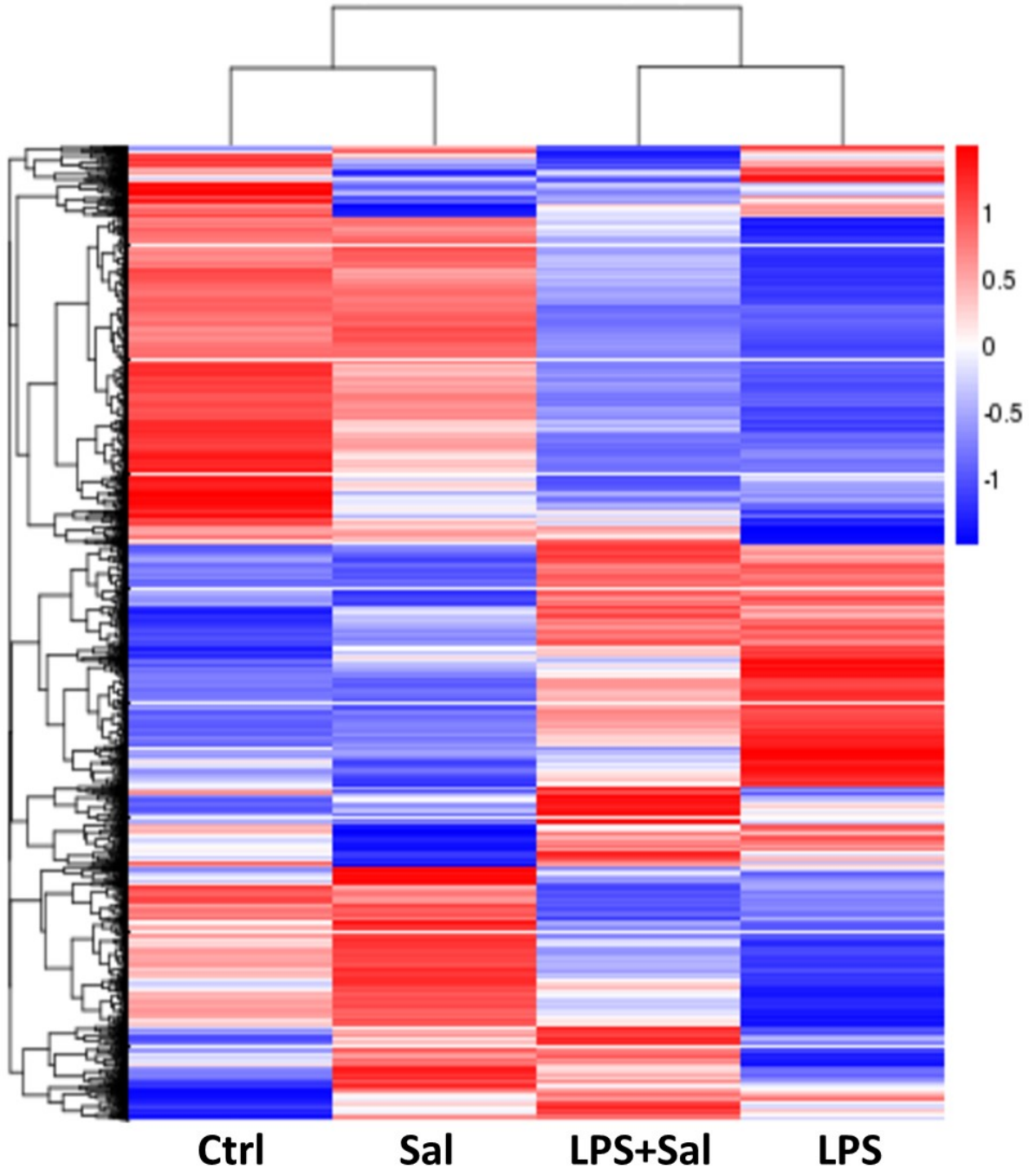


Figure 6. 2: Cluster analysis of differentially expressed genes.

Hierarchical clustering was used to estimate expression pattern of DEGs under different experimental conditions and heat map was created. Red represents the higher expression of genes while blue represents the low expression of genes. Color descending from red to blue. Indicated log10(FPKM+1) from higher to lower value.

6.3.2. Salmeterol suppresses inflammatory response in LPS-stimulated BV2 cells. Our previous observations clearly suggest the anti-inflammatory properties of Salmeterol in activated macrophages and microglia. We have shown that Salmeterol suppresses the expression and release of pro-inflammatory mediators such as TNF-α, IL-1β, ROS and nitric oxide (20,260,263). To further investigate whether Salmeterol is a broad-spectrum, anti-inflammatory agent, its effect on other inflammatory genes was examined. Most of the LPS-activated genes were significantly suppressed by Salmeterol. A list of 40 genes that were suppressed by Salmeterol is presented in **Table 6.1**. Genes were selected on basis of their biological processes and molecular function from their gene ontology. Salmeterol reduces the expression of inflammation-related genes including *TNFA, IL1A, IL1B, iNOS*, *CCL2, CCL3, CCL4, CXCL10, IL-18* etc. Further validation of these was performed by qRT-PCR analysis and results showed the similar effect of Salmeterol (presented as part of chapter 5 of this thesis). A key transcription factor for expression of inflammatory genes, NF-κB was significantly suppressed by Salmeterol which is consistent with our previous findings (20,263). In addition, *Irf1, Irf5, Irf7 and Stat1* transcription factor genes were also downregulated by Salmeterol in LPS-activated BV2 cells. These genes and other inflammation-related genes were clustered in a heat map showing a clear difference between experimental groups **(Figure 6.3)**. Top inflammatory genes regulated by Salmeterol are listed in **Table 6.1.**

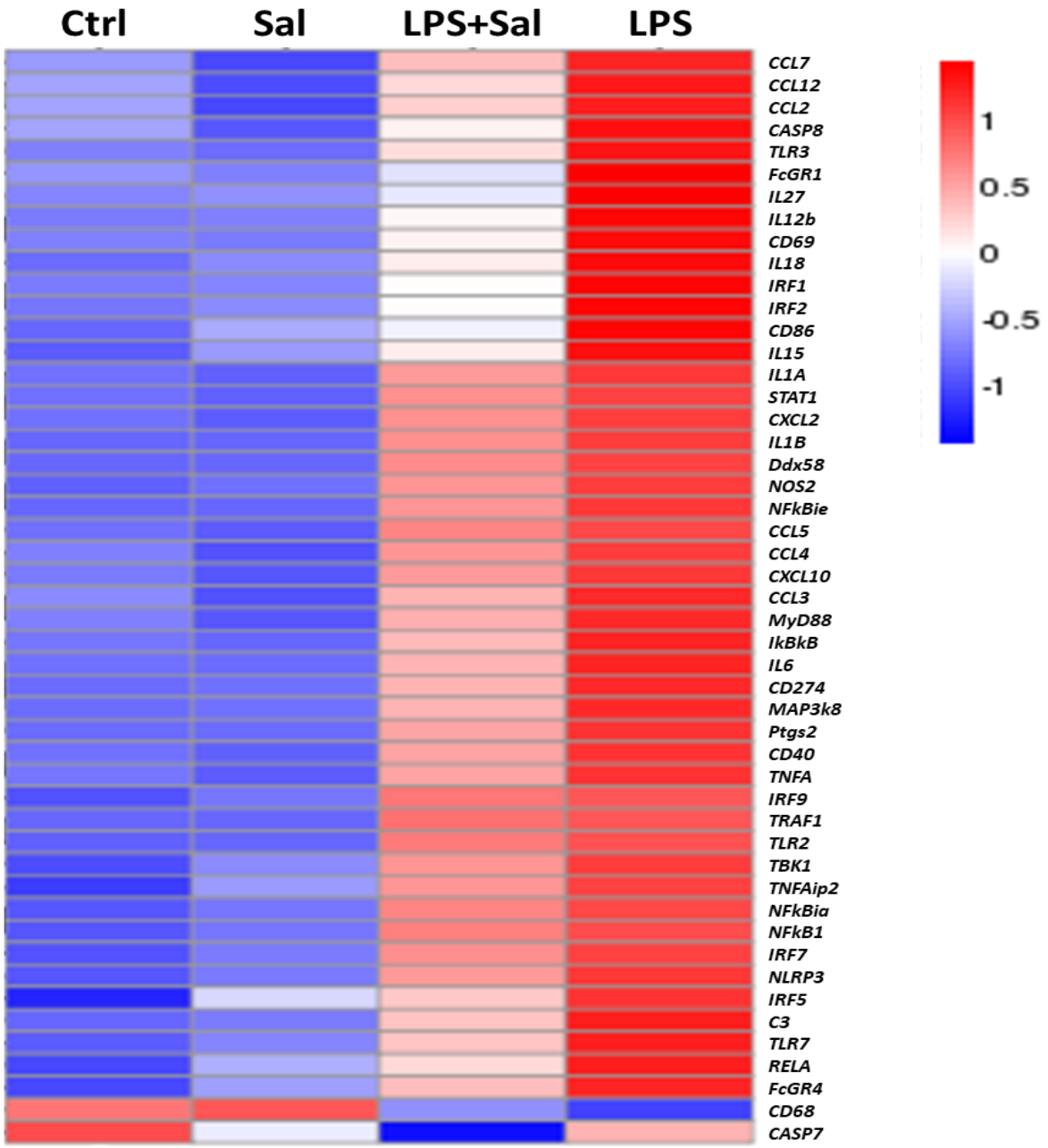


Figure 6. 3: Salmeterol suppresses a subset of LPS-induced inflammatory response-related genes in BV2 cells.

Heat map is depicting the inflammatory genes (cytokines, chemokines and other immune response-related genes) that were significantly downregulated by Salmeterol in LPS-stimulated BV2 cells. Heat map was generated using *'pheatmap'* library in R and $P<0.05$ considered significant. Color descending from red to blue. Indicated log10(FPKM+1) from higher to lower value.

Table 6. 1: Top inflammatory genes down-regulated by Salmeterol in LPS-activated BV2 cells.

Fold change was compared between Sal+LPS and LPS alone experimental group.

Gene	Gene Name	Down regulated genes (log_2 Fold Change)	P-value
NFkBie	Nuclear factor kappa-B	-0.98119	2.05E-15
TNFA	Tumor necrosis factor-alpha	-1.45	1.37E-222
IL1A	Interleukin-1 alpha	-1.7562	3.37E-171
IL1B	Interleukin-1 beta	-1.814	6.76E-207
IL6	Interleukin-6	-2.6539	1.06E-171
IL12b	Interleukin-12 beta	-1.9234	9.37E-16
IL18	Interleukin-18	-1.4641	8.26E-18
IL27	Interleukin-27	-2.8216	6.41E-34
CXCL10	Chemokine (C-X-C) motif ligand 10	-2.6672	0.0
CCL2	Chemokine (C-C) motif ligand2	-1.6761	0.0
CCL3	Chemokine (C-C) motif ligand3	-1.6555	0.0
CCL4	Chemokine (C-C) motif ligand4	-1.0858	1.33E-137
CCL5/RANTES	Chemokine (C-C) motif ligand5	-1.3583	8.04E-93
CXCL2	Chemokine (C-X-C) motif ligand2	-2.0216	7.32E-282
NOS2	Nitric oxide synthase 2 inducible	-1.6437	2.12E-145
IRF5	Interferon regulatory factor 5	-0.45326	6.03E-16
IRF7	Interferon regulatory factor 7	-1.148	4.78E-121
MyD88	Myeloid differentiation primary response gene	-0.73766	1.34E-29
IKKb/ ikbkb	Inhibitor of kappa B kinase beta	-0.69417	9.83E-07
CD86	CD 86 antigen	-0.46215	1.46E-08
TLR2	Toll-like receptor 2	-0.37191	5.20E-23
TLR3	Toll-like receptor 3	-1.7251	1.83E-66
TLR7	Toll-like receptor 7	-0.5125	1.37E-11
CASP8	Caspase 8	-0.4442	1.27E-12
RIG-I	Retinoic acid inducible gene 1	-0.96925	3.46E-59
COX2	Cyclooxygenase 2	-2.0063	0.0
TBK1	TANK-binding kinase 1	-0.3618	3.28E-07
NLRP3	NLR-family pyrin domain containing 3	-0.87158	5.96E-98
CD40	CD 40 antigen	-1.8114	0.0
STAT1	Signal transducer and activator of transcription	-0.71919	0.0
CASP7	Caspase 7	-1.0348	1.68E-10
TRAF1	TNF-receptor associated factor	-0.48415	5.42E-12
CD69	CD 69 antigen	-2.9819	1.21E-84

6.3.3. Effect of Salmeterol alone on unstimulated/resting BV2 microglia. After binding to β2-AR, Salmeterol by itself can activate the downstream signaling pathway and related genes. Therefore, we thought it was important to examine the effect of Salmeterol alone on the resting microglia. Results show that Salmeterol can alter the expression of various genes in the absence of LPS-activation and list of genes is shown in **Table 6.2**. Interestingly, we have found that genes related to inflammation and immune response were not significantly affected. However, the expression of CD14 which orchestrates functions of TLR4, was enhanced significantly compared to untreated resting BV2 cells. Salmeterol also enhances the expression of neurotrophic factors such as *Vegfa and Fgf1*.

Table 6. 2: List of top genes regulated by Salmeterol alone in resting BV2 cells.

Gene	Gene Name	DEGs (log_2 Fold Change)	P - value
Crebrf	*CREB3_regulatory_factor*	0.63591	5.37E-05
Crem	*cAMP_responsive_element_modulator*	0.98312	7.04E-09
S100a11	S100_calcium_binding_protein_A11	0.74237	4.69E-10
Vegfa	vascular_endothelial_growth_factor_A	0.70186	8.81E-09
Stat3	signal_transducer_and_activator_of_transcription_3	0.50112	5.63E-07
Abca1	ATP-binding_cassette_sub-family A_(ABC1)_member_1	0.76531	8.57E-14
Arrb2	arrestin_beta_2	0.45036	0.01193
CD14	CD 40_antigen	1.1017	5.83E-25
Vim	vimentin	0.355	7.65E-05
Syn1	synapsin_I	0.58253	4.78E-05
Cebpe	CCAAT/enhancer_binding_protein_(C/EBP)epsilon	0.63756	3.49E-05
Foxo3	forkhead_box_O3	0.50303	1.76E-05
Thbs1	thrombospondin_1	2.3903	1.75E-69
Ube2h	ubiquitin-conjugating_enzyme_E2H	0.66766	2.40E-08
Pde10a	phosphodiesterase_10A	0.75922	8.98E-05
Vdr	vitamin_D_receptor	1.4354	7.85E-12
Csf2rb	colony_stimulating_factor_2_receptor_beta	1.1224	1.36E-27
Creb5	cAMP_responsive_element_binding_protein_5	0.44029	0.014505
Socs3	Suppressor of cytokine signaling_3	0.47574	0.00650
Timm9	translocase_of_inner_mitochondrial_membrane_9	-0.61208	2.75E-05
Timm10	translocase_of_inner_mitochondrial_membrane_10	-0.58783	1.00E-04
Pcna	proliferating_cell_nuclear_antigen	-0.66706	1.25E-11
Usp1	ubiquitin_specific_peptidase_1	-0.51566	6.54E-07
Aen	apoptosis_enhancing_nuclease	-0.81698	1.61E-08
Cdc45	Cell division cycle_45	-0.91161	1.68E-14
Uchl5	**Ubiquitin carboxyl-terminal hydrolase isozyme L5**	-0.45739	0.000115
Trim37	tripartite_motif-containing_37	-0.47712	8.64E-05

6.3.4. Effect of Salmeterol treatment and LPS activation on neurodegenerative disease-related genes. *Vegfa, Vegfb, Fgf 1, Gdnf, Fgf 11and Egf* stand out among neurotrophic factors (NF) gene which were upregulated in Salmeterol+LPS treated group **(Figure 6.4)**. These NFs have shown to be neuroprotective and neurorestorative during neuro-inflammatory and degenerative conditions. Interestingly, many Rab proteins including Rab5, Rab10, Rab 32, Rab7, and Rab 11 which regulate protein trafficking and vesicle formation (380), were also upregulated by Salmeterol treatment in activated BV2 cells. In addition, *Park7, Sncaip, and Syn* genes involved in PD pathogenesis were also either positively or negatively affected by Salmeterol treatment (381–383). Also, *Timm23, Timm50, Timm44, Psen1, Psen2, Casp9, and Casp1* involved in mitochondrial dysfunction and mitophagy, proteolytic cleavage and apoptosis are found among the Alzheimer's disease (384–386) specific gene affected by Salmeterol treatment **(Figure 6.4)**.

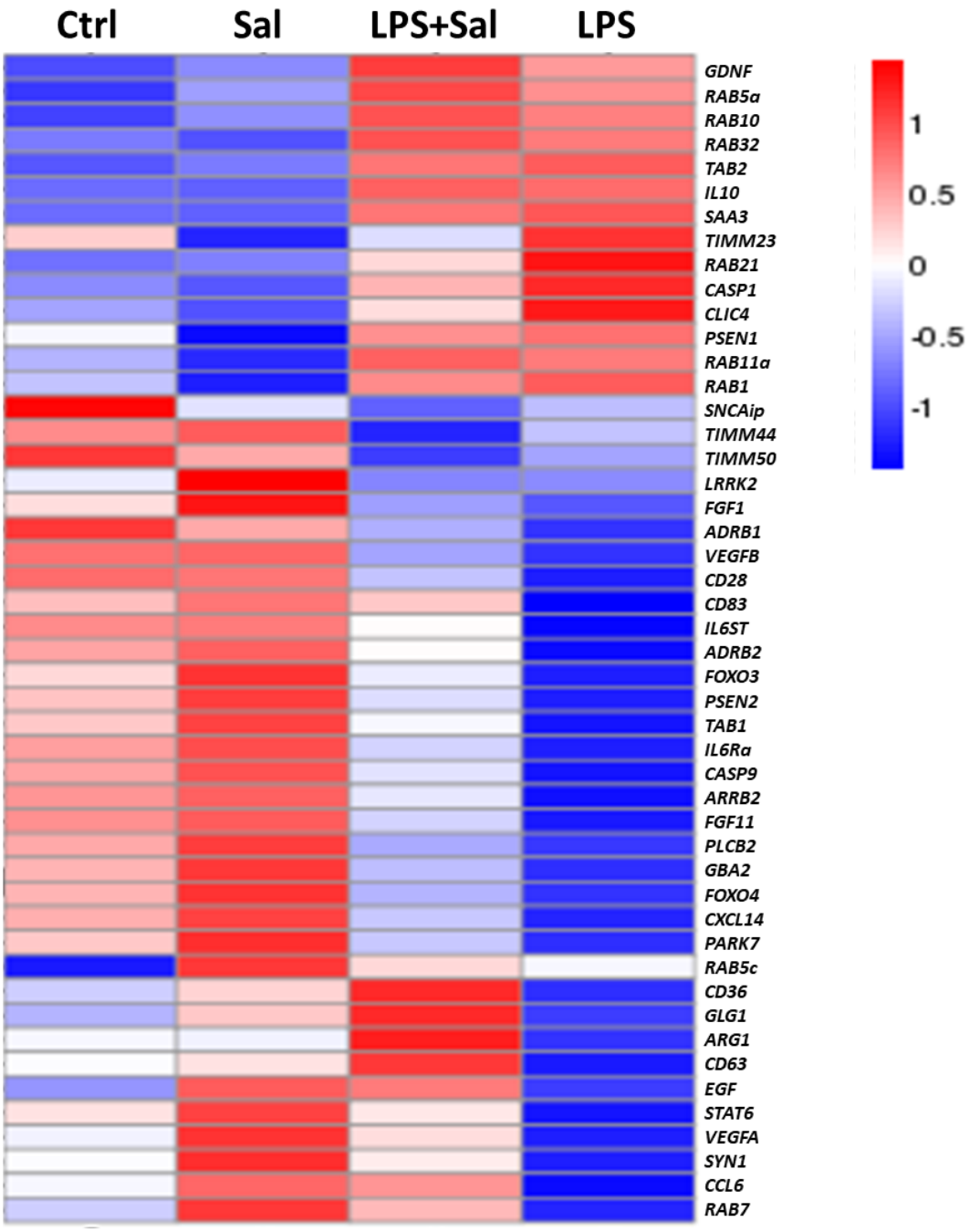
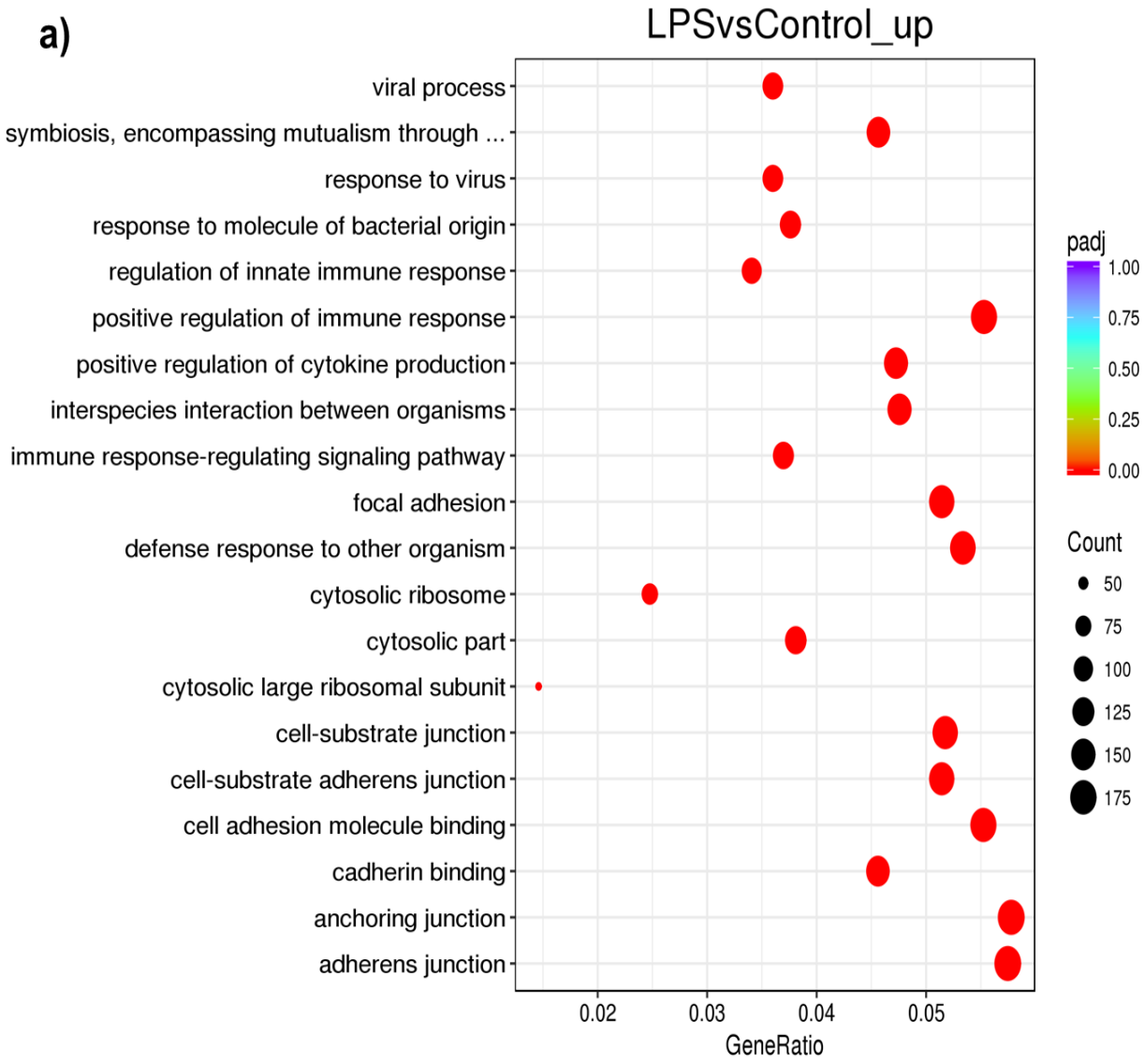


Figure 6. 4: Salmeterol regulates a specific subset of genes-related to neuroinflammation and degeneration.

A heat map representation of differentially expressed genes that were significantly suppressed or upregulated by Salmeterol treatment in LPS-activated BV2 cells. Color descending from red to blue. Indicated log10(FPKM+1) from higher to lower value.

6.3.5. Functional annotation and pathway analysis of Salmeterol treatment in LPS-stimulated BV2 microglia. Furthermore, the enrichment analysis of DEGs were performed to find out which biological functions and pathways are significantly associated with DEGs. The functional annotation was performed using GO (gene ontology) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis. Data shows the GO of DEGs between the control and LPS stimulated group **(Figure 6.5)**. The major biological processes and molecular functions within GO for LPS-stimulated genes were associated with response to molecules of bacterial origin, regulation of the innate immune response and cytokine production and the defense response to other organisms (Figure 6.5A). Furthermore, genes downregulated by Salmeterol in response to LPS-stimulation were functionally classified. Interestingly, we have found that majority of genes came under similar biological processes: positive regulation of innate immune response, defense response to other organisms and response to molecules of bacterial origin (Figure 6.5B). P-value $p<0.05$ were considered as significant enrichment. Additionally, DEGs were subjected to KEGG pathway analysis **(Figure 6.6)** and total of 58 pathways were significantly regulated by Sal in activated BV2 cells (LPS alone vs. Sal+LPS group were compared). The top 20 pathways regulated by Salmeterol in LPS-activated BV2 cells are listed in **Table 6.3**. The TLR-signaling pathway is a key pathway which is activated in response to LPS or other endotoxins. Genes of the TLR-pathway were upregulated in BV2 cells after LPS stimulation (Figure 6.6A) and significantly regulated in BV2 cells treated with both Salmeterol and LPS (Figure 6.6B). Most of the elevated cytokines and chemokines were suppressed in Sal+LPS-treated experimental group. These result show the anti-inflammatory potential of Salmeterol and its therapeutic use against inflammatory diseases.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viral process
symbiosis, encompassing mutualism through ...
response to virus
response to molecule of bacterial origin
regulation of innate immune response
positive regulation of immune response
positive regulation of cytokine production
interspecies interaction between organisms
immune response-regulating signaling pathway
focal adhesion
defense response to other organism
cytosolic ribosome
cytosolic part
cytosolic large ribosomal subunit
cell-substrate junction
cell-substrate adherens junction
cell adhesion molecule binding
cadherin binding
anchoring junction
adherens junction
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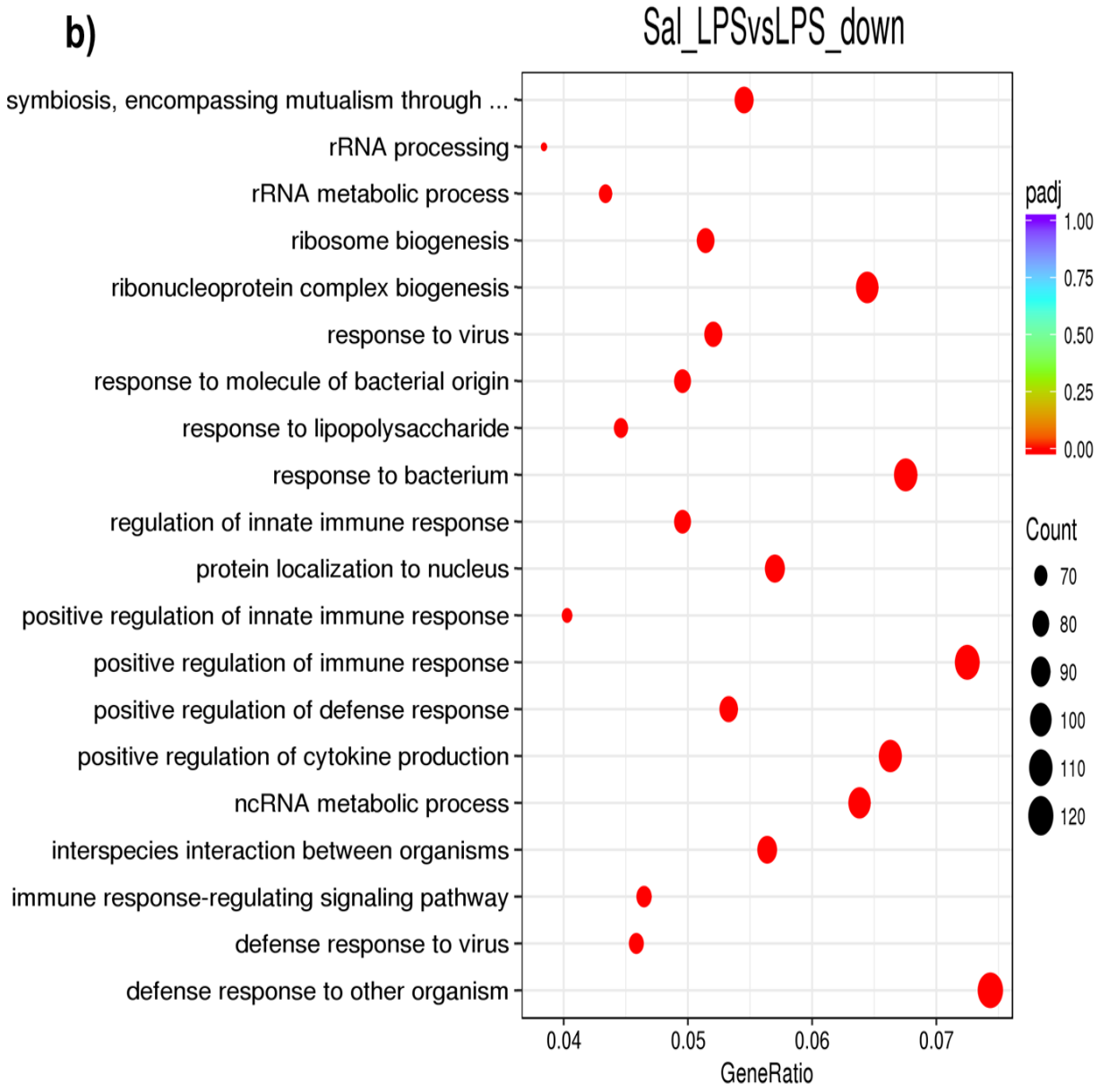
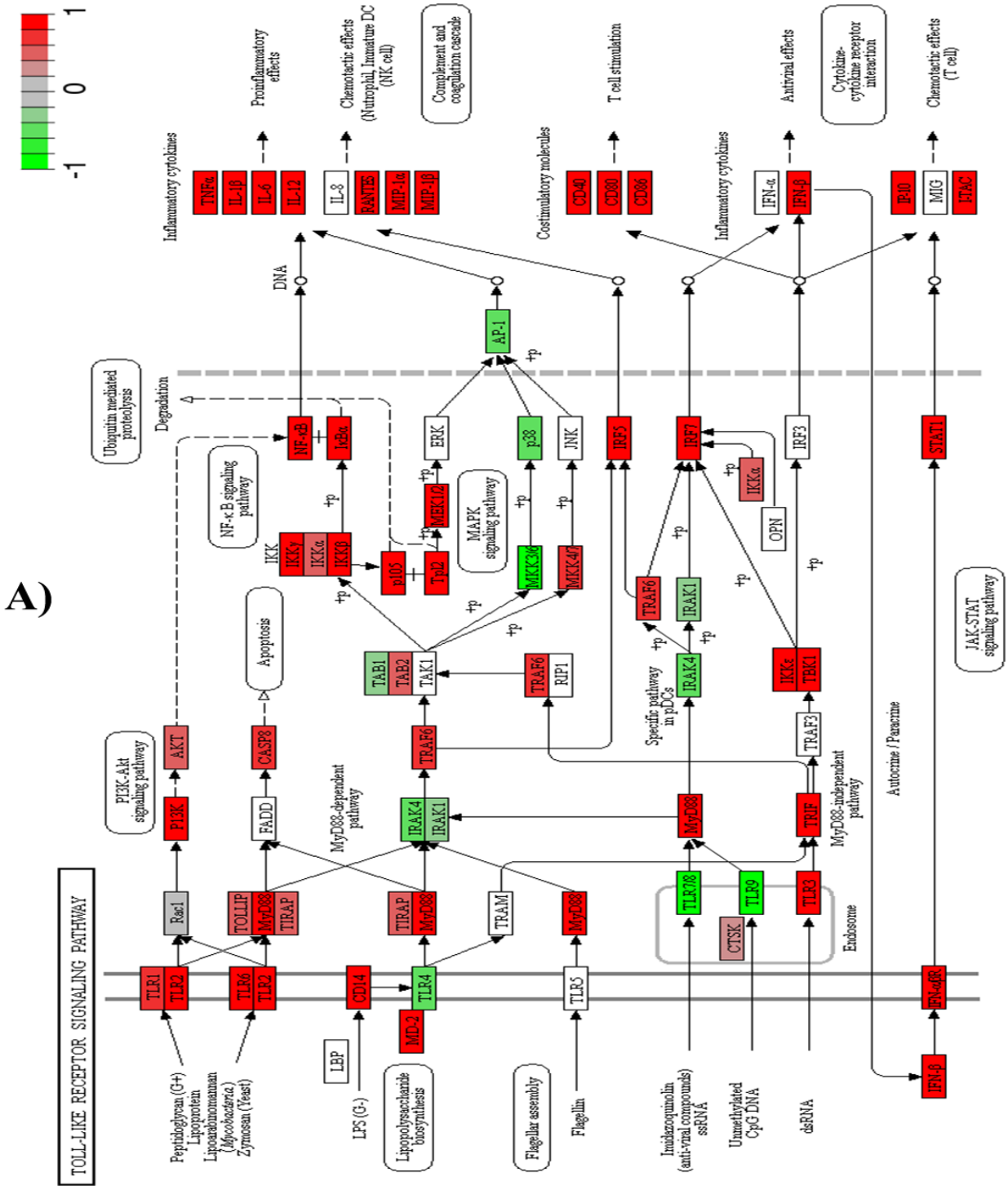


Figure 6. 5: Functional annotation of Salmeterol-mediated down-regulated genes.

GO-term (gene ontology) enrichment analysis for the 'biological process' category of the LPS up-regulated genes (A) and Salmeterol+LPS down-regulated genes (B). Vertical axis represent the functional description of gene ontology. Scatter plot displaying top-20 significant enriched in the GO-enrichment analysis. Horizontal axis indicates gene ratio (ratio of differentially expressed genes to all genes for this GO-term. The size and color of the points depict the gene count (number of differentially expressed gene in respective GO term) and corrected p-value respectively.

Table 6. 3: List of top 20 signaling pathway regulated by Salmeterol in LPS-activated BV2 cells.

	ID	Description	No. of DEGs	p-value
1	Mmu04380	Osteoclast differentiation	42	1.16E-13
2	Mmu03008	Ribosome biogenesis in eukaryotes	32	5.01E-13
3	Mmu05140	Leishmaniasis	24	7.11E-09
4	Mmu04621	NOD-like receptor signaling pathway	22	1.63E-08
5	Mmu00970	Aminoacyl-tRNA biosynthesis	18	4.91E-08
6	Mmu04623	Cytosolic DNA-sensing pathway	21	6.28E-08
7	Mmu04666	Fc gamma R-mediated phagocytosis	27	1.14E-07
8	Mmu04620	Toll-like receptor signaling pathway	29	1.93E-07
9	Mmu04722	Neurotrophin signaling pathway	35	5.55E-07
10	Mmu03040	Spliceosome	32	6.61E-07
11	Mmu04210	Apoptosis	25	8.22E-07
12	Mmu04612	Antigen processing and presentation	23	1.68E-06
13	Mmu05160	Hepatitis C	33	2.37E-06
14	Mmu05145	Toxoplasmosis	31	2.98E-06
15	Mmu04622	RIG-I-like receptor signaling pathway	21	3.41E-06
16	Mmu04062	Chemokine signaling pathway	37	3.38E-05
17	Mmu04010	MAPK signaling pathway	64	3.82E-05
18	Mmu04145	Phagosome	49	4.14E-05
19	Mmu03050	Proteasome	14	8.57E-05
20	Mmu05020	Prion diseases	12	0.000122


A)
TOLL-LIKE RECEPTOR SIGNALING PATHWAY
-1
0
1
Inflammatory cytokines
TNFα
IL-1β
IL-6
IL-12
Proinflammatory effects
IL-8
RANTES
MIP-1α
MIP-1β
Chemotactic effects (Neutrophil, Immature DC (NK cell)
Complement and coagulation cascade
Costimulatory molecules
CD40
CD80
CD86
T cell stimulation
Inflammatory cytokines
IFN-α
IFN-β
Antiviral effects
Cytokine-cytokine receptor interaction
IP-10
MIG
ITAC
Chemotactic effects (T cell)
DNA
Ubiquitin mediated proteolysis
Degradation
AP-1
NF-κB signaling pathway
NF-κB
IκBα
IKK
IKKγ
IKKα
IKKβ
p105
Tpl2
ERK
MEK1/2
MAPK signaling pathway
p38
JNK
MKK3/6
MKK4/7
IRF5
IRF7
IRF3
IKKε
OPN
PI3K-Akt signaling pathway
PI3K
AKT
Apoptosis
CASP8
FADD
MyD88-dependent pathway
TAB1
TAB2
TAK1
TRAF6
RIP1
IRAK4
IRAK1
Specific pathway in pDCs
MyD88
TLR7/8
TLR9
CTSK
Endosome
TRIF
TRAF3
TLR3
MyD88-independent pathway
Autocrine / Paracrine
JAK-STAT signaling pathway
STAT1
IFN-αβR
IFN-β
TLR1
TLR2
TLR6
TLR2
CD14
TLR4
MD-2
TLR5
Rac1
TOLLIP
MyD88
TIRAP
TRAM
Peptidoglycan (G+)
Lipoprotein
Lipoarabinomannan (Mycobacteria)
Zymosan (Yeast)
LBP
LPS (G-)
Lipopolysaccharide biosynthesis
Flagellar assembly
Flagellin
Imidazoquinolin (anti-viral compounds) ssRNA
Unmethylated CpG DNA
dsRNA

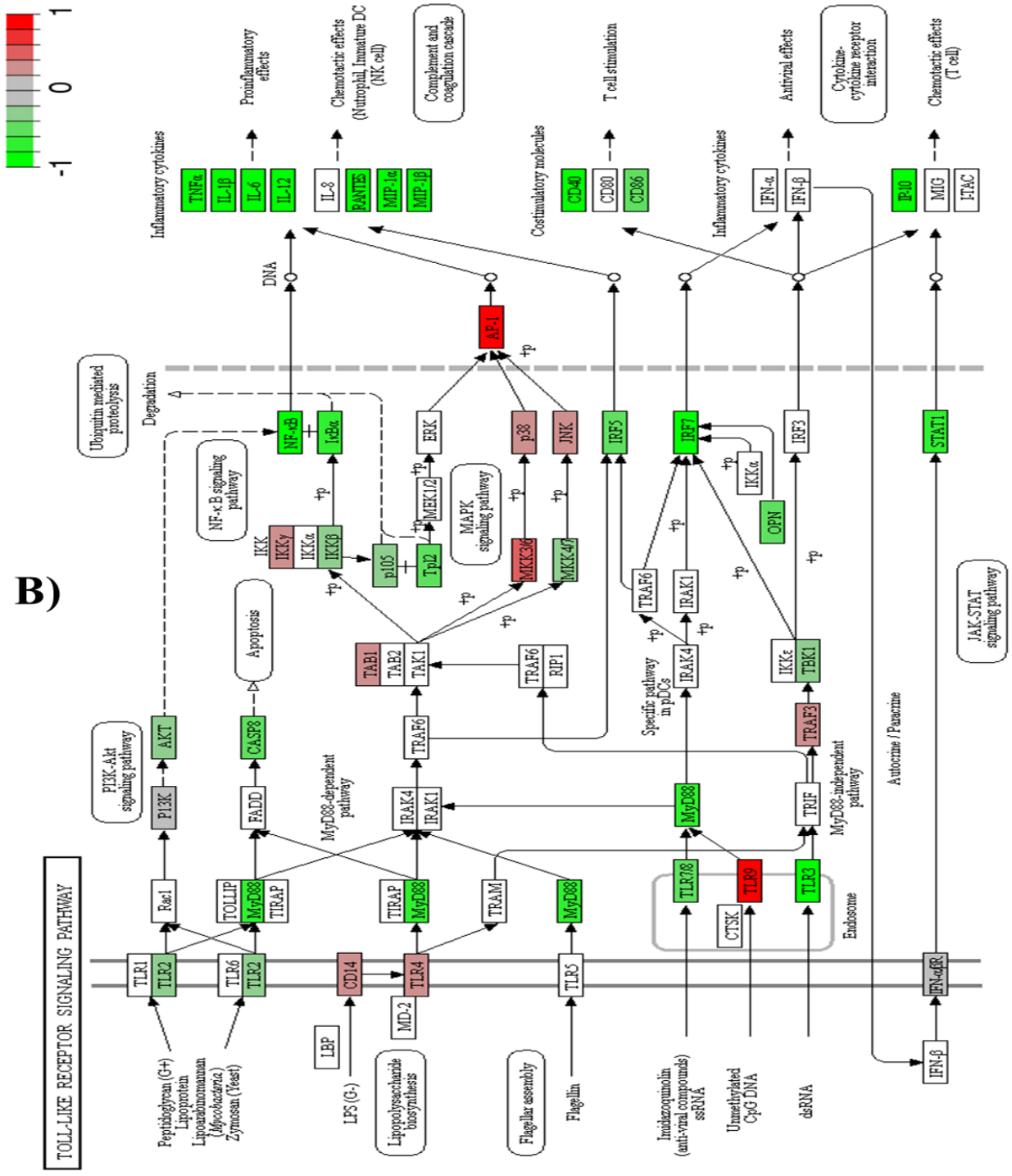


Figure 6. 6: KEGG pathway analysis.
Toll-like receptor (TLR) signaling pathway shows significant difference between LPS-activated and LPS+Sal-treated group. (A) Genes involved in TLR-signaling affected by LPS in BV2 cells (B) Graph shows the genes within the TLR-pathway significantly altered in Sal+LPS experimental group when compared with LPS-treated group.

6.4. DISCUSSION

β2-AR agonists such as Salbutamol and Salmeterol, are FDA-approved drugs that are used primarily as a bronchodilator for the treatment of COPD. However, many studies have now reported a wider prospective for the use of β2-AR agonists as a therapeutic modality in other disease conditions such as acute lung injury (325), IBD (inflammatory bowel disease) (328), systemic inflammation (375) and even PD (237). We have studied the neuroprotective and anti-inflammatory effects of β2-AR in *in vitro* and *in vivo* models of PD, and found it to be a highly effective therapeutic treatment in animal models of PD (20). In this chapter, we present RNA-Seq data that shows Salmeterol downregulates expression of key inflammatory mediators including cytokines and chemokines. Our data also verified some of our previous finding as well as revealing new inflammatory genes regulated by Salmeterol. Inflammatory genes such as *Tnfα, Il1a, Il1b, Il6, Il15, Il18, Il27, Il12b* and also *NF-κB* were significantly downregulated by Salmeterol. These cytokines have been associated with many neurodegenerative diseases including Parkinson's, Alzheimer's, Huntington's, multiple sclerosis, stroke and many others (342). IL-1α and IL-1β plays critical role in early-onset of PD and AD and also serve as pathological hallmark of CNS inflammation (387). These pro-inflammatory cytokines are critical regulator of microglial activation and neuroinflammation. In addition, Salmeterol also downregulates many inflammatory chemokines such as *Ccl2/Mcp-1, Ccl3, Ccl4, Ccl5, Ccl7, Ccl12, Cxcl2, and Cxcl10* which have been associated with progression of neurodegenerative diseases (354). MCP-1, CCL3, CCL4 and Cxcl10 influences neuronal loss, progression of the diseases and cognitive impairments in PD patients (355,356). Similarly, Salmeterol suppresses TLR-activated increased expression of CD40 which is critical for immune response and also negatively regulates TLR-mediated inflammasome

activation in microglia (388). In addition, other key inflammatory genes like *Fcgr1, Ikbkb, Myd88*, *CD86* and *CD69* were also significantly downregulated by Salmeterol in LPS-activated BV2 cells. It has been shown that NF-κB, IRFs and STATs are key transcription factors involved immune responses. Interestingly, our results show that Salmeterol reduces the expression of *Irf1, Irf2, Irf5, Irf7, NF-κB* and *Stat1*. IRF1 and IRF7 are associated with viral response in neuroinflammation (389) and M1-like microglia polarization switch (390,391). Similarly, STAT1 is also involved in IFN-γ-mediated degeneration of dopaminergic neurons (392). Furthermore, the role of Nlrp3-associated inflammatory response in PD has been studied by many researchers (393–395) and targeting Nlrp3 inflammasome signaling has therapeutic benefits against dopaminergic neurodegeneration (396,397) and other inflammation-associated neurological disorders (398). In support of these findings, our finding shows that Salmeterol significantly suppresses the expression of *Nlrp3* and its associated genes such as *Il1a* and *Il1b*. Similar to our results, Song et al have shown that Salmeterol suppressed LPS-induced systemic inflammation via inhibiting the Nlrp3 inflammasome (375). In addition, our data also shows that Salmeterol downregulates the expression of *Casp7* and *Casp8* which have been associated with neuronal apoptosis and inflammation (399,400). Caspase 8 also mediates amyloid-beta-induced apoptosis in AD model (401). Thus, these findings suggest that Salmeterol can be used as therapy against neuroinflammation associated with CNS diseases.

Salmeterol also regulates the expression of variety of Rab proteins including *Rab1, Rab5, Rab7*, *Rab10*, and *Rab32*. Over-expression of Rab5 and Rab7 induces the clearance of α-synuclein aggregates in A53T mutant mice model of PD (402). Enhanced expression of Rab5 and Rab7 by Salmeterol, suggests its therapeutic potential for targeting the synucleopathy. Similar to our results, Mittal et has shown that β2-AR agonist Salbutamol and Clenbuterol reduces the expression of

SNCA, the gene responsible for expression of α-synuclein in PD models. They have shown that β2-AR agonist reduces SNCA transcription via histone 3 lysine 27 acetylation of its promoter and enhancer (237). Our results suggest another potential mechanism by which Salmeterol can regulate the over-expression of α-synuclein. Similarly, role of Rab1, Rab 10, and Rab 32 has been studied extensively in LRRK2 model of PD (403–405). Interestingly, Salmeterol downregulates the expression of Sncaip or Synphilin-1 which inhibits degradation of α-synuclein and has implication in PD pathogenesis (406). In addition, the KEGG pathway analysis reveals the potential role of Salmeterol in regulating the several important pathways related to human diseases. Data also shows that Salmeterol-treatment in activated BV2 cells regulates the proteasome and phagosome pathway which is highly associated with several neurodegenerative conditions including PD and AD (407). The prion pathway which has been linked with tau pathology and synucleopathy (408), was also regulated by Salmeterol treatment.

The verification of the effect of Salmeterol on select inflammatory gene expression by qPCR analysis has been included in the chapter 5 of this thesis document. Altogether, this genome-wide analysis by RNA-seq shows that Salmeterol-treatment of LPS-activated BV2 cells regulates the expression of inflammatory mediators as well genes-related to neurodegenerative diseases. However, further validation of genes regulated by Salmeterol still needs to be performed. Also, *in vivo* experimentation is required to investigate the molecular mechanisms by which Salmeterol regulates disease-related genes which have been implicated in inflammation-mediated neurodegenerative diseases. In summary, this study is the first to perform a genome-wide analysis of Salmeterol's effect on resting and LPS-activated BV2 cells, and suggests its efficacy in the therapeutic application toward the treatment of neurodegenerative diseases.

CHAPTER 7

Salmeterol; A long-acting β2-adrenergic receptor agonist inhibits macrophage activation by lipopolysaccharide from *Porphyromonas Gingivalis*

This chapter is modified from the published article:

Sharma, M., Patterson, L., Chapman, E., Flood, P.M., (2017). ***Salmeterol, a long-acting β2-adrenergic receptor agonist, inhibits macrophage activation by lipopolysaccharide from Porphyromonas gingivalis***. J. Periodontology. 88 (7), 681–692.

7.1. ABSTRACT

Background: Salmeterol is a long-acting β2-adrenergic receptor agonist used to treat chronic obstructive pulmonary disease. The authors of the current study previously showed that pre-incubation of primary microglial-enriched cells with Salmeterol could inhibit the inflammatory response induced by *Escherichia coli* lipopolysaccharide (LPS), a Toll-like receptor (TLR)-4 agonist. In this study, the authors sought to determine if Salmeterol had a similar inhibitory effect on the inflammatory response of the murine macrophage cell line RAW264.7 and human monocyte THP-1 to LPS from *Porphyromonas gingivalis* (*Pg*LPS), an oral microbe implicated in the pathogenesis of periodontal disease.

Methods: RAW264.7 and THP-1 cells were pretreated with Salmeterol, followed by *Pg*LPS, and monitored for production of inflammatory mediators by enzyme-linked immunosorbent assay. The nitric oxide concentration and nuclear factor-kappa B (NF-κB) activity were measured by Griess method and secretory alkaline phosphatase reporter activity assay, respectively. Reverse-transcriptase polymerase chain reaction and immunoblot analysis were used to measure messenger RNA and protein levels. Nuclear translocation of NF-κB was detected by immunofluorescence.

Results: Pre-treatment with Salmeterol significantly inhibited production of pro-inflammatory mediators by RAW264.7 and THP-1 cells. Salmeterol downregulated *Pg*LPS-mediated phosphorylation of the extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase but not p38 mitogen-activated protein kinases (MAPKs). Salmeterol also attenuated activation of NF-κB via inhibition of nuclear translocation of p65-NF-κB, the transcriptional activity of NF-κB and IκBα phosphorylation.

Conclusion: Salmeterol can significantly inhibit activation of macrophage-mediated inflammation by *Pg*LPS, suggesting that use of Salmeterol may be an effective treatment in inhibiting or lessening the inflammatory response mediated through TLR pathway activation.

7.2. INTRODUCTION

Periodontitis is the most common chronic oral inflammatory disease in humans (409). Periodontal disease is usually caused by accumulation of bacterial biofilm and inflammation in tooth surrounding tissues. Inflammation spreads deep into tissue, causing loss of tissue and alveolar bone, leading to periodontal pocket formation (410). It is well known that the oral anaerobic Gram-negative bacterium *Porphyromonas gingivalis* (*Pg*) is strongly associated with onset of moderate and severe periodontitis, and it can also increase the risk of systemic chronic inflammatory conditions such as atherosclerosis (411), rheumatoid arthritis (412,413), cancer (414,415), chronic kidney disease (416), pneumonia (417,418), COPD (419), and adverse pregnancy outcomes (420). Macrophages in gingiva are known for phagocytosis of periodontal bacteria. The LPS from microorganisms is recognized by Toll-like receptors (TLRs) on the cell surface, which further initiates the immune response against pathogens in macrophages; these activated macrophages can produce inflammatory mediators in response to pathogens (421,422). One of the virulence factors of *Pg* is LPS (*Pg*LPS), which activates immunity by targeting TLRs on macrophages to initiate the inflammatory cascade, resulting in the secretion of inflammatory mediators (423,424). These mediators, including TNF-α, and NO help mediate immune protection against pathogenic infection, but also play a role in tissue destruction (425–427) allowing entry of immune cells, such as neutrophils, into the infection site (428), and bone resorption associated with periodontal disease (429). Production of inflammatory mediators is regulated by intracellular signaling cascades, the most important of which are the NF-κB and MAPK pathways (430,431). TLR activation by *Pg*LPS can stimulate both the canonical NF-κB pathway and the MAPK pathway, and regulation of these pathways can be an important therapeutic approach to treatment of periodontal disease (432). LPS-activated macrophages also induce production of reactive oxygen species, which can also act as a

secondary messenger for NF-κB activation and further lead to production of inflammatory mediators including interleukin (IL)-1β, TNF-α, and NO (426,433). NO is a ubiquitous free radical that is involved in various pathophysiologic processes and also is associated with tissue injury (427,433). Immune cells, including gingival macrophages, have a high density of β2-ARs on their surface, and engagement of these receptors by β2-AR agonists can result in immunomodulatory actions (434). Therefore, the authors have studied the ability of Salmeterol to suppress the pro-inflammatory response in a range of immune cells, including murine RAW264.7 macrophages and human THP-1 monocytes. These β2-AR agonists have been shown to regulate both the NF-κB and MAPK pathways, resulting in either activation or inhibition of chronic inflammation (435). Salmeterol is a long-acting β2-AR agonist that is currently being used as a bronchodilator in the treatment of COPD (285,293). Previously, the authors have shown that Salmeterol can inhibit the inflammatory activity of microglial cells and can be used as an effective therapy in the treatment of chronic inflammatory diseases (20). In a previous study by the authors, LPS from *Escherichia coli* (a TLR-4 agonist) was used to initiate chronic neurodegeneration in a murine model of Parkinson disease, and it was determined that Salmeterol can inhibit dopaminergic neurodegeneration by inhibiting production of inflammatory mediators through an NF-κB- and MAPK-dependent mechanism (20). Similarly, other β2-AR agonists like salbutamol and albuterol have also been found to be inhibiting the inflammatory cytokine production in human monocytes (436). Taken together, these studies demonstrated that Salmeterol and other β2-AR agonists could inhibit LPS-mediated activation of macrophages through a TLR-4 dependent pathway. On the basis of previous studies, the authors hypothesized that the long-acting β2-AR agonist Salmeterol could exhibit anti-inflammatory effects on TLR-mediated inflammation by stimulating the murine macrophage cell line RAW264.7 and human monocytes cell line THP-1 with *Pg*LPS. It was found

that Salmeterol can significantly suppress the *Pg*LPS induced production of inflammatory cytokines and NO in both murine macrophages and human monocytes by inhibiting the extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and NF-κB pathways.

7.3. RESULTS

7.3.1. Salmeterol inhibits *Pg*LPS-stimulated release of inflammatory cytokines from RAW264.7 cells and THP-1 cells.

To investigate the effect of Salmeterol on *Pg*LPS stimulated inflammatory response, concentration of pro-inflammatory cytokines was measured by ELISA. Release of pro-inflammatory cytokines was significantly increased upon stimulation with *Pg*LPS in RAW and THP-1 cells. **Figure 7.1** shows elevated concentrations of pro-inflammatory cytokines were significantly decreased by pretreatment with Salmeterol in both RAW (TNF-α by 1.5-fold, $P < 0.01$; IL-6 by 2.0-fold, $P < 0.05$; IL-1β by 2.5-fold, $P < 0.01$; Figs. 7.1A through 1C) and THP-1 cells (TNF-α by 1.7-fold, $P < 0.05$; IL-6 by 1.8-fold, $P < 0.05$; IL-1β by 1.5-fold, $P < 0.01$; and IL-8 by 1.6- fold, $P < 0.05$; Figs. 7.1D through 1G).

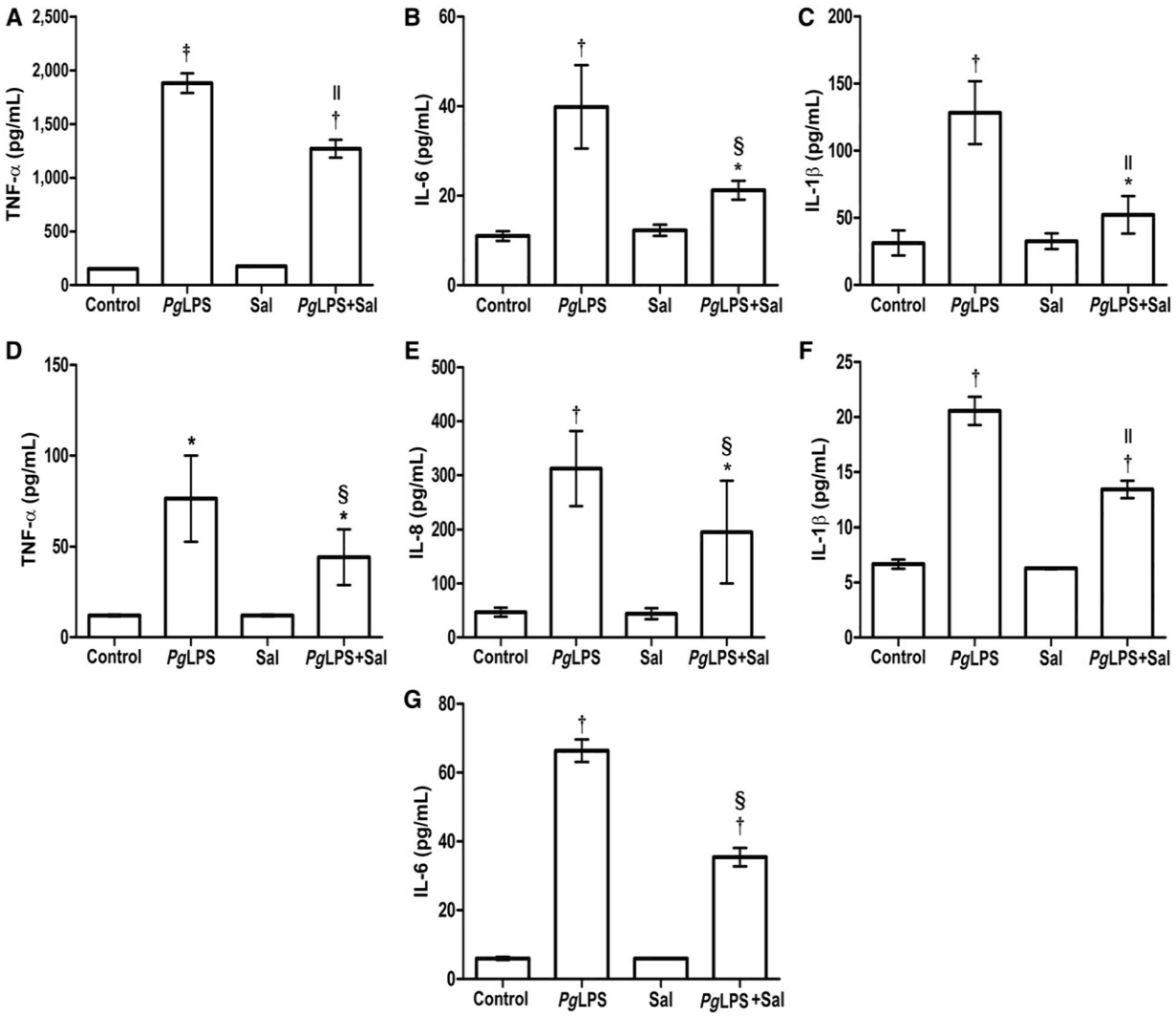


Figure 7. 1: Effect of Salmeterol (Sal) on production of pro-inflammatory cytokines in *Pg*LPS-stimulated RAW264.7 and THP-1 cells.

RAW and THP-1 cells were pretreated with Salmeterol (10^{-9} M) for 45 minutes and stimulated with *Pg*LPS (500 ng/mL). After 24 hours incubation, the levels of TNF-α, IL-6, and IL-1β (A through C) in RAW264.7 cell culture supernatant and TNF-α, IL-6, IL-1β, and IL-8 (D through G) in THP-1 cell culture supernatant were measured by ELISA kits. Data represented as mean ± SD of three individual experiments. $^{*}P$ <0.05; $^{\dagger}P$ <0.01; $^{\ddagger}P$ <0.001 represent a significant difference from the unstimulated control group. $^{\S}P$ <0.05; $^{\|}P$ <0.01 represent significant difference from the *Pg*LPS-treated group.

As shown in **Figure 7.2**, messenger RNA (mRNA) expression level of cytokines was upregulated upon stimulation with *Pg*LPS in RAW and THP-1 cells. Similar suppressive effects were observed on cytokine production when mRNA expression level of cytokines by RT-PCR was measured. Data shows that Salmeterol could significantly suppress mRNA level of TNF-α (2.3 to 1.1- fold, $P < 0.01$), IL-1β (2.1 to 1.4-fold, $P < 0.05$), and IL-6 (1.9 to 1.3-fold, $P < 0.05$) in RAW cells (Figs. 7.2A through C). Data also showed that Salmeterol could significantly suppress mRNA level of TNF-α (2.0 to 1.2-fold, $P < 0.01$), IL-1β (2.2 to 1.5-fold, $P < 0.01$), IL-6 (1.9 to 1.3-fold, $P < 0.05$), and IL-8 (3.0 to 2.1-fold, $P < 0.05$) in THP-1 cells (Figs. 7.2D through G). This showed significant anti-inflammatory effects of Salmeterol against *Pg*LPS-stimulated inflammatory response in both RAW and THP-1 cells (Figs. 7.1 and 7.2).

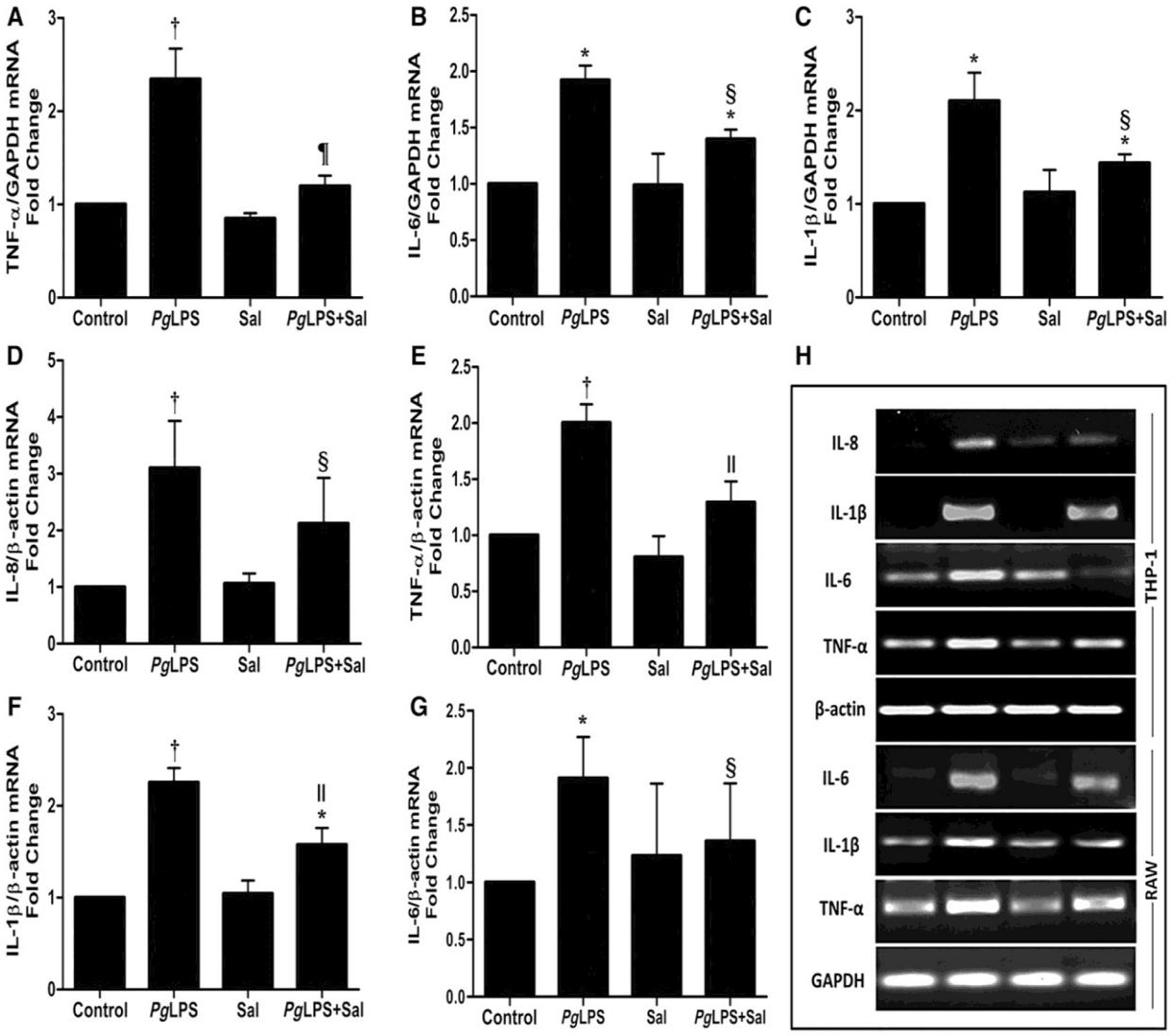


Figure 7. 2: Effect of Salmeterol (Sal) on *Pg*LPS-induced inflammatory mediators.

TNF-α, IL-6, IL-1β mRNA expression in RAW264.7cells (A through C) and TNF-α, IL-6, IL-8, and IL-1β mRNA expression in THP-1 cells (D through G). Cells were pretreated with Salmeterol (10^{-9} M) for 45 minutes and stimulated with *Pg*LPS (500 ng/mL) for 6 hours. Cells were harvested, and the extracted RNA of cells was subjected to semi-quantitative RT-PCR (H). The relative mRNA levels were normalized to β-actin and GAPDH. Data represents outcome of three individual experiments (as mean ± SD). mRNA data expressed as fold change compared with control. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ indicate significant difference from the unstimulated control group. $^{\S}P < 0.05$; $^{\|}P < 0.01$; $^{\P}P < 0.001$ represent significant difference from the *Pg*LPS-treated group.

7.3.1. Salmeterol inhibits *Pg*LPS-induced NO production in RAW264.7 and THP-1 Cells

Effect of Salmeterol on *Pg*LPS-induced NO production in RAW and THP-1 cells was assessed by measuring NO_2 concentrations using Griess assay. Cells were pretreated with Salmeterol and stimulated with *Pg*LPS for 24 hours. Concentration of nitrite in control groups (untreated and Salmeterol alone) was undetectable (<1 mM), and it was found that *Pg*LPS stimulation elevated the level of NO_2 (≤3.1 ± 0.182 SEM mM in RAW cells; $P < 0.001$, and 3.3 ± 0.018 SEM mM in THP-1 cells; $P < 0.001$ cells). **Figure 7.3** shows that pre-incubation with Salmeterol significantly inhibited *Pg*LPS-induced nitrite production in both RAW (down to 1.8 ± 0.125 SEM mM; $P < 0.01$ [Fig. 7.3A]) and THP-1 (down to 1.9 ± 0.150 SEM mM; $P < 0.01$ [Fig. 7.3B]) cells. RT-PCR was used to measure the effect of Salmeterol on expression of mRNA for the regulatory enzyme for NO synthesis, the inducible nitric oxide synthase (iNOS). Data showed *Pg*LPS upregulated iNOS expression (by 2.0-fold, $P < 0.01$). This upregulation was significantly reduced (down to 1.5-fold, $P < 0.05$) by pretreatment with Salmeterol (Fig. 7.3C).

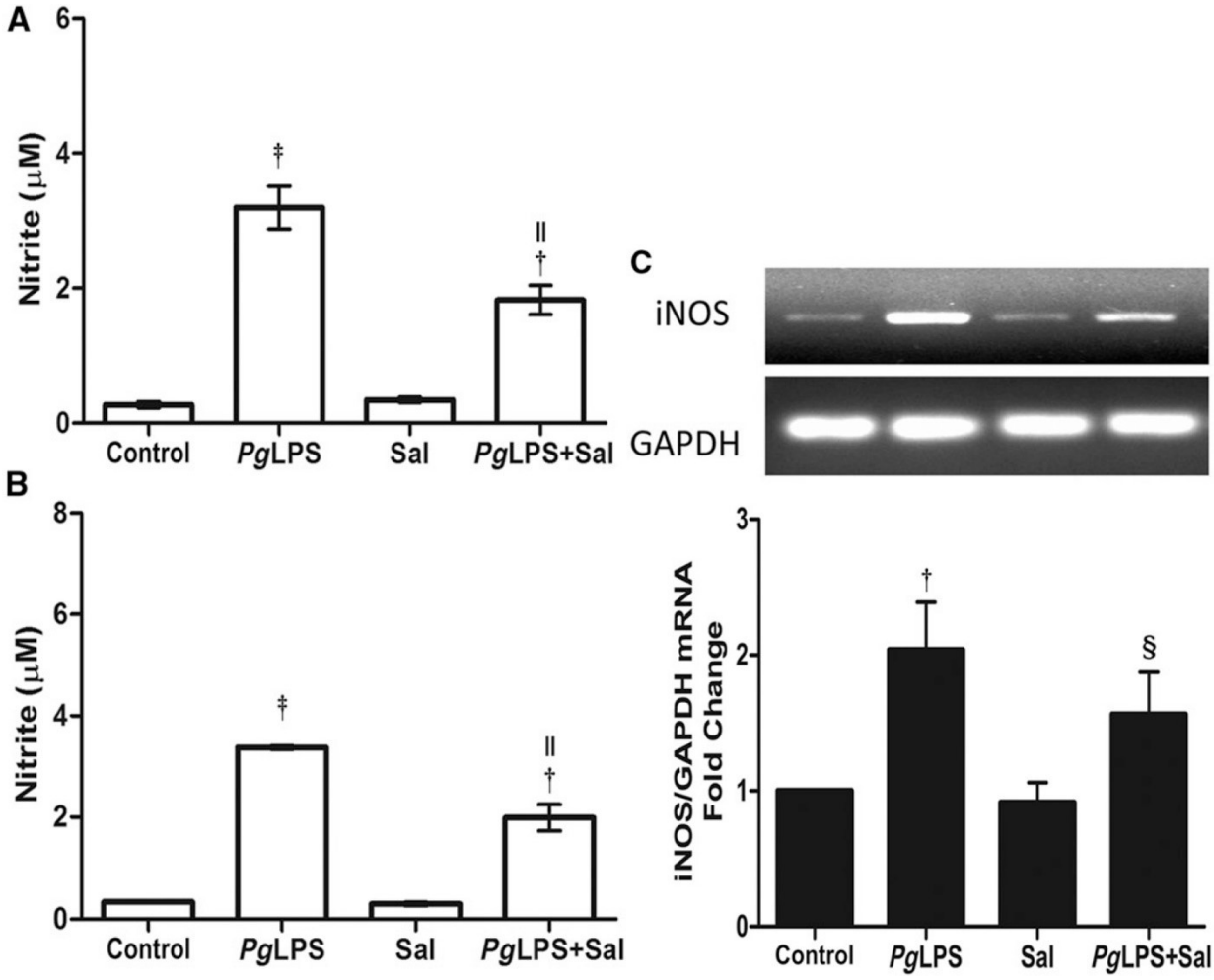


Figure 7. 3: Inhibitory effect of Salmeterol (Sal) on production of NO in *Pg*LPS-stimulated RAW264.7 macrophages and THP-1 monocytes.

Cells were pretreated with Salmeterol (10^{-9} M) for 45 minutes and stimulated with *Pg*LPS (500 ng/mL) for 24 hours. Supernatant was collected and the level of NO was measured by Griess reaction assay. Bar graphs represent the concentration of NO in supernatant of A) RAW and B) THP-1 cells. C) The mRNA level of iNOS in RAW264.7 macrophages was also measured by real-time PCR. Bar graph represents iNOS mRNA levels in indicated conditions and GAPDH mRNA levels were used to normalize iNOS mRNA level. Data expressed as mean ± SD of three individual experiments. ‡P <0.01; ‖P <0.001 indicate significant difference from the unstimulated control group. †P <0.05; §P <0.01 indicate significant difference from *Pg*LPS-stimulated group.

7.3.3. Salmeterol inhibits activation of the MAPK pathway in *Pg*LPS-stimulated RAW264.7 cells. The mechanism by which Salmeterol inhibits cytokine and NO production in RAW cells was examined by investigating the effect of Salmeterol on activation of the major inflammatory signaling pathways MAPK. In **Figure 7.4**, the immunoblot analysis indicated that the *Pg*LPS stimulated group showed significant upregulation of phosphorylation of ERK1/2 (7.1-fold, $P < 0.01$), JNK (2.2-fold, $P < 0.01$), and p38 (11.3-fold, $P < 0.05$) MAPKs, whereas the group pretreated with Salmeterol attenuated the *Pg*LPS-induced phosphorylation of ERK1/2 (down to 5.3-fold, $P < 0.05$) and JNK (down to 1.5-fold, $P < 0.05$) significantly. Interestingly, phosphorylation of p38 was not significantly inhibited (Fig. 7.4).

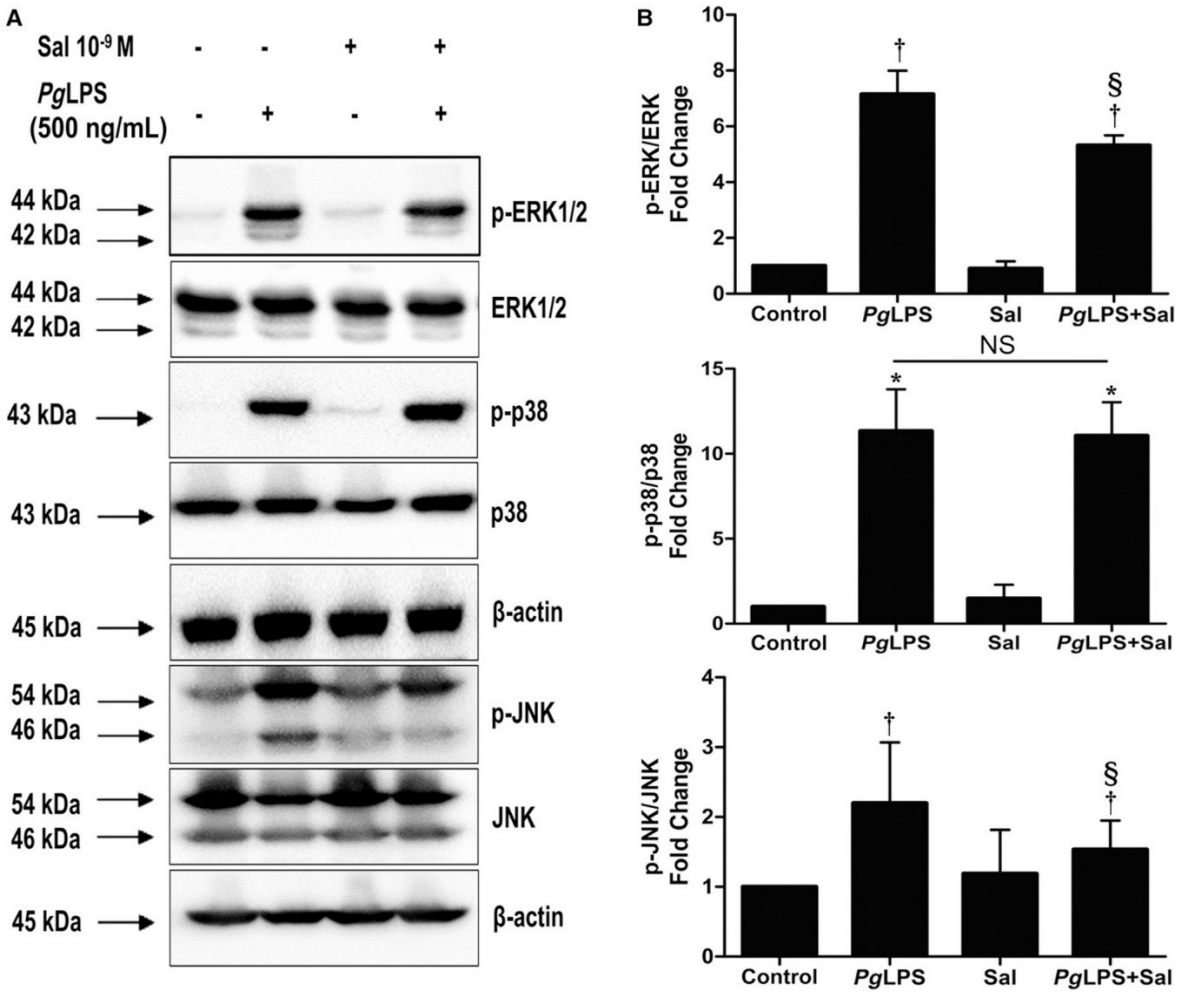
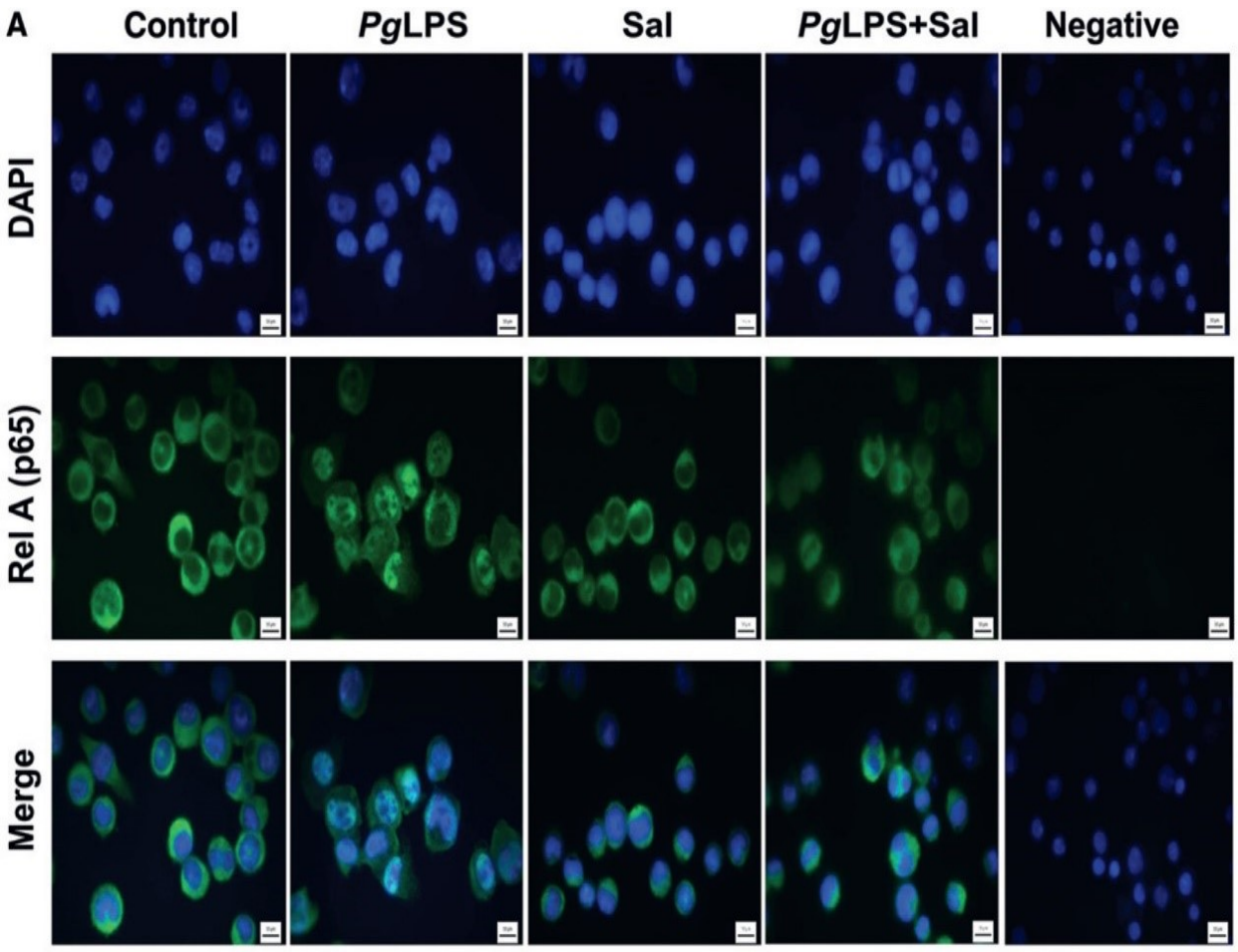


Figure 7. 4: Pre-treatment with the β-adrenergic receptor agonist Salmeterol (Sal) suppresses *Pg*LPS-induced activation of MAPKs in RAW264.7cells.

A) Cells were treated with Salmeterol 45 minutes prior to *Pg*LPS (500 ng/mL) and harvested 45 minutes after *Pg*LPS stimulation. MAPKs and phosphorylation of MAPKs were detected by Western blot analysis. B) Bar graphs represent fold change compared with control, where p-ERK, p-p38, p-JNK signals were normalized with ERK, p38, JNK signals respectively. Data expressed as fold change compared with control. Results are representative of four individual experiments (mean ± SD). †P <0.05; *P <0.01 indicate significant difference from the unstimulated control group. §P <0.05 represents significant difference from the *Pg*LPS-treated group. NS = not significant.

7.3.4. Salmeterol inhibits activation and expression of NF-κB pathway in PgLPS-stimulated RAW264.7 cells. Immunofluorescence staining was performed to image NF-κB (p65) translocation from cytoplasm to nucleus in RAW cells treated with *Pg*LPS and Salmeterol, and data represents the decreased nuclear translocation of NF-κB in response to pretreatment with Salmeterol **(Figure 7.5A)**. The suppressive effect of Salmeterol on the activation of NF-κB transcriptional activity was examined using RAW cells. It was found that the increased (≤0.5 OD 635 nm ± 0.020 SEM; P <0.001) SEAP production observed in *Pg*LPS-treated cells was significantly suppressed (≤0.1 OD 635 nm ± 0.024 SEM; P <0.001) by pre-incubation with Salmeterol (Fig. 7.5B). The effect of Salmeterol on activation of the NF-κB pathway induced by *Pg*LPS was examined by measuring IκBα phosphorylation and nuclear translocation of NF-κB (p65). RAW cells stimulated with *Pg*LPS showed a 13-fold increase (P <0.001) in phosphorylation of IκBα, and pretreatment with Salmeterol showed a significant inhibition (by 1.6-fold, P <0.01) of phosphorylation of IκBα (Fig. 7.5C). Similarly, the *Pg*LPS-stimulated group showed increased nuclear translocation of NF-κB (p65) (by ≤2.5-fold, P <0.05), and pretreatment with Salmeterol reduced nuclear translocation to 1.7-fold (P <0.05; Fig. 7.5D). In addition, expression of NF-κB was reduced (to 0.6-fold, P <0.01) in cytosol in response to *Pg*LPS stimulation, and pretreatment with Salmeterol abolished the effect of *Pg*LPS by enhancing (≤0.9-fold, P <0.01) expression of NF-κB in cytosol (Fig. 7.5E). Taken together, these results indicate that Salmeterol suppresses phosphorylation and degradation of IκBα and nuclear translocation of NF-κB (p65).


A
Control
PgLPS
Sal
PgLPS+Sal
Negative
DAPI
Rel A (p65)
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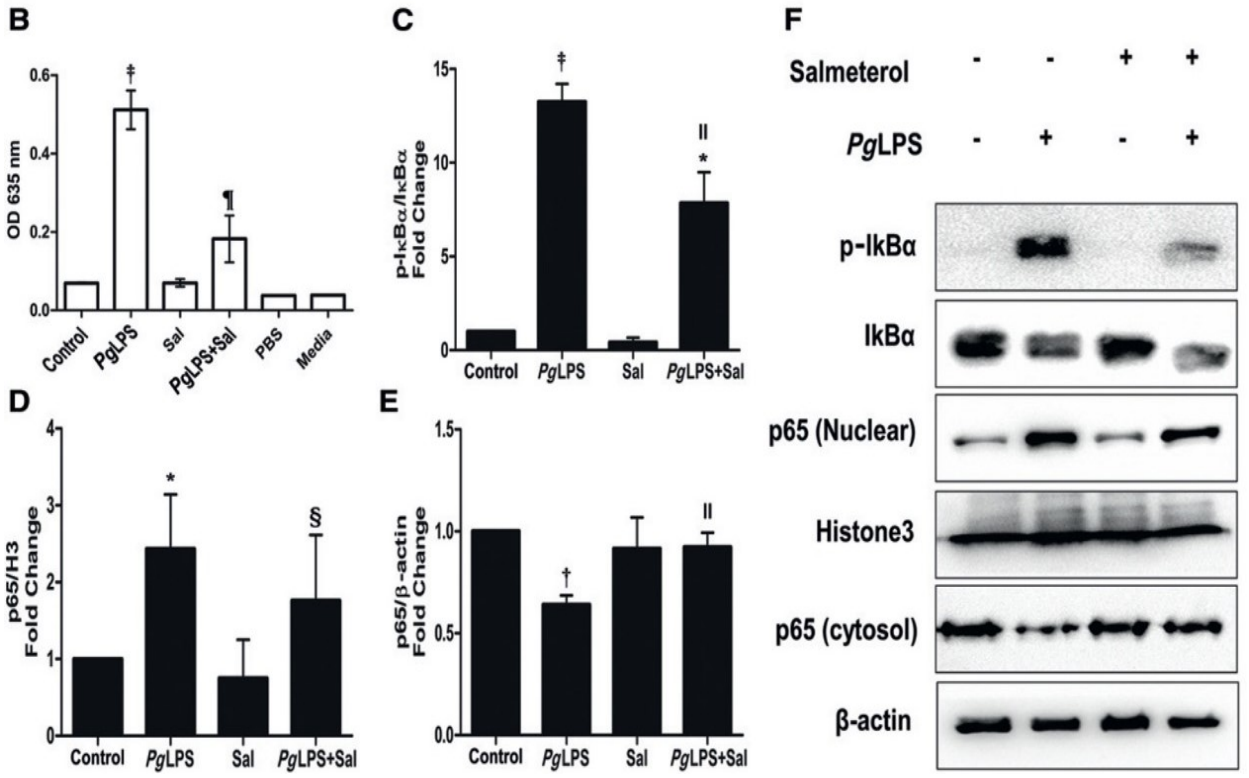

Figure 7. 5: Effect of Salmeterol (Sal) on *Pg*LPS-mediated activation of NF-κB in RAW cells:

(A) Immunofluorescence staining (nuclear and cytoplasmic p65) demonstrate NF-κB (p65) nuclear translocation. Scale bar, 10 μm. (B) NF-κB activation by reporter gene assay (SEAP activity): RAW cells were incubated with *Pg*LPS after 45 minutes pre-incubation with Salmeterol. Supernatant was collected after 24 hours, and SEAP activity was measured spectrophotometrically at 635 nm. Bar graph represents colorimetric change in culture supernatant of RAW cells after *Pg*LPS stimulation. PBS = phosphate-buffered saline. (C through F) Western blots and quantitative analysis of effect of Salmeterol on NF-κB p65 nuclear translocation and IκBα phosphorylation in RAW264.7 cells. The expression of IκBα and p-IκBα protein measured from whole cell lysate. Cells were pre-incubated with Salmeterol and stimulated with *Pg*LPS. Bar graphs represent fold change compared with control. The H3 and β-actin were used as the loading control for nuclear and cytoplasmic p65, respectively. Data represent the average of three replicates and are mean ± SD of three different experiments. †P <0.05; *P <0.01; ‡P <0.001 indicate significant difference from the unstimulated control group. ‖P <0.05; §P <0.01; ¶P <0.001 represent significant difference from the *Pg*LPS-treated group.

7.4. DISCUSSION

Pg is a Gram-negative anaerobic bacterium that has been found to be a major oral pathogen associated with periodontal disease (437). *Pg* can activate innate immune cells by production of LPS, and this LPS has been found to activate primarily via the TLR signaling pathway (423,424,438). The exact TLR-mediated mechanism of *Pg*LPS stimulation is still somewhat controversial, with previous results suggesting that *Pg*LPS can stimulate both TLR-2 and TLR 4 (438–441). A previous study by the authors has shown that a number of β2-AR agonists, such as Salmeterol, can significantly inhibit the inflammatory response of primary microglia-enriched cell culture induced by the TLR-4 agonist LPS from *E. coli* (20). In the results presented here, it is shown that Salmeterol can also significantly inhibit the *Pg*-LPS-induced inflammatory response of monocytes/macrophages, both murine and human, by inhibiting production of inflammatory cytokines as well as NO, and by reducing activation of two major inflammatory signaling pathways, MAPK and NF-κB. These results are identical to the anti-inflammatory effects the authors have observed with Salmeterol using *E. coli* LPS and suggests the effect of Salmeterol may not be limited to inhibition of only TLR-4-mediated inflammation on murine microglial cells, but may be a more general anti-inflammatory agent that also inhibits the TLR-2/4-mediated inflammatory responses in both murine and human macrophage/ monocytes.

The increase in expression of pro-inflammatory cytokines and NO is one of the essential features of the inflammatory response by innate immune cells. Exposure of bacterial products, such as LPS, can trigger an inflammatory response, which includes release of cytokines and other inflammatory mediators (442). It is thought that release of LPS by Gram-negative bacteria in the gingival biofilm is a significant risk factor in the progression of periodontal disease. Consequently, *Pg*LPS has been widely used in the study of inflammatory etiology of periodontal diseases and on the effectiveness

of anti-inflammatory agents used to treat periodontitis. It is well known that TNF-α, IL-1β, IL-6, and IL-8 are associated with periodontal inflammation and help recruit inflammatory cells to the site of infection (443,444). Results from the current study indicate that Salmeterol, a long-acting β2-AR agonist, may have significant efficacy in controlling release of pro-inflammatory mediators TNF-α, IL-1β, IL-6, and IL-8 **(Figure 7.1)**. Salmeterol appears to reduce expression of these pro-inflammatory cytokines through its regulation of inflammatory gene production at the transcriptional level since mRNA expression for these cytokines is also greatly reduced **(Figure 7.2)**. NO is another important inflammatory mediator involved in periodontitis and plays a critical role in macrophage activation (445,446). NO is generated from L-arginine by iNOS during inflammatory reactions in macrophages (447,448). iNOS-derived NO has been indicated in regulating progression of bone resorption in a murine model of apical periodontitis (449). It has been proven that NO modulates release of pro-inflammatory cytokines such as TNF-α and interferon-γ during inflammation (450). In concordance with results obtained here, it is suggested that Salmeterol may be a highly effective anti-inflammatory agent in controlling production, not only of inflammatory cytokines, but also NO. Results from the current study suggest that one mechanism for this inhibition of NO is through its effect on the expression of iNOS mRNA at the transcriptional level. Previous work of the authors using *E. coli* LPS on primary microglia-enriched cultures has shown that Salmeterol, and other long-acting β2-AR agonists, exert a strong anti-inflammatory effect on microglia when used at concentrations of 10^{-8} to 10^{-10} M by inhibiting activation of two key pro-inflammatory signaling pathways, the NF-κB pathway and the MAPK pathway (20). It is well known that MAPKs and NF-κB are involved in *Pg*LPS-induced cytokine expression (450,451), so it seemed logical that Salmeterol would mediate its anti-inflammatory effect on murine and human macrophages via the same basic cellular mechanism. It has been well

documented that production of pro-inflammatory cytokines and NO is primarily regulated at the transcriptional level, and expression of genes, including TNF-α, IL-1β, IL-6, IL-8, and iNOS, in macrophages and microglial cells is strongly regulated by the NF-κB classic pathway (452).

Previous results from studies with Salmeterol by the authors have shown that TLR-4-driven activation of microglia, the target of inhibition for Salmeterol is p65 phosphorylation and nuclear translocation. Determination of IκBα protein by immunoblotting analysis showed that pretreatment with Salmeterol prevented the stimulated phosphorylation and degradation of IκBα. After *Pg*LPS stimulation IκBα was phosphorylated and degraded, but the immunoblotting analysis of cytosolic and nuclear NF-κB (p65) showed that Salmeterol also prevented nuclear translocation of NF-κB stimulated by *Pg*LPS in macrophages (Figs. 7.5C and 7.5D). In addition, when the transcriptional activity of NF-κB was measured with an NF-κB reporter gene assay, it was found that Salmeterol also inhibited *Pg*LPS-mediated NF-κB transcriptional activity (Fig. 7.5B).

Therefore, data from the current study, using murine macrophages, is consistent with the observations that Salmeterol inhibits *Pg*LPS-mediated inflammatory mediator production through inactivation of NF-κB by reducing IκBα phosphorylation and degradation. The differential inhibitory effect of Salmeterol on the MAPK pathways was surprising. MAPKs are conserved family of proteins, which includes ERK1/2, p38, and JNK. Results from the current study show that Salmeterol significantly inhibited phosphorylation of ERK1/2 and JNK MAPK, but not p38 in *Pg*LPS stimulated RAW macrophages **(Figure 7.4)**. The authors have previously found that Salmeterol mediates its inhibitory effect on the MAPK pathway through inhibition of TAK-1 (20), whose activation leads to the downstream phosphorylation of ERK1/2, JNK, and p38. Therefore, it is not clear why p38 phosphorylation is not affected by Salmeterol inhibition. In addition, given the relationship between the NF-κB and MAPK pathways in inflammation, both pathways activate

and promote the nuclear translocation of transcription factors (NF-κB [p65] and AP- 1), which are essential for transcription of inflammatory genes and further release of cytokines and chemokines in response to stimuli. Activation of the NF-κB pathway is a common downstream component of the cellular response to many different innate immune stimuli and is used frequently in these cells as a biochemical detection and quantification method to study innate immune cellular activation (453). In the present study, it was found that Salmeterol inhibited activation of both NF-κB and MAPK pathways, and the mechanism of action of Salmeterol in response to *Pg*LPS in murine macrophages is summarized in **Figure 7.6.**

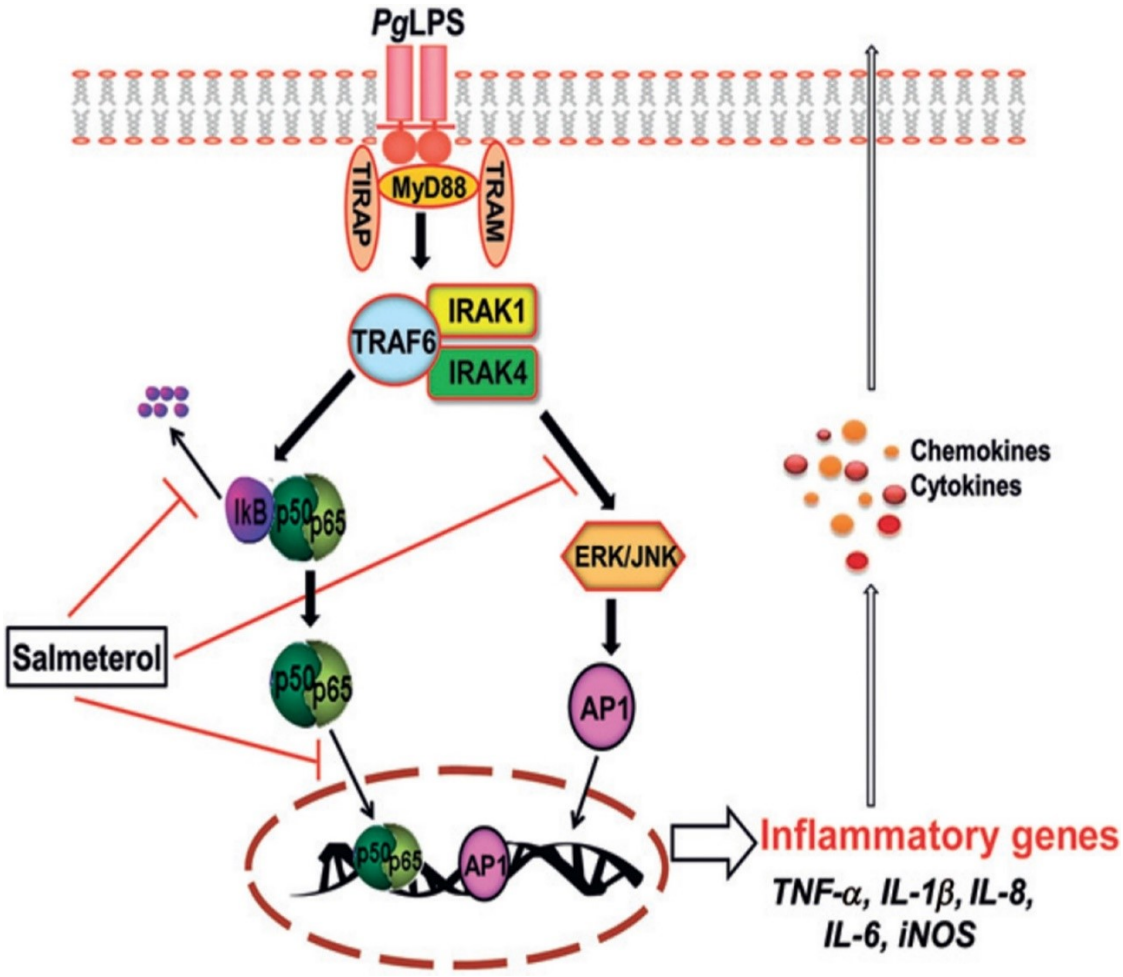

Figure 7. 6: Schematic layout of anti-inflammatory effects of Salmeterol in *Pg*LPS-induced inflammatory response in murine macrophages.

*Pg*LPS activates macrophages through its interaction with TLR-2/4. It results in the binding with key adaptor proteins (MyD88, TRAM, or TIRAP) and leads to activation of downstream signaling molecules (TRAF6, IRAK1/4). This further activates both NF-κB and MAPK pathways. Salmeterol suppresses phosphorylation and activation of ERK1/2 and JNK MAPK, which inhibits nuclear translocation of AP1 (not examined in this study). It also inhibits the phosphorylation and degradation of IκBα, which attenuates the activation and nuclear translocation of NF-κB (p65). This collectively inhibits the transcription of inflammatory genes such as *TNF-α, IL-1β, IL-6, IL-8, and iNOS* and eventually leads to inhibition of the inflammatory cytokine/chemokine production and suppressed immune response against *Pg*LPS.

Another important observation made in this study is that Salmeterol has the same inhibitory effect on human monocytes/macrophages as has been observed with murine responses. Previous work of the authors has shown that the use of Salmeterol *in vivo* can be very effective in the treatment of LPS-induced chronic dopaminergic neurodegeneration (20), a murine model for Parkinson disease. Results from the current study demonstrate that this effectiveness is also found in human cells; this suggests that Salmeterol may be a very effective treatment for a number of human chronic inflammatory conditions, including periodontal disease. Salmeterol and other long-acting β2-AR agonists are normally used to treat COPD, and results from the current study suggest that chronic use of long-acting β2-AR agonists like Salmeterol or Formoterol, or even the new generation of super long-acting agonists such as Vilanterol or Indacaterol, can be used to help control the inflammatory component of periodontal disease. Experimentation to determine the effects of β2-AR treatment on murine models of periodontal disease is now underway.

These results demonstrate the ability of the β2-AR agonist Salmeterol to inhibit the inflammatory response of both the murine macrophage cell line RAW264.7 and the human monocytes cell line THP-1 stimulated with *Pg*LPS. The mechanism of action, which includes inhibition of two important signaling pathways in the activation of inflammation, the MAPK and NF-κB pathways, is consistent with previous observations on the mode of action of Salmeterol on murine microglial cells. These results suggest that Salmeterol can inhibit chronic inflammation and that Salmeterol may be an effective treatment for chronic inflammatory disease such as periodontal disease.

CHAPTER 8

General discussion and future directions

8.1. Summary of the most important findings

Research studies supporting the repurposing of β2-AR agonist towards neurodegenerative conditions including PD are rapidly growing. Our previous and current observations clearly suggest the therapeutic efficacy of β2-AR agonists, specifically Salmeterol, for the treatment of PD. We have shown the neuroprotective and anti-inflammatory properties of Salmeterol in PD models. Further, results from this thesis show the mechanisms underlying the anti-inflammatory effects of Salmeterol. We have found that Salmeterol not only suppress the pro-inflammatory response, but also enhances the anti-inflammatory response using distinct signaling pathways **(Figure 8.1)**. The suppression of pro-inflammatory response or M1-like phenotype is dependent on the non-canonical/β-arrestin pathway of GPCR signaling (263). In contrast, the enhancement of anti-inflammatory response is dependent on the classical or cAMP/PKA/CREB pathway of GPCR signaling, but also with contribution from the PI3K and p38 MAPK signaling pathways. Furthermore, RNA-seq analysis also shows the differential expression of inflammatory and non-inflammatory genes regulated by Salmeterol in microglial cells, and reaffirms our findings that Salmeterol is a potent anti-inflammatory drug. We have confirmed that Salmeterol exerts similar anti-inflammatory effects on other chronic inflammatory conditions by using a periodontal disease model, suggesting the use of Salmeterol and by extension other β2-AR agonists as a class of universal anti-inflammatory drugs.

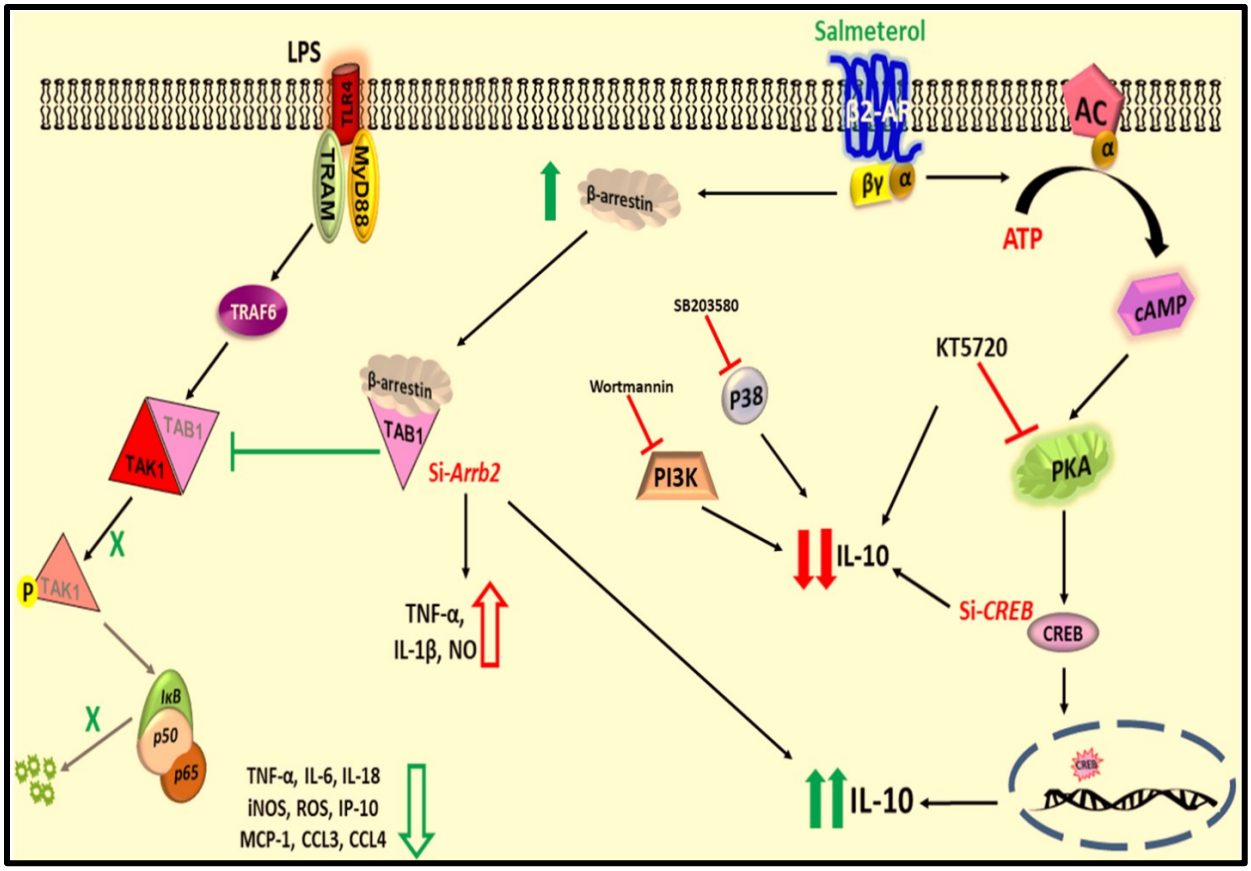

Figure 8. 1: Schematic of mechanisms underlying immunomodulatory effects by Salmeterol in activated BV2 cells.

Stimulation of microglia with LPS triggers TLR4 signaling pathway through TAK1, leading to the activation of NF-κB microglia, which further enhances the production of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-18, MCP-1, CCL3, CCL4, ROS and CXCL10/IP-10. However, microglial cells treated with Salmeterol enhances the expression of β-arrestin2 and leads to its increased interaction with TAB1, *preventing* formation of the TAB1/TAK1 complex, and *inhibiting* the activation of NF-κB and production of inflammatory mediators. Lack of β-arrestin2 (knockdown using siRNA against *Arrb2*) in BV2 cells abrogates the anti-inflammatory effects of the Sal/β2-AR/β-arrestin2 pathway and leads to increased production of TNF-α, IL-1β and NO in response to inflammatory stimuli. In contrast, treatment of

LPS-activated microglia with Salmeterol also increases the production of anti-inflammatory cytokines and chemokines IL-10, arginase-1, and CXCL14. Inhibition of classical signaling (cAMP/PKA/CREB) pathway of β2-AR via silencing *CREB* by siRNA and inhibiting protein kinase A (PKA) abrogates Salmeterol-mediated production of IL-10. Similarly, blockade of PI3K and p38 also inhibit the Salmeterol mediated IL-10 enhancement. In contrast, silencing of β-arrestin2 via siRNA does not affect the Salmeterol-mediated production of IL-10.

From bench to bedside: challenges in translation to the clinic

The β2-AR agonists are FDA and Health Canada-approved for the treatment of respiratory diseases such as asthma and COPD, but none of these β2-AR agonists are specifically developed for PD. In one landmark study, Mittal et al. have found in a Norwegian population that long-term use of Salbutamol, a SABA, lowers the risk of developing PD whereas the long-term use of Propranolol, a β2-AR antagonist (commonly used to treat hypertension and certain other forms of heart disease) was associated with increased risk of PD. Specifically, this risk of developing PD was dependent on the duration of Salbutamol intake in those patients (237). In the patient population who used Salbutamol for at least 6 months, it was expected that 43 would develop PD, but only 23 patients were ultimately diagnosed with the disease (rate ratio 0.66). On the other hand, in the cohort who used Salbutamol for 2 months or less, there was no decreased risk of developing PD in this population. In contrast, patients on Propranolol (which is also used as therapeutic for tremors in PD) for at least 1 year showed a significantly *increased* risk of developing PD compared to patients not on propranolol (rate ratio 2.2) (237). Similar results were found by Gronich et al (377) on a population of patients in Israel. Therefore, it is clear that patients on long-term Salbutamol (a β2-AR agonist) had significantly decreased the risk of developing PD, while patients on long-term propranolol (a β2-AR antagonist) therapy had significantly higher rates of PD, suggesting that β2-AR inhibition is a highly significant risk factor in developing PD. When we compared the effectiveness of Salbutamol to Salmeterol (a more lipophilic drug) in animal models of PD, Salmeterol was much more effective both *in vitro* and *in vivo* in dopaminergic neuroprotection (20). More importantly, we found that animals given Salmeterol treatment well before the appearance of symptoms in a long-term model of PD showed little evidence of dopaminergic neurodegeneration compared to untreated animals (20). Taken together, this data

suggests that administration of β2-AR agonists may have a profound preventative effect on the development of PD. Since the blood-brain-barrier penetration is a major obstacle in the development of therapeutics targeting CNS disorders, it will be important to consider the importance of lipophilic properties, concentration within the CNS, as well as the specificity, half-life and safety in using β2-AR agonists in older patients before and after the initial appearance of symptoms associated with PD. Consequently, these drugs require further investigation in a large cohort study to assess their utility as a potential therapeutic for PD and other neurodegenerative diseases.

8.2 Future Directions

The findings from this thesis study introduce several intriguing questions and hypotheses for the exploration of neuroprotective effects of β2-AR agonists. The following are the possible steps forward to test the therapeutic potential of Salmeterol which can lead to clinical trials in patients with symptoms of PD.

8.2.1. The immunological conversion by Salmeterol is required for the protection of dopaminergic neurons

One of the important findings from this study is that Salmeterol not only shows neuroprotective effects but also elicit anti-inflammatory effects via suppression of TNF-α and upregulation of IL-10, termed as phenotypic conversion. IL-10 also promotes the M1-like to M2-like phenotypic transition of macrophages and has been found as a neurotrophic and anti-inflammatory agent. IL-10 has been shown to prevent glutamate-induced excitotoxicity in brain ischemia (454). IL-10 is also known to protect LPS-induced dopaminergic neurodegeneration in SN and mesencephalic culture (301). We have found the increased levels of IL-10 in Salmeterol treated and LPS-stimulated microglial cells **(Figure 5.3)**, and these findings give further credence to the idea that

the neuroprotective role of Salmeterol is not only due to the inhibition of the pro-inflammatory phenotype in microglia but also to the enhanced production of IL-10. Therefore, to confirm this, we would propose to use *in vitro* approaches which inhibit IL-10 production and/or function, and mouse models which express a targeted genetic deletion of IL-10, to examine the effects of Salmeterol on neuroprotection in these mice. Since both *in vitro* and *in vivo* models lacking IL-10 activity would no longer exhibit the anti-inflammatory and neurotrophic properties normally associated with this cytokine, these experiments would help delineate the central role of IL-10 in Salmeterol-mediated neuroprotection. Furthermore, we can examine the role of inflammatory conversion by using the *IL-10-/-* KO mice model by looking at the loss of the M1-type phenotype and the expression of M2-like markers such as arginase-1 and CXCL14 in nigral microglia following initiation of neurodegeneration, and elucidate the effect of Salmeterol on PD pathology and neuroinflammation by conducting molecular, structural and behavioral studies. As we have hypothesized, if inflammatory conversion is critical for the protection of DA-neurons by Salmeterol and one of the key cytokine involved in inflammatory conversion is IL-10, as we would expect significant neurodegeneration and impaired motor skills in Salmeterol-treated *IL-10* knockout mice compared to their respective control mice. Results from these studies will show another mechanism underlying the neuroprotective effects mediated by Salmeterol.

8.2.2. The effects of Salmeterol on dopaminergic neuronal protection in genetic mouse models of PD.

It is clear that inflammation plays a major role in the progressive degeneration of neurons (314), but there are other associated factors which may also play an important role in the etiology of the disease such as Lewy body formation (aggregated α-synuclein) (57) and genetic alteration of key proteins involved in normal dopaminergic neuronal function (e.g. mutations in *LRRK2*, *SNCA,*

PINK1, *DJ-1*, and PARK-genes) (160). A number of animal models have been developed with genetic alterations in these critical genes, but and all these current animal models of PD do not completely mimic all clinical and neuropathological symptoms of human PD. Here, we propose to use different animal models of PD, other than inflammation model, to test the efficacy of Salmeterol on all major neuropathological features of PD.

Findings from previous and current studies clearly show the neuroprotective effects of Salmeterol in LPS and neurotoxin-based (MPTP) models of PD (20,263). We can next investigate the effects of Salmeterol in genetic mouse models of PD such as *SNCA**A53T and *LRRK2* to examine whether Salmeterol has similar protective effects in these models as the LPS and MPTP models of PD. These are the well-established models to study the pathogenesis of PD. These genetic mouse models more represent the familial form of PD, which represent only about 15% of all cases of human PD, but it is also important to know whether Salmeterol can protect against neurodegeneration in these forms of PD. To perform the *in vivo* studies we can propose the use of the *SNCA**A53T and *LRRK2**G2019S mice models, given the central role of α-synuclein in most forms of disease, and the role of LRRK2 in the pathogenesis of several forms of familial PD (45,81). Similar to our previous work, molecular and behavioral studies would be conducted to test the effects of Salmeterol on PD pathology in these models.

8.2.3. The effect of Salmeterol on neurogenesis of dopaminergic neurons.

Enhancing neurogenesis would be another key step to halt the progression of PD. We have shown that Salmeterol protects DA-neurons form degeneration; next it is important to study if and how β2-AR agonists can promote neurogenesis. It has previously been found that the activation of β2-AR by the agonist Clenbuterol enhances hippocampal neurogenesis in mouse model of Alzheimer's disease (236). We can look for expression of markers of neurogenesis such as NeuN

(neuronal nuclei), BrdU (bromodeoxyuridine) and DCX (doublecortin) in mouse models of PD. Another important observation would be to examine the long-term effects of Salmeterol. To test this, mice should first be treated with Salmeterol and after recovery from the disease symptoms, Salmeterol treatment would be terminated. These animals would then be kept under observation to determine whether they suffer a relapse in symptomology by observing whether they exhibit the behavioral changes of neurodegeneration or the reoccurrence of dopaminergic neurodegeneration in absence of continued Salmeterol treatment. These experiments would provide some insight as to the long-term protective of the acute Salmeterol treatment against degeneration of DA-neurons.

8.2.4. Investigate other mechanisms by which Salmeterol exhibits neuroprotective effects.

Results from our RNA-seq analysis have revealed that Salmeterol regulates several genes which have been associated with PD pathology. One of the genes is Rab7 which was significantly upregulated by Salmeterol in resting and LPS-activated microglia. Rab7 has been shown to induce the clearance of α-synuclein in SNCA*A53T model of PD and over-expression of Rab7 is beneficial in PD (402). Therefore, it will be interesting to investigate whether Salmeterol can reduce the α-synuclein aggregation by regulating the expression of Rab proteins. Findings from this study would result in a mechanism by which Salmeterol can protect dopaminergic neurons from abnormal protein aggregation and degeneration. We know that Salmeterol has therapeutic potential against PD due to its immunoregulatory properties but results from this study may provide another perspective of the therapeutic potential of Salmeterol other than one of immunologic conversion.

8.3. Study limitations

As an immune cell of the CNS, microglial cells are widely used to study the neuroinflammation. In our study we have used an immortalised murine microglial cell line BV2 which is a standard *in vitro* model to investigate the biology of primary microglia cells. This cell line also reduces the rigorous procedures involved in the isolation and maintenance of primary microglia and animal experimentation models. Transcriptome and proteome analysis of LPS-stimulated BV2 cells showed high similarity when compared with LPS-stimulated primary microglia. However, the average up-regulation of some inflammatory genes was less pronounced in BV2 cells compare to primary microglia. Overall, results from this study suggest BV2 cells as a valid substitute for primary microglia cell cultures for studying the molecular mechanism of LPS and Salmeterol action (455).

In contrast, another transcriptome sequencing analysis reveals that primary microglia express a unique cluster of transcripts in response to LPS stimulation while this distinct pattern was not observed in BV2 microglia. Das et. al have identified that a large number of transcripts including immunoregulatory genes, transcription factors and epigenetic regulators which were significantly altered in primary microglia when compared to BV2 cells. Furthermore, they have showed several specific genes involved in immune response that were altered only in primary microglia and not BV2 cells after LPS stimulation (456). However, the genes we primarily investigated in this thesis were found by Das et. al to show parallel immunoregulatory effects in both primary microglia and BV2 cells when stimulated by LPS (456). These results suggest that BV2 cells may not be a complete representation of primary microglia, and therefore verification of our results in primary microglia and *in vivo* settings still needs to be addressed.

8.4. Conclusion

Extensive previous investigations into the etiology of PD demonstrate a central role for the inflammatory microglial cell in the progression of PD. Thus, targeting neuroinflammation mediated by microglia may serve as a potential therapeutic benefit in the treatment of PD. Traditional treatment for PD is aimed at controlling the disease symptoms. Therefore, the search for effective neuroprotective therapies is receiving significant attention. This study was aimed to promote the development of a novel therapeutic approach and aid in the drug discovery for neurodegenerative conditions such as PD. Natural or synthetic activation or inhibition of the β2-AR can have profound effects on the development and progression of Parkinson's disease, a chronic neurodegenerative disorder which involves both neuroinflammatory and cellular mechanisms in dopaminergic neurotoxicity. Results from this study clearly suggest that the therapeutic use of β2-AR agonists can both inhibit the cause of neurodegeneration and activate a mechanism that can enhance recovery of patients with this disease, and serves as an important new therapeutic approach for the treatment of chronic neurodegenerative disorders.

Bibliography

1. Hirsch EC, Hunot S, Damier P, Faucheux B. Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? Ann Neurol. 1998 Sep;44(3 Suppl 1):S115-120.

2. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J Neurol Sci. 1973 Dec;20(4):415–55.

3. Bezard E, Dovero S, Prunier C, Ravenscroft P, Chalon S, Guilloteau D, et al. Relationship between the appearance of symptoms and the level of nigrostriatal degeneration in a progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned macaque model of Parkinson's disease. J Neurosci Off J Soc Neurosci. 2001 Sep 1;21(17):6853–61.

4. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev. 2000 Dec;52(4):595–638.

5. Huang Y, Halliday GM. Aspects of innate immunity and Parkinson's disease. Front Pharmacol. 2012;3:33.

6. Hsieh J-C, Lue K-H, Lee Y-L. Parkinson-like syndrome as the major presenting symptom of Epstein-Barr virus encephalitis. Arch Dis Child. 2002 Oct;87(4):358.

7. Stojkovska I, Wagner BM, Morrison BE. Parkinson's disease and enhanced inflammatory response. Exp Biol Med Maywood NJ. 2015 Nov;240(11):1387–95.

8. Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol. 2007 Mar;28(3):138–45.

9. Damier P, Hirsch EC, Zhang P, Agid Y, Javoy-Agid F. Glutathione peroxidase, glial cells and Parkinson's disease. Neuroscience. 1993 Jan;52(1):1–6.

10. Wilhelmsson U, Bushong EA, Price DL, Smarr BL, Phung V, Terada M, et al. Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. Proc Natl Acad Sci U S A. 2006 Nov 14;103(46):17513–8.

11. Knott C, Wilkin GP, Stern G. Astrocytes and microglia in the substantia nigra and caudate-putamen in Parkinson's disease. Parkinsonism Relat Disord. 1999 Sep;5(3):115–22.

12. Teismann P, Schulz JB. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. Cell Tissue Res. 2004 Oct;318(1):149–61.

13. Kohutnicka M, Lewandowska E, Kurkowska-Jastrzebska I, Członkowski A, Członkowska A. Microglial and astrocytic involvement in a murine model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Immunopharmacology. 1998 Jun;39(3):167–80.

14. Nomura T, Yabe T, Rosenthal ES, Krzan M, Schwartz JP. PSA-NCAM distinguishes reactive astrocytes in 6-OHDA-lesioned substantia nigra from those in the striatal terminal fields. J Neurosci Res. 2000 Sep 15;61(6):588–96.

15. Rodrigues RW, Gomide VC, Chadi G. Astroglial and microglial reaction after a partial nigrostriatal degeneration induced by the striatal injection of different doses of 6-hydroxydopamine. Int J Neurosci. 2001 Jul;109(1–2):91–126.

16. Depino AM, Earl C, Kaczmarczyk E, Ferrari C, Besedovsky H, del Rey A, et al. Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. Eur J Neurosci. 2003 Nov;18(10):2731–42.

17. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology. 1988 Aug;38(8):1285–91.

18. Qian L, Hu X, Zhang D, Snyder A, Wu H-M, Li Y, et al. beta2 Adrenergic receptor activation induces microglial NADPH oxidase activation and dopaminergic neurotoxicity through an ERK-dependent/protein kinase A-independent pathway. Glia. 2009 Nov 15;57(15):1600–9.

19. Mount MP, Lira A, Grimes D, Smith PD, Faucher S, Slack R, et al. Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. J Neurosci Off J Soc Neurosci. 2007 Mar 21;27(12):3328–37.

20. Qian L, Wu H, Chen S-H, Zhang D, Ali SF, Peterson L, et al. β2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. J Immunol Baltim Md 1950. 2011 Apr 1;186(7):4443–54.

21. Xing B, Bachstetter AD, Van Eldik LJ. Microglial p38α MAPK is critical for LPS-induced neuron degeneration, through a mechanism involving TNFα. Mol Neurodegener. 2011 Dec 20;6:84.

22. Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, O'Callaghan JP. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. FASEB J Off Publ Fed Am Soc Exp Biol. 2002 Sep;16(11):1474–6.

23. Peterson L, Ismond KP, Chapman E, Flood P. Potential benefits of therapeutic use of β2-adrenergic receptor agonists in neuroprotection and Parkinsonμs disease. J Immunol Res. 2014;2014:103780.

24. Kannarkat GT, Boss JM, Tansey MG. The role of innate and adaptive immunity in Parkinson's disease. J Park Dis. 2013;3(4):493–514.

25. Ahmed I, Tamouza R, Delord M, Krishnamoorthy R, Tzourio C, Mulot C, et al. Association between Parkinson's disease and the HLA-DRB1 locus. Mov Disord Off J Mov Disord Soc. 2012 Aug;27(9):1104–10.

26. Orr CF, Rowe DB, Halliday GM. An inflammatory review of Parkinson's disease. Prog Neurobiol. 2002 Dec;68(5):325–40.

27. Reynolds AD, Banerjee R, Liu J, Gendelman HE, Mosley RL. Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease. J Leukoc Biol. 2007 Nov;82(5):1083–94.

28. Le WD, Engelhardt J, Xie WJ, Schneider L, Smith RG, Appel SH. Experimental autoimmune nigral damage in guinea pigs. J Neuroimmunol. 1995 Mar;57(1–2):45–53.

29. McRae-Degueurce A, Rosengren L, Haglid K, Bööj S, Gottfries CG, Granérus AC, et al. Immunocytochemical investigations on the presence of neuron-specific antibodies in the CSF of Parkinson's disease cases. Neurochem Res. 1988 Jul;13(7):679–84.

30. Appel SH, Le WD, Tajti J, Haverkamp LJ, Engelhardt JI. Nigral damage and dopaminergic hypofunction in mesencephalon-immunized guinea pigs. Ann Neurol. 1992 Oct;32(4):494–501.

31. Fahn S, Cohen G. The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. Ann Neurol. 1992 Dec;32(6):804–12.

32. Zigmond MJ, Burke RE. Pathophysiology of Parkinson's disease. 2002. 1781 p.

33. Wang Q, Liu Y, Zhou J. Neuroinflammation in Parkinson's disease and its potential as therapeutic target. Transl Neurodegener. 2015;4:19.

34. Lull ME, Block ML. Microglial activation and chronic neurodegeneration. Neurother J Am Soc Exp Neurother. 2010 Oct;7(4):354–65.

35. Wood-Kaczmar A, Gandhi S, Wood NW. Understanding the molecular causes of Parkinson's disease. Trends Mol Med. 2006 Nov;12(11):521–8.

36. Dzamko N, Geczy CL, Halliday GM. Inflammation is genetically implicated in Parkinson's disease. Neuroscience. 2015 Aug 27;302:89–102.

37. Su X, Federoff HJ. Immune responses in Parkinson's disease: interplay between central and peripheral immune systems. BioMed Res Int. 2014;2014:275178.

38. Van Limbergen J, Wilson DC, Satsangi J. The genetics of Crohn's disease. Annu Rev Genomics Hum Genet. 2009;10:89–116.

39. Cardoso CC, Pereira AC, de Sales Marques C, Moraes MO. Leprosy susceptibility: genetic variations regulate innate and adaptive immunity, and disease outcome. Future Microbiol. 2011 May;6(5):533–49.

40. Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. Mol Cell. 2011 Oct 7;44(1):9–16.

41. Dzamko N, Inesta-Vaquera F, Zhang J, Xie C, Cai H, Arthur S, et al. The IkappaB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling. PloS One. 2012;7(6):e39132.

42. Kim JS, Cho JW, Shin H, Lee WY, Ki C-S, Cho AR, et al. A Korean Parkinson's disease family with the LRRK2 p.Tyr1699Cys mutation showing clinical heterogeneity. Mov Disord Off J Mov Disord Soc. 2012 Feb;27(2):320–4.

43. Moehle MS, Webber PJ, Tse T, Sukar N, Standaert DG, DeSilva TM, et al. LRRK2 inhibition attenuates microglial inflammatory responses. J Neurosci Off J Soc Neurosci. 2012 Feb 1;32(5):1602–11.

44. Gillardon F, Schmid R, Draheim H. Parkinson's disease-linked leucine-rich repeat kinase 2(R1441G) mutation increases proinflammatory cytokine release from activated primary microglial cells and resultant neurotoxicity. Neuroscience. 2012 Apr 19;208:41–8.

45. Russo I, Bubacco L, Greggio E. LRRK2 and neuroinflammation: partners in crime in Parkinson's disease? J Neuroinflammation. 2014 Mar 21;11:52.

46. Frank-Cannon TC, Tran T, Ruhn KA, Martinez TN, Hong J, Marvin M, et al. Parkin deficiency increases vulnerability to inflammation-related nigral degeneration. J Neurosci Off J Soc Neurosci. 2008 Oct 22;28(43):10825–34.

47. Sha D, Chin L-S, Li L. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum Mol Genet. 2010 Jan 15;19(2):352–63.

48. Narendra D, Tanaka A, Suen D-F, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol. 2008 Dec 1;183(5):795–803.

49. Chen ZJ. Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol. 2005 Aug;7(8):758–65.

50. Chung J-Y, Park HR, Lee S-J, Lee S-H, Kim JS, Jung Y-S, et al. Elevated TRAF2/6 expression in Parkinson's disease is caused by the loss of Parkin E3 ligase activity. Lab Investig J Tech Methods Pathol. 2013 Jun;93(6):663–76.

51. Marques O, Outeiro TF. Alpha-synuclein: from secretion to dysfunction and death. Cell Death Dis. 2012 Jul 19;3:e350.

52. Recchia A, Debetto P, Negro A, Guidolin D, Skaper SD, Giusti P. Alpha-synuclein and Parkinson's disease. FASEB J Off Publ Fed Am Soc Exp Biol. 2004 Apr;18(6):617–26.

53. Fellner L, Irschick R, Schanda K, Reindl M, Klimaschewski L, Poewe W, et al. Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia. Glia. 2013 Mar;61(3):349–60.

54. Kim C, Ho D-H, Suk J-E, You S, Michael S, Kang J, et al. Neuron-released oligomeric α-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. Nat Commun. 2013;4:1562.

55. Forsyth CB, Shannon KM, Kordower JH, Voigt RM, Shaikh M, Jaglin JA, et al. Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. PloS One. 2011;6(12):e28032.

56. Alvarez-Erviti L, Couch Y, Richardson J, Cooper JM, Wood MJA. Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. Neurosci Res. 2011 Apr;69(4):337–42.

57. Lema Tomé CM, Tyson T, Rey NL, Grathwohl S, Britschgi M, Brundin P. Inflammation and α-synuclein's prion-like behavior in Parkinson's disease--is there a link? Mol Neurobiol. 2013 Apr;47(2):561–74.

58. Reynolds AD, Stone DK, Mosley RL, Gendelman HE. Nitrated {alpha}-synuclein-induced alterations in microglial immunity are regulated by CD4+ T cell subsets. J Immunol Baltim Md 1950. 2009 Apr 1;182(7):4137–49.

59. Tran HT, Chung CH-Y, Iba M, Zhang B, Trojanowski JQ, Luk KC, et al. A-synuclein immunotherapy blocks uptake and templated propagation of misfolded α-synuclein and neurodegeneration. Cell Rep. 2014 Jun 26;7(6):2054–65.

60. Kazlauskaite A, Muqit MMK. PINK1 and Parkin – mitochondrial interplay between phosphorylation and ubiquitylation in Parkinson's disease. FEBS J. 2015 Jan;282(2):215–23.

61. Akundi RS, Huang Z, Eason J, Pandya JD, Zhi L, Cass WA, et al. Increased mitochondrial calcium sensitivity and abnormal expression of innate immunity genes precede dopaminergic defects in Pink1-deficient mice. PloS One. 2011 Jan 13;6(1):e16038.

62. Lee HJ, Chung KC. PINK1 positively regulates IL-1β-mediated signaling through Tollip and IRAK1 modulation. J Neuroinflammation. 2012 Dec 17;9:271.

63. Waak J, Weber SS, Waldenmaier A, Görner K, Alunni-Fabbroni M, Schell H, et al. Regulation of astrocyte inflammatory responses by the Parkinson's disease-associated gene DJ-1. FASEB J Off Publ Fed Am Soc Exp Biol. 2009 Aug;23(8):2478–89.

64. Trudler D, Weinreb O, Mandel SA, Youdim MBH, Frenkel D. DJ-1 deficiency triggers microglia sensitivity to dopamine toward a pro-inflammatory phenotype that is attenuated by rasagiline. J Neurochem. 2014 May;129(3):434–47.

65. Mitsumoto A, Nakagawa Y. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res. 2001 Dec;35(6):885–93.

66. Jackson-Lewis V, Przedborski S. Protocol for the MPTP mouse model of Parkinson's disease. Nat Protoc. 2007;2(1):141–51.

67. Kurkowska-Jastrzebska I, Wrońska A, Kohutnicka M, Członkowski A, Członkowska A. The inflammatory reaction following 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine intoxication in mouse. Exp Neurol. 1999 Mar;156(1):50–61.

68. Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, et al. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci Off J Soc Neurosci. 2002 Mar 1;22(5):1763–71.

69. Thiele SL, Warre R, Nash JE. Development of a unilaterally-lesioned 6-OHDA mouse model of Parkinson's disease. J Vis Exp JoVE. 2012 Feb 14;(60).

70. Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM. Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. Brain Res Dev Brain Res. 2002 Jan 31;133(1):27–35.

71. Gao H-M, Hong J-S, Zhang W, Liu B. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. J Neurosci Off J Soc Neurosci. 2002 Feb 1;22(3):782–90.

72. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003 Sep 11;39(6):889–909.

73. Bae E-J, Lee H-J, Rockenstein E, Ho D-H, Park E-B, Yang N-Y, et al. Antibody-aided clearance of extracellular α-synuclein prevents cell-to-cell aggregate transmission. J Neurosci Off J Soc Neurosci. 2012 Sep 26;32(39):13454–69.

74. Watson MB, Richter F, Lee SK, Gabby L, Wu J, Masliah E, et al. Regionally-specific microglial activation in young mice over-expressing human wildtype alpha-synuclein. Exp Neurol. 2012 Oct;237(2):318–34.

75. Cebrián C, Loike JD, Sulzer D. Neuroinflammation in Parkinson's disease animal models: a cell stress response or a step in neurodegeneration? Curr Top Behav Neurosci. 2015;22:237–70.

76. Fernagut P-O, Chesselet M-F. Alpha-synuclein and transgenic mouse models. Neurobiol Dis. 2004 Nov;17(2):123–30.

77. Dauer W, Kholodilov N, Vila M, Trillat A-C, Goodchild R, Larsen KE, et al. Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP. Proc Natl Acad Sci U S A. 2002 Oct 29;99(22):14524–9.

78. Theodore S, Cao S, McLean PJ, Standaert DG. Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease. J Neuropathol Exp Neurol. 2008 Dec;67(12):1149–58.

79. Van der Perren A, Macchi F, Toelen J, Carlon MS, Maris M, de Loor H, et al. FK506 reduces neuroinflammation and dopaminergic neurodegeneration in an α-synuclein-based rat model for Parkinson's disease. Neurobiol Aging. 2015 Mar;36(3):1559–68.

80. Daher JPL, Volpicelli-Daley LA, Blackburn JP, Moehle MS, West AB. Abrogation of α-synuclein-mediated dopaminergic neurodegeneration in LRRK2-deficient rats. Proc Natl Acad Sci U S A. 2014 Jun 24;111(25):9289–94.

81. Hinkle KM, Yue M, Behrouz B, Dächsel JC, Lincoln SJ, Bowles EE, et al. LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors. Mol Neurodegener. 2012 May 30;7:25.

82. Lee BD, Shin J-H, VanKampen J, Petrucelli L, West AB, Ko HS, et al. Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. Nat Med. 2010 Sep;16(9):998–1000.

83. Manthripragada AD, Schernhammer ES, Qiu J, Friis S, Wermuth L, Olsen JH, et al. Non-steroidal anti-inflammatory drug use and the risk of Parkinson's disease. Neuroepidemiology. 2011;36(3):155–61.

84. Castaño A, Herrera AJ, Cano J, Machado A. The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF-alpha, IL-1beta and IFN-gamma. J Neurochem. 2002 Apr;81(1):150–7.

85. Szekely CA, Green RC, Breitner JCS, Østbye T, Beiser AS, Corrada MM, et al. No advantage of A beta 42-lowering NSAIDs for prevention of Alzheimer dementia in six pooled cohort studies. Neurology. 2008 Jun 10;70(24):2291–8.

86. Gao X, Chen H, Schwarzschild MA, Ascherio A. Use of ibuprofen and risk of Parkinson disease. Neurology. 2011 Mar 8;76(10):863–9.

87. Casper D, Yaparpalvi U, Rempel N, Werner P. Ibuprofen protects dopaminergic neurons against glutamate toxicity in vitro. Neurosci Lett. 2000 Aug 11;289(3):201–4.

88. Tribl GG, Wöber C, Schönborn V, Brücke T, Deecke L, Panzer S. Amantadine in Parkinson's disease: lymphocyte subsets and IL-2 secreting T cell precursor frequencies. Exp Gerontol. 2001 Nov;36(10):1761–71.

89. Gangemi S, Basile G, Merendino RA, Epifanio A, Di Pasquale G, Ferlazzo B, et al. Effect of levodopa on interleukin-15 and RANTES circulating levels in patients affected by Parkinson's disease. Mediators Inflamm. 2003 Aug;12(4):251–3.

90. Bi W, Zhu L, Wang C, Liang Y, Liu J, Shi Q, et al. Rifampicin inhibits microglial inflammation and improves neuron survival against inflammation. Brain Res. 2011 Jun 13;1395:12–20.

91. Xu J, Wei C, Xu C, Bennett MC, Zhang G, Li F, et al. Rifampicin protects PC12 cells against MPP+-induced apoptosis and inhibits the expression of an alpha-Synuclein multimer. Brain Res. 2007 Mar 30;1139:220–5.

92. Ruzza P, Siligardi G, Hussain R, Marchiani A, Islami M, Bubacco L, et al. Ceftriaxone blocks the polymerization of α-synuclein and exerts neuroprotective effects in vitro. ACS Chem Neurosci. 2014 Jan 15;5(1):30–8.

93. Chotibut T, Davis RW, Arnold JC, Frenchek Z, Gurwara S, Bondada V, et al. Ceftriaxone increases glutamate uptake and reduces striatal tyrosine hydroxylase loss in 6-OHDA Parkinson's model. Mol Neurobiol. 2014 Jun;49(3):1282–92.

94. Ho Y-J, Ho S-C, Pawlak CR, Yeh K-Y. Effects of D-cycloserine on MPTP-induced behavioral and neurological changes: potential for treatment of Parkinson's disease dementia. Behav Brain Res. 2011 Jun 1;219(2):280–90.

95. Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, Whitworth AJ. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. Nat Neurosci. 2009 Sep;12(9):1129–35.

96. Malagelada C, Jin ZH, Jackson-Lewis V, Przedborski S, Greene LA. Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. J Neurosci Off J Soc Neurosci. 2010 Jan 20;30(3):1166–75.

97. Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14669–74.

98. Schildknecht S, Pape R, Müller N, Robotta M, Marquardt A, Bürkle A, et al. Neuroprotection by minocycline caused by direct and specific scavenging of peroxynitrite. J Biol Chem. 2011 Feb 18;286(7):4991–5002.

99. Lamine A, Létourneau M, Doan ND, Maucotel J, Couvineau A, Vaudry H, et al. Characterizations of a synthetic pituitary adenylate cyclase-activating polypeptide analog displaying potent neuroprotective activity and reduced in vivo cardiovascular side effects in a Parkinson's disease model. Neuropharmacology. 2016;108:440–50.

100. Qin X, Sun Z-Q, Dai X-J, Mao S-S, Zhang J-L, Jia M-X, et al. Toll-like receptor 4 signaling is involved in PACAP-induced neuroprotection in BV2 microglial cells under OGD/reoxygenation. Neurol Res. 2012 May;34(4):379–89.

101. Delgado M, Ganea D. Vasoactive intestinal peptide prevents activated microglia-induced neurodegeneration under inflammatory conditions: potential therapeutic role in brain trauma. FASEB J Off Publ Fed Am Soc Exp Biol. 2003 Oct;17(13):1922–4.

102. Jin X, Liu Q, Jia L, Li M, Wang X. Pinocembrin attenuates 6-OHDA-induced neuronal cell death through Nrf2/ARE pathway in SH-SY5Y cells. Cell Mol Neurobiol. 2015 Apr;35(3):323–33.

103. Lou H, Jing X, Wei X, Shi H, Ren D, Zhang X. Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. Neuropharmacology. 2014 Apr;79:380–8.

104. Chandran G, Muralidhara null. Insights on the neuromodulatory propensity of Selaginella (Sanjeevani) and its potential pharmacological applications. CNS Neurol Disord Drug Targets. 2014 Feb;13(1):82–95.

105. Girish C, Muralidhara null. Propensity of Selaginella delicatula aqueous extract to offset rotenone-induced oxidative dysfunctions and neurotoxicity in Drosophila melanogaster: Implications for Parkinson's disease. Neurotoxicology. 2012 Jun;33(3):444–56.

106. Meesarapee B, Thampithak A, Jaisin Y, Sanvarinda P, Suksamrarn A, Tuchinda P, et al. Curcumin I mediates neuroprotective effect through attenuation of quinoprotein formation, p-p38 MAPK expression, and caspase-3 activation in 6-hydroxydopamine treated SH-SY5Y cells. Phytother Res PTR. 2014 Apr;28(4):611–6.

107. Lee E, Park HR, Ji ST, Lee Y, Lee J. Baicalein attenuates astroglial activation in the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine-induced Parkinson's disease model by downregulating the activations of nuclear factor-κB, ERK, and JNK. J Neurosci Res. 2014 Jan;92(1):130–9.

108. Anandhan A, Tamilselvam K, Radhiga T, Rao S, Essa MM, Manivasagam T. Theaflavin, a black tea polyphenol, protects nigral dopaminergic neurons against chronic MPTP/probenecid induced Parkinson's disease. Brain Res. 2012 Jan 18;1433:104–13.

109. Lofrumento DD, Nicolardi G, Cianciulli A, De Nuccio F, La Pesa V, Carofiglio V, et al. Neuroprotective effects of resveratrol in an MPTP mouse model of Parkinson's-like disease: possible role of SOCS-1 in reducing pro-inflammatory responses. Innate Immun. 2014 Apr;20(3):249–60.

110. Chang CY, Choi D-K, Lee DK, Hong YJ, Park EJ. Resveratrol confers protection against rotenone-induced neurotoxicity by modulating myeloperoxidase levels in glial cells. PloS One. 2013;8(4):e60654.

111. Qian L, Block ML, Wei S-J, Lin C-F, Reece J, Pang H, et al. Interleukin-10 protects lipopolysaccharide-induced neurotoxicity in primary midbrain cultures by inhibiting the function of NADPH oxidase. J Pharmacol Exp Ther. 2006 Oct;319(1):44–52.

112. Johnston LC, Su X, Maguire-Zeiss K, Horovitz K, Ankoudinova I, Guschin D, et al. Human interleukin-10 gene transfer is protective in a rat model of Parkinson's disease. Mol Ther J Am Soc Gene Ther. 2008 Aug;16(8):1392–9.

113. Gonzalez-Aparicio R, Flores JA, Fernandez-Espejo E. Antiparkinsonian trophic action of glial cell line-derived neurotrophic factor and transforming growth factor β1 is enhanced after co-infusion in rats. Exp Neurol. 2010 Nov;226(1):136–47.

114. Zhu Y, Yang G-Y, Ahlemeyer B, Pang L, Che X-M, Culmsee C, et al. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci Off J Soc Neurosci. 2002 May 15;22(10):3898–909.

115. Qian L, Wei S-J, Zhang D, Hu X, Xu Z, Wilson B, et al. Potent anti-inflammatory and neuroprotective effects of TGF-beta1 are mediated through the inhibition of ERK and p47phox-Ser345 phosphorylation and translocation in microglia. J Immunol Baltim Md 1950. 2008 Jul 1;181(1):660–8.

116. Dehmer T, Heneka MT, Sastre M, Dichgans J, Schulz JB. Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B alpha induction and block of NF kappa B and iNOS activation. J Neurochem. 2004 Jan;88(2):494–501.

117. Ji H, Wang H, Zhang F, Li X, Xiang L, Aiguo S. PPARγ agonist pioglitazone inhibits microglia inflammation by blocking p38 mitogen-activated protein kinase signaling pathways. Inflamm Res Off J Eur Histamine Res Soc Al. 2010 Nov;59(11):921–9.

118. Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, et al. GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. Proc Natl Acad Sci U S A. 2009 Jan 27;106(4):1285–90.

119. Qian L, Tan KS, Wei S-J, Wu H-M, Xu Z, Wilson B, et al. Microglia-mediated neurotoxicity is inhibited by morphine through an opioid receptor-independent reduction of NADPH oxidase activity. J Immunol Baltim Md 1950. 2007 Jul 15;179(2):1198–209.

120. Qian L, Xu Z, Zhang W, Wilson B, Hong J-S, Flood PM. Sinomenine, a natural dextrorotatory morphinan analog, is anti-inflammatory and neuroprotective through inhibition of microglial NADPH oxidase. J Neuroinflammation. 2007 Sep 19;4:23.

121. Zhang W, Qin L, Wang T, Wei S-J, Gao H, Liu J, et al. 3-hydroxymorphinan is neurotrophic to dopaminergic neurons and is also neuroprotective against LPS-induced neurotoxicity. FASEB J Off Publ Fed Am Soc Exp Biol. 2005 Mar;19(3):395–7.

122. Zhang W, Shin E-J, Wang T, Lee PH, Pang H, Wie M-B, et al. 3-Hydroxymorphinan, a metabolite of dextromethorphan, protects nigrostriatal pathway against MPTP-elicited damage both in vivo and in vitro. FASEB J Off Publ Fed Am Soc Exp Biol. 2006 Dec;20(14):2496–511.

123. Ghosh A, Roy A, Liu X, Kordower JH, Mufson EJ, Hartley DM, et al. Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A. 2007 Nov 20;104(47):18754–9.

124. Zhang F, Qian L, Flood PM, Shi J-S, Hong J-S, Gao H-M. Inhibition of IkappaB kinase-beta protects dopamine neurons against lipopolysaccharide-induced neurotoxicity. J Pharmacol Exp Ther. 2010 Jun;333(3):822–33.

125. Wang Q, Chu C-H, Oyarzabal E, Jiang L, Chen S-H, Wilson B, et al. Subpicomolar diphenyleneiodonium inhibits microglial NADPH oxidase with high specificity and shows great potential as a therapeutic agent for neurodegenerative diseases. Glia. 2014 Dec;62(12):2034–43.

126. Qian L, Gao X, Pei Z, Wu X, Block M, Wilson B, et al. NADPH oxidase inhibitor DPI is neuroprotective at femtomolar concentrations through inhibition of microglia over-activation. Parkinsonism Relat Disord. 2007;13 Suppl 3:S316-320.

127. Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, et al. Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. Arch Neurol. 2002 Oct;59(10):1541–50.

128. Holmay MJ, Terpstra M, Coles LD, Mishra U, Ahlskog M, Öz G, et al. N-Acetylcysteine boosts brain and blood glutathione in Gaucher and Parkinson diseases. Clin Neuropharmacol. 2013 Aug;36(4):103–6.

129. Cheng B, Guo Y, Li C, Ji B, Pan Y, Chen J, et al. Edaravone protected PC12 cells against MPP(+)-cytoxicity via inhibiting oxidative stress and up-regulating heme oxygenase-1 expression. J Neurol Sci. 2014 Aug 15;343(1–2):115–9.

130. Yim G, Wang HH, Davies J. Antibiotics as signalling molecules. Philos Trans R Soc Lond B Biol Sci. 2007 Jul 29;362(1483):1195–200.

131. Gordon RA, Mays R, Sambrano B, Mayo T, Lapolla W. Antibiotics used in nonbacterial dermatologic conditions. Dermatol Ther. 2012 Feb;25(1):38–54.

132. Yulug B, Hanoglu L, Kilic E, Schabitz WR. RIFAMPICIN: an antibiotic with brain protective function. Brain Res Bull. 2014 Aug;107:37–42.

133. Bi W, Zhu L, Jing X, Zeng Z, Liang Y, Xu A, et al. Rifampicin improves neuronal apoptosis in LPS-stimulated co-cultured BV2 cells through inhibition of the TLR-4 pathway. Mol Med Rep. 2014 Oct;10(4):1793–9.

134. Matsui T, Svensson CI, Hirata Y, Mizobata K, Hua X-Y, Yaksh TL. Release of prostaglandin E(2) and nitric oxide from spinal microglia is dependent on activation of p38 mitogen-activated protein kinase. Anesth Analg. 2010 Aug;111(2):554–60.

135. Aron L, Klein R. Repairing the parkinsonian brain with neurotrophic factors. Trends Neurosci. 2011 Feb;34(2):88–100.

136. Miyamoto K, Tsumuraya T, Ohtaki H, Dohi K, Satoh K, Xu Z, et al. PACAP38 suppresses cortical damage in mice with traumatic brain injury by enhancing antioxidant activity. J Mol Neurosci MN. 2014 Nov;54(3):370–9.

137. Delgado M, Leceta J, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit the production of inflammatory mediators by activated microglia. J Leukoc Biol. 2003 Jan;73(1):155–64.

138. Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, et al. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev. 2001 Nov;33(3–4):255–71.

139. Siddique YH, Khan W, Singh BR, Naqvi AH. Synthesis of alginate-curcumin nanocomposite and its protective role in transgenic Drosophila model of Parkinson's disease. ISRN Pharmacol. 2013;2013:794582.

140. Jantaratnotai N, Utaisincharoen P, Sanvarinda P, Thampithak A, Sanvarinda Y. Phytoestrogens mediated anti-inflammatory effect through suppression of IRF-1 and pSTAT1 expressions in lipopolysaccharide-activated microglia. Int Immunopharmacol. 2013 Oct;17(2):483–8.

141. Nash KR, Moran P, Finneran DJ, Hudson C, Robinson J, Morgan D, et al. Fractalkine over expression suppresses α-synuclein-mediated neurodegeneration. Mol Ther J Am Soc Gene Ther. 2015 Jan;23(1):17–23.

142. Reynolds AD, Stone DK, Hutter JAL, Benner EJ, Mosley RL, Gendelman HE. Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease. J Immunol Baltim Md 1950. 2010 Mar 1;184(5):2261–71.

143. Hölscher C. Insulin, incretins and other growth factors as potential novel treatments for Alzheimer's and Parkinson's diseases. Biochem Soc Trans. 2014 Apr;42(2):593–9.

144. Culmsee C, Junker V, Thal S, Kremers W, Maier S, Schneider HJ, et al. Enantio-selective effects of clenbuterol in cultured neurons and astrocytes, and in a mouse model of cerebral ischemia. Eur J Pharmacol. 2007 Dec 1;575(1–3):57–65.

145. Culmsee C, Stumm RK, Schäfer MK, Weihe E, Krieglstein J. Clenbuterol induces growth factor mRNA, activates astrocytes, and protects rat brain tissue against ischemic damage. Eur J Pharmacol. 1999 Aug 20;379(1):33–45.

146. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, et al. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J Biol Chem. 2006 Jan 13;281(2):1261–73.

147. Sakurai H. Targeting of TAK1 in inflammatory disorders and cancer. Trends Pharmacol Sci. 2012 Oct;33(10):522–30.

148. Anrather J, Racchumi G, Iadecola C. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. J Biol Chem. 2006 Mar 3;281(9):5657–67.

149. Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol. 2003;53 Suppl 3:S26-36; discussion S36-38.

150. Przedborski S, Jackson-Lewis V. Mechanisms of MPTP toxicity. Mov Disord Off J Mov Disord Soc. 1998;13 Suppl 1:35–8.

151. Parkinson Study Group QE3 Investigators, Beal MF, Oakes D, Shoulson I, Henchcliffe C, Galpern WR, et al. A randomized clinical trial of high-dosage coenzyme Q10 in early Parkinson disease: no evidence of benefit. JAMA Neurol. 2014 May;71(5):543–52.

152. Lapchak PA. A critical assessment of edaravone acute ischemic stroke efficacy trials: is edaravone an effective neuroprotective therapy? Expert Opin Pharmacother. 2010 Jul;11(10):1753–63.

153. Gao H-M, Hong J-S. Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. Trends Immunol. 2008 Aug;29(8):357–65.

154. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, et al. Parkinson disease. Nat Rev Dis Primer. 2017 Mar 23;3:17013.

155. Nolan YM, Sullivan AM, Toulouse A. Parkinson's disease in the nuclear age of neuroinflammation. Trends Mol Med. 2013 Mar;19(3):187–96.

156. Moustafa AA, Chakravarthy S, Phillips JR, Gupta A, Keri S, Polner B, et al. Motor symptoms in Parkinson's disease: A unified framework. Neurosci Biobehav Rev. 2016;68:727–40.

157. Kalia LV, Lang AE. Parkinson's disease. Lancet Lond Engl. 2015 Aug 29;386(9996):896–912.

158. Löhle M, Storch A, Reichmann H. Beyond tremor and rigidity: non-motor features of Parkinson's disease. J Neural Transm Vienna Austria 1996. 2009 Nov;116(11):1483–92.

159. Gasser T. Molecular pathogenesis of Parkinson disease: insights from genetic studies. Expert Rev Mol Med. 2009 Jul 27;11:e22.

160. Spatola M, Wider C. Genetics of Parkinson's disease: the yield. Parkinsonism Relat Disord. 2014 Jan;20 Suppl 1:S35-38.

161. Chade AR, Kasten M, Tanner CM. Nongenetic causes of Parkinson's disease. J Neural Transm Suppl. 2006;(70):147–51.

162. Wei Z, Li X, Li X, Liu Q, Cheng Y. Oxidative Stress in Parkinson's Disease: A Systematic Review and Meta-Analysis. Front Mol Neurosci. 2018;11:236.

163. Doehner J, Genoud C, Imhof C, Krstic D, Knuesel I. Extrusion of misfolded and aggregated proteins--a protective strategy of aging neurons? Eur J Neurosci. 2012 Jun;35(12):1938–50.

164. Blaylock RL. Parkinson's disease: Microglial/macrophage-induced immunoexcitotoxicity as a central mechanism of neurodegeneration. Surg Neurol Int. 2017;8:65.

165. Ryan BJ, Hoek S, Fon EA, Wade-Martins R. Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. Trends Biochem Sci. 2015 Apr;40(4):200–10.

166. Molteni M, Rossetti C. Neurodegenerative diseases: The immunological perspective. J Neuroimmunol. 2017 Dec 15;313:109–15.

167. Lehnardt S. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia. 2010 Feb;58(3):253–63.

168. Hirsch EC, Hunot S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol. 2009 Apr;8(4):382–97.

169. Funk N, Wieghofer P, Grimm S, Schaefer R, Bühring H-J, Gasser T, et al. Characterization of peripheral hematopoietic stem cells and monocytes in Parkinson's disease. Mov Disord Off J Mov Disord Soc. 2013 Mar;28(3):392–5.

170. Ginhoux F, Garel S. The mysterious origins of microglia. Nat Neurosci. 2018 Jul;21(7):897–9.

171. Li Q, Barres BA. Microglia and macrophages in brain homeostasis and disease. Nat Rev Immunol. 2018 Apr;18(4):225–42.

172. Glezer I, Simard AR, Rivest S. Neuroprotective role of the innate immune system by microglia. Neuroscience. 2007 Jul 29;147(4):867–83.

173. Block ML, Zecca L, Hong J-S. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007 Jan;8(1):57–69.

174. Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol (Berl). 2003 Dec;106(6):518–26.

175. Koshimori Y, Ko J-H, Mizrahi R, Rusjan P, Mabrouk R, Jacobs MF, et al. Imaging Striatal Microglial Activation in Patients with Parkinson's Disease. PloS One. 2015;10(9):e0138721.

176. Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nat Rev Neurol. 2014 Apr;10(4):217–24.

177. Tambasco N, Romoli M, Calabresi P. Levodopa in Parkinson's disease: current status and future Developments. Curr Neuropharmacol. 2017 May 10;

178. Connolly BS, Lang AE. Pharmacological treatment of Parkinson disease: a review. JAMA. 2014 Apr 23;311(16):1670–83.

179. You H, Mariani L-L, Mangone G, Le Febvre de Nailly D, Charbonnier-Beaupel F, Corvol J-C. Molecular basis of dopamine replacement therapy and its side effects in Parkinson's disease. Cell Tissue Res. 2018 Jul;373(1):111–35.

180. Flood P, Arbabzada N, Sharma M. Inflammation: Role in Parkinson's Disease and Target for Therapy. Chall Park Dis [Internet]. 2016 Aug 24.

181. Casper D, Yaparpalvi U, Rempel N, Werner P. Ibuprofen protects dopaminergic neurons against glutamate toxicity in vitro. Neurosci Lett. 2000 Aug 11;289(3):201–4.

182. Zhu Y, Liu Z, Peng Y-P, Qiu Y-H. Interleukin-10 inhibits neuroinflammation-mediated apoptosis of ventral mesencephalic neurons via JAK-STAT3 pathway. Int Immunopharmacol. 2017 Sep;50:353–60.

183. Li G, Cui G, Tzeng N-S, Wei S-J, Wang T, Block ML, et al. Femtomolar concentrations of dextromethorphan protect mesencephalic dopaminergic neurons from inflammatory damage. FASEB J Off Publ Fed Am Soc Exp Biol. 2005 Apr;19(6):489–96.

184. Ahlquist RP. The adrenergic receptor. J Pharm Sci. 1966 Apr;55(4):359–67.

185. Ahlquist RP. Historical perspective. Classification of adrenoreceptors. J Auton Pharmacol. 1980 Nov;1(1):101–6.

186. Ahlquist RP. Adrenergic receptors: a personal and practical view. Perspect Biol Med. 1973;17(1):119–22.

187. Berthelsen S, Pettinger WA. A functional basis for classification of alpha-adrenergic receptors. Life Sci. 1977 Sep 1;21(5):595–606.

188. Fraser CM, Venter JC. Monoclonal antibodies to beta-adrenergic receptors: use in purification and molecular characterization of beta receptors. Proc Natl Acad Sci U S A. 1980 Dec;77(12):7034–8.

189. Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, et al. Molecular characterization of the human beta 3-adrenergic receptor. Science. 1989 Sep 8;245(4922):1118–21.

190. Basic Neurochemistry. 6th ed. Lippincott-Raven; 1999.

191. Fredriksson R, Lagerström MC, Lundin L-G, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 2003 Jun;63(6):1256–72.

192. Garland SL. Are GPCRs still a source of new targets? J Biomol Screen. 2013 Oct;18(9):947–66.

193. Kobilka BK, MacGregor C, Daniel K, Kobilka TS, Caron MG, Lefkowitz RJ. Functional activity and regulation of human beta 2-adrenergic receptors expressed in Xenopus oocytes. J Biol Chem. 1987 Nov 15;262(32):15796–802.

194. Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, et al. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature. 1986 May 1;321(6065):75–9.

195. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science. 2007 Nov 23;318(5854):1258–65.

196. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: A G protein-coupled receptor. Science. 2000 Aug 4;289(5480):739–45.

197. Collins S, Lohse MJ, O'Dowd B, Caron MG, Lefkowitz RJ. Structure and regulation of G protein-coupled receptors: the beta 2-adrenergic receptor as a model. Vitam Horm. 1991;46:1–39.

198. Rands E, Candelore MR, Cheung AH, Hill WS, Strader CD, Dixon RA. Mutational analysis of beta-adrenergic receptor glycosylation. J Biol Chem. 1990 Jun 25;265(18):10759–64.

199. O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ, Bouvier M. Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. J Biol Chem. 1989 May 5;264(13):7564–9.

200. Dohlman HG, Caron MG, DeBlasi A, Frielle T, Lefkowitz RJ. Role of extracellular disulfide-bonded cysteines in the ligand binding function of the beta 2-adrenergic receptor. Biochemistry. 1990 Mar 6;29(9):2335–42.

201. Carstairs JR, Nimmo AJ, Barnes PJ. Autoradiographic visualization of beta-adrenoceptor subtypes in human lung. Am Rev Respir Dis. 1985 Sep;132(3):541–7.

202. Barnes PJ. Beta-adrenoceptors on smooth muscle, nerves and inflammatory cells. Life Sci. 1993;52(26):2101–9.

203. Simon V, Robin M-T, Legrand C, Cohen-Tannoudji J. Endogenous G protein-coupled receptor kinase 6 triggers homologous beta-adrenergic receptor desensitization in primary uterine smooth muscle cells. Endocrinology. 2003 Jul;144(7):3058–66.

204. Tanaka KF, Kashima H, Suzuki H, Ono K, Sawada M. Existence of functional beta1- and beta2-adrenergic receptors on microglia. J Neurosci Res. 2002 Oct 15;70(2):232–7.

205. Lorton D, Bellinger DL. Molecular mechanisms underlying β-adrenergic receptor-mediated cross-talk between sympathetic neurons and immune cells. Int J Mol Sci. 2015 Mar 11;16(3):5635–65.

206. Barisione G, Baroffio M, Crimi E, Brusasco V. Beta-Adrenergic Agonists. Pharm Basel Switz. 2010 Mar 30;3(4):1016–44.

207. Shukla AK, Xiao K, Lefkowitz RJ. Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. Trends Biochem Sci. 2011 Sep;36(9):457–69.

208. Luttrell LM, Gesty-Palmer D. Beyond desensitization: physiological relevance of arrestin-dependent signaling. Pharmacol Rev. 2010 Jun;62(2):305–30.

209. Claing A, Laporte SA, Caron MG, Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. Prog Neurobiol. 2002 Feb;66(2):61–79.

210. Szelenyi J, Selmeczy Z, Brozik A, Medgyesi D, Magocsi M. Dual beta-adrenergic modulation in the immune system: stimulus-dependent effect of isoproterenol on MAPK activation and inflammatory mediator production in macrophages. Neurochem Int. 2006 Jul;49(1):94–103.

211. Chu EK, Drazen JM. Asthma: one hundred years of treatment and onward. Am J Respir Crit Care Med. 2005 Jun 1;171(11):1202–8.

212. Brittain RT. A comparison of the pharmacology of salbutamol with that of isoprenaline, orciprenaline and trimetoquinol. Postgrad Med J. 1971 Mar;47:Suppl:11-16.

213. Billington CK, Penn RB, Hall IP. β2 Agonists. Handb Exp Pharmacol. 2017;237:23–40.

214. Hizawa N. Pharmacogenetics of β2-agonists. Allergol Int Off J Jpn Soc Allergol. 2011 Sep;60(3):239–46.

215. Morgan DJ. Clinical pharmacokinetics of beta-agonists. Clin Pharmacokinet. 1990 Apr;18(4):270–94.

216. Johnson M. Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation. J Allergy Clin Immunol. 2006 Jan;117(1):18–24; quiz 25.

217. Johnson M. Beta2-adrenoceptors: mechanisms of action of beta2-agonists. Paediatr Respir Rev. 2001 Mar;2(1):57–62.

218. Chowdhury BA, Dal Pan G. The FDA and safe use of long-acting beta-agonists in the treatment of asthma. N Engl J Med. 2010 Apr 1;362(13):1169–71.

219. Schwarz LA, Luo L. Organization of the locus coeruleus-norepinephrine system. Curr Biol CB. 2015 Nov 2;25(21):R1051–6.

220. Foote SL, Bloom FE, Aston-Jones G. Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. Physiol Rev. 1983 Jul;63(3):844–914.

221. Peterson AC, Li C-SR. Noradrenergic Dysfunction in Alzheimer's and Parkinson's Diseases-An Overview of Imaging Studies. Front Aging Neurosci. 2018;10:127.

222. Schildkraut JJ. The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am J Psychiatry. 1965 Nov;122(5):509–22.

222. Zorec R, Parpura V, Verkhratsky A. Preventing neurodegeneration by adrenergic astroglial excitation. FEBS J. 2018 Apr 6;

224. Feinstein DL, Kalinin S, Braun D. Causes, consequences, and cures for neuroinflammation mediated via the locus coeruleus: noradrenergic signaling system. J Neurochem. 2016 Oct;139 Suppl 2:154–78.

225. Heneka MT, Nadrigny F, Regen T, Martinez-Hernandez A, Dumitrescu-Ozimek L, Terwel D, et al. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc Natl Acad Sci U S A. 2010 Mar 30;107(13):6058–63.

226. Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003 Apr;24(2):197–211.

227. Weinshenker D. Long Road to Ruin: Noradrenergic Dysfunction in Neurodegenerative Disease. Trends Neurosci. 2018 Apr;41(4):211–23.

228. Lauder JM. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci. 1993 Jun;16(6):233–40.

229. Day JS, O'Neill E, Cawley C, Aretz NK, Kilroy D, Gibney SM, et al. Noradrenaline acting on astrocytic β_2-adrenoceptors induces neurite outgrowth in primary cortical neurons. Neuropharmacology. 2014 Feb;77:234–48.

230. Traver S, Salthun-Lassalle B, Marien M, Hirsch EC, Colpaert F, Michel PP. The neurotransmitter noradrenaline rescues septal cholinergic neurons in culture from degeneration caused by low-level oxidative stress. Mol Pharmacol. 2005 Jun;67(6):1882–91.

231. Troadec JD, Marien M, Darios F, Hartmann A, Ruberg M, Colpaert F, et al. Noradrenaline provides long-term protection to dopaminergic neurons by reducing oxidative stress. J Neurochem. 2001 Oct;79(1):200–10.

232. Musazzi L, Rimland JM, Ieraci A, Racagni G, Domenici E, Popoli M. Pharmacological characterization of BDNF promoters I, II and IV reveals that serotonin and norepinephrine input is sufficient for transcription activation. Int J Neuropsychopharmacol. 2014 May;17(5):779–91.

233. Chen MJ, Nguyen TV, Pike CJ, Russo-Neustadt AA. Norepinephrine induces BDNF and activates the PI-3K and MAPK cascades in embryonic hippocampal neurons. Cell Signal. 2007 Jan;19(1):114–28.

234. Juric DM, Loncar D, Carman-Krzan M. Noradrenergic stimulation of BDNF synthesis in astrocytes: mediation via alpha1- and beta1/beta2-adrenergic receptors. Neurochem Int. 2008 Jan;52(1–2):297–306.

235. Alexander GM, Schwartzman RJ, Nukes TA, Grothusen JR, Hooker MD. Beta 2-adrenergic agonist as adjunct therapy to levodopa in Parkinson's disease. Neurology. 1994 Aug;44(8):1511–3.

236. Chai G-S, Wang Y-Y, Yasheng A, Zhao P. Beta 2-adrenergic receptor activation enhances neurogenesis in Alzheimer's disease mice. Neural Regen Res. 2016 Oct;11(10):1617–24.

237. Mittal S, Bjørnevik K, Im DS, Flierl A, Dong X, Locascio JJ, et al. β2-Adrenoreceptor is a regulator of the α-synuclein gene driving risk of Parkinson's disease. Science. 2017 01;357(6354):891–8.

238. Dang V, Medina B, Das D, Moghadam S, Martin KJ, Lin B, et al. Formoterol, a long-acting β2 adrenergic agonist, improves cognitive function and promotes dendritic complexity in a mouse model of Down syndrome. Biol Psychiatry. 2014 Feb 1;75(3):179–88.

239. Abdelmotilib H, West AB. Breathing new life into an old target: pulmonary disease drugs for Parkinson's disease therapy. Genome Med. 2017 Oct 19;9(1):88.

240. Schilero GJ, Hobson JC, Singh K, Spungen AM, Bauman WA, Radulovic M. Bronchodilator effects of ipratropium bromide and albuterol sulfate among subjects with tetraplegia. J Spinal Cord Med. 2018 Jan;41(1):42–7.

241. Phillips C, Fahimi A, Das D, Mojabi FS, Ponnusamy R, Salehi A. Noradrenergic System in Down Syndrome and Alzheimer's Disease A Target for Therapy. Curr Alzheimer Res. 2016;13(1):68–83.

242. Khoury SJ, Healy BC, Kivisäkk P, Viglietta V, Egorova S, Guttmann CRG, et al. A randomized controlled double-masked trial of albuterol add-on therapy in patients with multiple sclerosis. Arch Neurol. 2010 Sep;67(9):1055–61.

243. de Quervain D, Schwabe L, Roozendaal B. Stress, glucocorticoids and memory: implications for treating fear-related disorders. Nat Rev Neurosci. 2017;18(1):7–19.

244. Salvat E, Yalcin I, Muller A, Barrot M. A comparison of early and late treatments on allodynia and its chronification in experimental neuropathic pain. Mol Pain. 2018 Dec;14:1744806917749683.

245. Sorarù G, Pegoraro E, Spinella P, Turra S, D'Ascenzo C, Baggio L, et al. A pilot trial with clenbuterol in amyotrophic lateral sclerosis. Amyotroph Lateral Scler Off Publ World Fed Neurol Res Group Mot Neuron Dis. 2006 Dec;7(4):246–8.

246. Kinali M, Mercuri E, Main M, De Biasia F, Karatza A, Higgins R, et al. Pilot trial of albuterol in spinal muscular atrophy. Neurology. 2002 Aug 27;59(4):609–10.

247. Querin G, D'Ascenzo C, Peterle E, Ermani M, Bello L, Melacini P, et al. Pilot trial of clenbuterol in spinal and bulbar muscular atrophy. Neurology. 2013 Jun 4;80(23):2095–8.

248. Cash R, Raisman R, Lanfumey L, Ploska A, Agid Y. Cellular localization of adrenergic receptors in rat and human brain. Brain Res. 1986 Apr 2;370(1):127–35.

249. Braun D, Madrigal JLM, Feinstein DL. Noradrenergic regulation of glial activation: molecular mechanisms and therapeutic implications. Curr Neuropharmacol. 2014 Jul;12(4):342–52.

250. Gyoneva S, Traynelis SF. Norepinephrine modulates the motility of resting and activated microglia via different adrenergic receptors. J Biol Chem. 2013 May 24;288(21):15291–302.

251. Madrigal JLM, Feinstein DL, Dello Russo C. Norepinephrine protects cortical neurons against microglial-induced cell death. J Neurosci Res. 2005 Aug 1;81(3):390–6.

252. Kizaki T, Izawa T, Sakurai T, Haga S, Taniguchi N, Tajiri H, et al. Beta2-adrenergic receptor regulates Toll-like receptor-4-induced nuclear factor-kappaB activation through beta-arrestin 2. Immunology. 2008 Jul;124(3):348–56.

253. Laureys G, Gerlo S, Spooren A, Demol F, De Keyser J, Aerts JL. β_2-adrenergic agonists modulate TNF-α induced astrocytic inflammatory gene expression and brain inflammatory cell populations. J Neuroinflammation. 2014 Jan 30;11:21.

254. Culmsee C, Semkova I, Krieglstein J. NGF mediates the neuroprotective effect of the beta2-adrenoceptor agonist clenbuterol in vitro and in vivo: evidence from an NGF-antisense study. Neurochem Int. 1999 Jul;35(1):47–57.

255. Gleeson LC, Ryan KJ, Griffin EW, Connor TJ, Harkin A. The β2-adrenoceptor agonist clenbuterol elicits neuroprotective, anti-inflammatory and neurotrophic actions in the kainic acid model of excitotoxicity. Brain Behav Immun. 2010 Nov;24(8):1354–61.

256. McNamee EN, Ryan KM, Griffin EW, González-Reyes RE, Ryan KJ, Harkin A, et al. Noradrenaline acting at central beta-adrenoceptors induces interleukin-10 and suppressor of cytokine signaling-3 expression in rat brain: implications for neurodegeneration. Brain Behav Immun. 2010 May;24(4):660–71.

257. Kin NW, Sanders VM. It takes nerve to tell T and B cells what to do. J Leukoc Biol. 2006 Jun;79(6):1093–104.

258. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. Pharmacol Rev. 2001 Dec;53(4):487–525.

259. Swanson MA, Lee WT, Sanders VM. IFN-gamma production by Th1 cells generated from naive CD4+ T cells exposed to norepinephrine. J Immunol Baltim Md 1950. 2001 Jan 1;166(1):232–40.

260. Sharma M, Patterson L, Chapman E, Flood PM. Salmeterol, a Long-Acting β2-Adrenergic Receptor Agonist, Inhibits Macrophage Activation by Lipopolysaccharide From Porphyromonas gingivalis. J Periodontol. 2017 Jul;88(7):681–92.

261. Feng X, Wu C-Y, Burton FH, Loh HH, Wei L-N. β-arrestin protects neurons by mediating endogenous opioid arrest of inflammatory microglia. Cell Death Differ. 2014 Mar;21(3):397–406.

262. Tan KS, Nackley AG, Satterfield K, Maixner W, Diatchenko L, Flood PM. Beta2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-kappaB-independent mechanisms. Cell Signal. 2007 Feb;19(2):251–60.

263. Sharma M, Flood PM. β-arrestin2 regulates the anti-inflammatory effects of Salmeterol in lipopolysaccharide-stimulated BV2 cells. J Neuroimmunol. 2018 Oct 4;325:10–9.

264. Drake MT, Violin JD, Whalen EJ, Wisler JW, Shenoy SK, Lefkowitz RJ. beta-arrestin-biased agonism at the beta2-adrenergic receptor. J Biol Chem. 2008 Feb 29;283(9):5669–76.

265. Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, et al. Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. Mol Cell. 2004 May 7;14(3):303–17.

266. Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ. beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha. Proc Natl Acad Sci U S A. 2004 Jun 8;101(23):8603–7.

267. Urs NM, Bido S, Peterson SM, Daigle TL, Bass CE, Gainetdinov RR, et al. Targeting β-arrestin2 in the treatment of L-DOPA-induced dyskinesia in Parkinson's disease. Proc Natl Acad Sci U S A. 2015 May 12;112(19):E2517-2526.

268. Zhou H-C, Sun Y-Y, Cai W, He X-T, Yi F, Li B-M, et al. Activation of β2-adrenoceptor enhances synaptic potentiation and behavioral memory via cAMP-PKA signaling in the medial prefrontal cortex of rats. Learn Mem Cold Spring Harb N. 2013 Apr 17;20(5):274–84.

269. Peterson YK, Cameron RB, Wills LP, Trager RE, Lindsey CC, Beeson CC, et al. β2-Adrenoceptor agonists in the regulation of mitochondrial biogenesis. Bioorg Med Chem Lett. 2013 Oct 1;23(19):5376–81.

270. Counts SE, Mufson EJ. Noradrenaline activation of neurotrophic pathways protects against neuronal amyloid toxicity. J Neurochem. 2010 May;113(3):649–60.

271. Theron AJ, Steel HC, Tintinger GR, Feldman C, Anderson R. Can the anti-inflammatory activities of β2-agonists be harnessed in the clinical setting? Drug Des Devel Ther. 2013;7:1387–98.

272. Carnevale D, De Simone R, Minghetti L. Microglia-neuron interaction in inflammatory and degenerative diseases: role of cholinergic and noradrenergic systems. CNS Neurol Disord Drug Targets. 2007 Dec;6(6):388–97.

273. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem. 1993 Oct;214(1):11–6.

274. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. Methods Mol Biol Clifton NJ. 2010;594:57–72.

275. Noursadeghi M, Tsang J, Haustein T, Miller RF, Chain BM, Katz DR. Quantitative imaging assay for NF-κB nuclear translocation in primary human macrophages. J Immunol Methods. 2008 Jan 1;329(1–2):194–200.

276. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinforma Oxf Engl. 2010 Jan 1;26(1):139–40.

277. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):R106.

278. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. Omics J Integr Biol. 2012 May;16(5):284–7.

279. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000 Jan 1;28(1):27–30.

280. Liu B, Gao H-M, Wang J-Y, Jeohn G-H, Cooper CL, Hong J-S. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann N Y Acad Sci. 2002 May;962:318–31.

281. Colton CA, Gilbert DL. Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett. 1987 Nov 2;223(2):284–8.

282. Sawada M, Kondo N, Suzumura A, Marunouchi T. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res. 1989 Jul 10;491(2):394–7.

283. Tanaka KF, Kashima H, Suzuki H, Ono K, Sawada M. Existence of functional beta1- and beta2-adrenergic receptors on microglia. J Neurosci Res. 2002 Oct 15;70(2):232–7.

284. Shenoy SK, Lefkowitz RJ. β-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci. 2011 Sep;32(9):521–33.

285. Blake K, Lima J. Pharmacogenomics of long-acting β2-agonists. Expert Opin Drug Metab Toxicol. 2015;11(11):1733–51.

286. Ryan KJ, Griffin É, Yssel JD, Ryan KM, McNamee EN, Harkin A, et al. Stimulation of central β2-adrenoceptors suppresses NFκB activity in rat brain: a role for IκB. Neurochem Int. 2013 Nov;63(5):368–78.

287. O'Sullivan JB, Ryan KM, Curtin NM, Harkin A, Connor TJ. Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. Int J Neuropsychopharmacol. 2009 Jun;12(5):687–99.

288. de Montmollin E, Aboab J, Mansart A, Annane D. Bench-to-bedside review: Beta-adrenergic modulation in sepsis. Crit Care Lond Engl. 2009;13(5):230.

289. Dello Russo C, Boullerne AI, Gavrilyuk V, Feinstein DL. Inhibition of microglial inflammatory responses by norepinephrine: effects on nitric oxide and interleukin-1beta production. J Neuroinflammation. 2004 Jun 30;1(1):9.

290. Jiang L, Chen S-H, Chu C-H, Wang S-J, Oyarzabal E, Wilson B, et al. A novel role of microglial NADPH oxidase in mediating extra-synaptic function of norepinephrine in regulating brain immune homeostasis. Glia. 2015 Jun;63(6):1057–72.

291. Anwar MM, El-Haggar RS, Zaghary WA. Salmeterol Xinafoate. Profiles Drug Subst Excip Relat Methodol. 2015;40:321–69.

292. Brogden RN, Faulds D. Salmeterol xinafoate. A review of its pharmacological properties and therapeutic potential in reversible obstructive airways disease. Drugs. 1991 Nov;42(5):895–912.

293. Calverley PMA, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, et al. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. N Engl J Med. 2007 Feb 22;356(8):775–89.

294. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-κB signaling pathways. Nat Immunol. 2011 Jul 19;12(8):695–708.

295. Fan H. β-Arrestins 1 and 2 are critical regulators of inflammation. Innate Immun. 2014 Jul;20(5):451–60.

296. Tansey MG, Goldberg MS. Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiol Dis. 2010 Mar;37(3):510–8.

297. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell. 2010 Mar 19;140(6):918–34.

298. Jhang KA, Lee EO, Kim H-S, Chong YH. Norepinephrine provides short-term neuroprotection against Aβ1-42 by reducing oxidative stress independent of Nrf2 activation. Neurobiol Aging. 2014 Nov;35(11):2465–73.

299. Madrigal JLM, Leza JC, Polak P, Kalinin S, Feinstein DL. Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline. J Neurosci Off J Soc Neurosci. 2009 Jan 7;29(1):263–7.

300. Qian L, Hong JS, Flood PM. Role of microglia in inflammation-mediated degeneration of dopaminergic neurons: neuroprotective effect of interleukin 10. J Neural Transm Suppl. 2006;(70):367–71.

301. Zhu Y, Chen X, Liu Z, Peng Y-P, Qiu Y-H. Interleukin-10 Protection against Lipopolysaccharide-Induced Neuro-Inflammation and Neurotoxicity in Ventral Mesencephalic Cultures. Int J Mol Sci. 2015 Dec 28;17(1).

302. Cameron B, Landreth GE. Inflammation, microglia, and Alzheimer's disease. Neurobiol Dis. 2010 Mar;37(3):503–9.

303. Kempuraj D, Thangavel R, Natteru PA, Selvakumar GP, Saeed D, Zahoor H, et al. Neuroinflammation Induces Neurodegeneration. J Neurol Neurosurg Spine. 2016;1(1).

304. Flood PM, Qian L, Peterson LJ, Zhang F, Shi J-S, Gao H-M, et al. Transcriptional Factor NF-κB as a Target for Therapy in Parkinson's Disease. Park Dis. 2011;2011:216298.

305. Latorraca NR, Wang JK, Bauer B, Townshend RJL, Hollingsworth SA, Olivieri JE, et al. Molecular mechanism of GPCR-mediated arrestin activation. Nature. 2018 May;557(7705):452–6.

306. Ma L, Pei G. Beta-arrestin signaling and regulation of transcription. J Cell Sci. 2007 Jan 15;120(Pt 2):213–8.

307. Han S-O, Kommaddi RP, Shenoy SK. Distinct roles for β-arrestin2 and arrestin-domain-containing proteins in β2 adrenergic receptor trafficking. EMBO Rep. 2013 Feb;14(2):164–71.

308. Du R-W, Du R-H, Bu W-G. β-Arrestin 2 mediates the anti-inflammatory effects of fluoxetine in lipopolysaccharide-stimulated microglial cells. J Neuroimmune Pharmacol Off J Soc NeuroImmune Pharmacol. 2014 Sep;9(4):582–90.

309. Matera MG, Page CP, Cazzola M. Novel bronchodilators for the treatment of chronic obstructive pulmonary disease. Trends Pharmacol Sci. 2011 Aug;32(8):495–506.

310. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, et al. Inflammation and Alzheimer's disease. Neurobiol Aging. 2000 Jun;21(3):383–421.

311. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nat Rev Immunol. 2015 Sep 15;15(9):545–58.

312. Kanazawa M, Ninomiya I, Hatakeyama M, Takahashi T, Shimohata T. Microglia and Monocytes/Macrophages Polarization Reveal Novel Therapeutic Mechanism against Stroke. Int J Mol Sci. 2017 Oct 13;18(10).

313. Zhao H, Alam A, Chen Q, A Eusman M, Pal A, Eguchi S, et al. The role of microglia in the pathobiology of neuropathic pain development: what do we know? Br J Anaesth. 2017 Apr 1;118(4):504–16.

314. Ransohoff RM. How neuroinflammation contributes to neurodegeneration. Science. 2016 Aug 19;353(6301):777–83.

315. Jin X, Yamashita T. Microglia in central nervous system repair after injury. J Biochem (Tokyo). 2016 May;159(5):491–6.

316. Grayson M. Parkinson's disease. Nature. 2016 27;538(7626):S1.

317. Rocha EM, De Miranda B, Sanders LH. Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. Neurobiol Dis. 2018 Jan;109(Pt B):249–57.

318. Subramaniam SR, Federoff HJ. Targeting Microglial Activation States as a Therapeutic Avenue in Parkinson's Disease. Front Aging Neurosci. 2017;9:176.

319. Walker DG, Lue L-F. Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. Alzheimers Res Ther. 2015 Aug 19;7(1):56.

320. Tang Y, Le W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol. 2016 Mar;53(2):1181–94.

321. Esposito E, Di Matteo V, Benigno A, Pierucci M, Crescimanno G, Di Giovanni G. Non-steroidal anti-inflammatory drugs in Parkinson's disease. Exp Neurol. 2007 Jun;205(2):295–312.

322. Moore AH, Bigbee MJ, Boynton GE, Wakeham CM, Rosenheim HM, Staral CJ, et al. Non-Steroidal Anti-Inflammatory Drugs in Alzheimer's Disease and Parkinson's Disease: Reconsidering the Role of Neuroinflammation. Pharm Basel Switz. 2010 Jun 2;3(6):1812–41.

323. Sharma M, Flood PM. Adrenergic Receptors as Pharmacological Targets for Neuroinflammation and Neurodegeneration in Parkinson's Disease. Neuroprotection [Internet]. 2018 Nov 26 .

324. Delaville C, Deurwaerdère PD, Benazzouz A. Noradrenaline and Parkinson's disease. Front Syst Neurosci. 2011;5:31.

325. Bosmann M, Grailer JJ, Zhu K, Matthay MA, Sarma JV, Zetoune FS, et al. Anti-inflammatory effects of β2 adrenergic receptor agonists in experimental acute lung injury. FASEB J Off Publ Fed Am Soc Exp Biol. 2012 May;26(5):2137–44.

326. Grailer JJ, Haggadone MD, Sarma JV, Zetoune FS, Ward PA. Induction of M2 regulatory macrophages through the β2-adrenergic receptor with protection during endotoxemia and acute lung injury. J Innate Immun. 2014;6(5):607–18.

327. Lamkin DM, Ho H-Y, Ong TH, Kawanishi CK, Stoffers VL, Ahlawat N, et al. β-Adrenergic-stimulated macrophages: Comprehensive localization in the M1-M2 spectrum. Brain Behav Immun. 2016 Oct;57:338–46.

328. Ağaç D, Estrada LD, Maples R, Hooper LV, Farrar JD. The β2-adrenergic receptor controls inflammation by driving rapid IL-10 secretion. Brain Behav Immun. 2018 Nov;74:176–85.

329. Keränen T, Hömmö T, Moilanen E, Korhonen R. β2-receptor agonists salbutamol and terbutaline attenuated cytokine production by suppressing ERK pathway through cAMP in macrophages. Cytokine. 2017;94:1–7.

330. Samarasinghe R, Tailor P, Tamura T, Kaisho T, Akira S, Ozato K. Induction of an anti-inflammatory cytokine, IL-10, in dendritic cells after toll-like receptor signaling. J Interferon Cytokine Res Off J Int Soc Interferon Cytokine Res. 2006 Dec;26(12):893–900.

331. Nijhuis LE, Olivier BJ, Dhawan S, Hilbers FW, Boon L, Wolkers MC, et al. Adrenergic β2 receptor activation stimulates anti-inflammatory properties of dendritic cells in vitro. PloS One. 2014;9(1):e85086.

332. Navarro G, Morales P, Rodríguez-Cueto C, Fernández-Ruiz J, Jagerovic N, Franco R. Targeting Cannabinoid CB2 Receptors in the Central Nervous System. Medicinal Chemistry Approaches with Focus on Neurodegenerative Disorders. Front Neurosci. 2016;10:406.

333. Wen AY, Sakamoto KM, Miller LS. The role of the transcription factor CREB in immune function. J Immunol Baltim Md 1950. 2010 Dec 1;185(11):6413–9.

334. Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cell Signal. 2005 Nov;17(11):1343–51.

335. Naqvi S, Martin KJ, Arthur JSC. CREB phosphorylation at Ser133 regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling. Biochem J. 2014 Mar 15;458(3):469–79.

336. Vergadi E, Ieronymaki E, Lyroni K, Vaporidi K, Tsatsanis C. Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization. J Immunol Baltim Md 1950. 2017 01;198(3):1006–14.

337. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? Nat Neurosci. 2016 26;19(8):987–91.

338. Chhor V, Le Charpentier T, Lebon S, Oré M-V, Celador IL, Josserand J, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain Behav Immun. 2013 Aug;32:70–85.

339. Nagatsu T, Mogi M, Ichinose H, Togari A. Changes in cytokines and neurotrophins in Parkinson's disease. J Neural Transm Suppl. 2000;(60):277–90.

340. Blum-Degen D, Müller T, Kuhn W, Gerlach M, Przuntek H, Riederer P. Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neurosci Lett. 1995 Dec 29;202(1–2):17–20.

341. Starhof C, Winge K, Heegaard NHH, Skogstrand K, Friis S, Hejl A. Cerebrospinal fluid pro-inflammatory cytokines differentiate parkinsonian syndromes. J Neuroinflammation. 2018 Nov 3;15(1):305.

342. Alam Q, Alam MZ, Mushtaq G, Damanhouri GA, Rasool M, Kamal MA, et al. Inflammatory Process in Alzheimer's and Parkinson's Diseases: Central Role of Cytokines. Curr Pharm Des. 2016;22(5):541–8.

343. Islam MT. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. Neurol Res. 2017 Jan;39(1):73–82.

344. Peterson LJ, Flood PM. Oxidative stress and microglial cells in Parkinson's disease. Mediators Inflamm. 2012;2012:401264.

345. Salim S. Oxidative Stress and the Central Nervous System. J Pharmacol Exp Ther. 2017 Jan;360(1):201–5.

346. Wadhwa R, Gupta R, Maurya PK. Oxidative Stress and Accelerated aging in Neurodegenerative and Neuropsychiatric disorder. Curr Pharm Des. 2019 Jan 15;

347. Chen H, Jacobs E, Schwarzschild MA, McCullough ML, Calle EE, Thun MJ, et al. Nonsteroidal antiinflammatory drug use and the risk for Parkinson's disease. Ann Neurol. 2005 Dec;58(6):963–7.

348. Etminan M, Carleton BC, Samii A. Non-steroidal anti-inflammatory drug use and the risk of Parkinson disease: a retrospective cohort study. J Clin Neurosci Off J Neurosurg Soc Australas. 2008 May;15(5):576–7.

349. Etminan M, Suissa S. NSAID use and the risk of Parkinson's disease. Curr Drug Saf. 2006 Aug;1(3):223–5.

350. Peña-Altamira E, Prati F, Massenzio F, Virgili M, Contestabile A, Bolognesi ML, et al. Changing paradigm to target microglia in neurodegenerative diseases: from anti-inflammatory strategy to active immunomodulation. Expert Opin Ther Targets. 2016;20(5):627–40.

351. Ferger B, Leng A, Mura A, Hengerer B, Feldon J. Genetic ablation of tumor necrosis factor-alpha (TNF-alpha) and pharmacological inhibition of TNF-synthesis attenuates MPTP toxicity in mouse striatum. J Neurochem. 2004 May;89(4):822–33.

352. Rousselet E, Callebert J, Parain K, Joubert C, Hunot S, Hartmann A, et al. Role of TNF-alpha receptors in mice intoxicated with the parkinsonian toxin MPTP. Exp Neurol. 2002 Sep;177(1):183–92.

353. Tanaka S, Ishii A, Ohtaki H, Shioda S, Yoshida T, Numazawa S. Activation of microglia induces symptoms of Parkinson's disease in wild-type, but not in IL-1 knockout mice. J Neuroinflammation. 2013 Dec 1;10:143.

354. Sawyer AJ, Tian W, Saucier-Sawyer JK, Rizk PJ, Saltzman WM, Bellamkonda RV, et al. The effect of inflammatory cell-derived MCP-1 loss on neuronal survival during chronic neuroinflammation. Biomaterials. 2014 Aug;35(25):6698–706.

355. Lindqvist D, Hall S, Surova Y, Nielsen HM, Janelidze S, Brundin L, et al. Cerebrospinal fluid inflammatory markers in Parkinson's disease--associations with depression, fatigue, and cognitive impairment. Brain Behav Immun. 2013 Oct;33:183–9.

356. Rocha NP, Scalzo PL, Barbosa IG, Souza MS, Morato IB, Vieira ELM, et al. Cognitive Status Correlates with CXCL10/IP-10 Levels in Parkinson's Disease. Park Dis. 2014;2014:903796.

357. Sugama S, Wirz SA, Barr AM, Conti B, Bartfai T, Shibasaki T. Interleukin-18 null mice show diminished microglial activation and reduced dopaminergic neuron loss following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment. Neuroscience. 2004;128(2):451–8.

358. Pawlik A, Kurzawski M, Czerny B, Gawronska-Szklarz B, Drozdzik M, Herczynska M. Interleukin-18 promoter polymorphism in patients with rheumatoid arthritis. Tissue Antigens. 2006 May;67(5):415–8.

359. Yu J-T, Tan L, Song J-H, Sun Y-P, Chen W, Miao D, et al. Interleukin-18 promoter polymorphisms and risk of late onset Alzheimer's disease. Brain Res. 2009 Feb 9;1253:169–75.

360. Fahmy E, Rabah A, Sharaf S, Helmy H, Kamal A. Interleukin-18 promoter polymorphisms and idiopathic Parkinson disease: an Egyptian study. Acta Neurol Belg. 2018 Apr 26;

361. Xu X, Li D, He Q, Gao J, Chen B, Xie A. Interleukin-18 promoter polymorphisms and risk of Parkinson's disease in a Han Chinese population. Brain Res. 2011 Mar 24;1381:90–4.

362. Burmeister AR, Marriott I. The Interleukin-10 Family of Cytokines and Their Role in the CNS. Front Cell Neurosci. 2018;12:458.

363. Lobo-Silva D, Carriche GM, Castro AG, Roque S, Saraiva M. Balancing the immune response in the brain: IL-10 and its regulation. J Neuroinflammation. 2016 24;13(1):297.

364. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. Cancer Lett. 2015 Oct 28;367(2):103–7.

365. Peñaloza HF, Schultz BM, Nieto PA, Salazar GA, Suazo I, Gonzalez PA, et al. Opposing roles of IL-10 in acute bacterial infection. Cytokine Growth Factor Rev. 2016;32:17–30.

366. Dambuza IM, He C, Choi JK, Yu C-R, Wang R, Mattapallil MJ, et al. IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. Nat Commun. 2017 28;8(1):719.

367. Hayakawa K, Wang X, Lo EH. CD200 increases alternatively activated macrophages through cAMP-response element binding protein - C/EBP-beta signaling. J Neurochem. 2016 Mar;136(5):900–6.

368. Baloyannis SJ, Costa V, Baloyannis IS. Morphological alterations of the synapses in the locus coeruleus in Parkinson's disease. J Neurol Sci. 2006 Oct 25;248(1–2):35–41.

369. Eser RA, Ehrenberg AJ, Petersen C, Dunlop S, Mejia MB, Suemoto CK, et al. Selective Vulnerability of Brainstem Nuclei in Distinct Tauopathies: A Postmortem Study. J Neuropathol Exp Neurol. 2018 Feb 1;77(2):149–61.

370. Cacabelos R. Parkinson's Disease: From Pathogenesis to Pharmacogenomics. Int J Mol Sci. 2017 Mar 4;18(3).

371. Gao V, Suzuki A, Magistretti PJ, Lengacher S, Pollonini G, Steinman MQ, et al. Astrocytic β2-adrenergic receptors mediate hippocampal long-term memory consolidation. Proc Natl Acad Sci U S A. 2016 26;113(30):8526–31.

372. Zorec R, Parpura V, Verkhratsky A. Preventing neurodegeneration by adrenergic astroglial excitation. FEBS J. 2018 Apr 6;

373. Shirshev SV. Role of Epac proteins in mechanisms of cAMP-dependent immunoregulation. Biochem Biokhimiia. 2011 Sep;76(9):981–98.

374. Ghosh M, Xu Y, Pearse DD. Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. J Neuroinflammation. 2016 Jan 13;13:9.

375. Song N, Fang Y, Sun X, Jiang Q, Song C, Chen M, et al. Salmeterol, agonist of β2-aderenergic receptor, prevents systemic inflammation via inhibiting NLRP3 inflammasome. Biochem Pharmacol. 2018;150:245–55.

376. Li H, Hu D, Fan H, Zhang Y, LeSage GD, Caudle Y, et al. β-Arrestin 2 negatively regulates Toll-like receptor 4 (TLR4)-triggered inflammatory signaling via targeting p38 MAPK and interleukin 10. J Biol Chem. 2014 Aug 15;289(33):23075–85.

377. Gronich N, Abernethy DR, Auriel E, Lavi I, Rennert G, Saliba W. β2-adrenoceptor agonists and antagonists and risk of Parkinson's disease. Mov Disord Off J Mov Disord Soc. 2018 Sep;33(9):1465–71.

378. Niranjan R. Recent advances in the mechanisms of neuroinflammation and their roles in neurodegeneration. Neurochem Int. 2018 Nov;120:13–20.

379. Schwartz M, Kipnis J, Rivest S, Prat A. How do immune cells support and shape the brain in health, disease, and aging? J Neurosci Off J Soc Neurosci. 2013 Nov 6;33(45):17587–96.

380. Gao Y, Wilson GR, Stephenson SEM, Bozaoglu K, Farrer MJ, Lockhart PJ. The emerging role of Rab GTPases in the pathogenesis of Parkinson's disease. Mov Disord Off J Mov Disord Soc. 2018;33(2):196–207.

381. Hijioka M, Inden M, Yanagisawa D, Kitamura Y. DJ-1/PARK7: A New Therapeutic Target for Neurodegenerative Disorders. Biol Pharm Bull. 2017;40(5):548–52.

382. Krüger R. The role of synphilin-1 in synaptic function and protein degradation. Cell Tissue Res. 2004 Oct;318(1):195–9.

383. Larson ME, Greimel SJ, Amar F, LaCroix M, Boyle G, Sherman MA, et al. Selective lowering of synapsins induced by oligomeric α-synuclein exacerbates memory deficits. Proc Natl Acad Sci U S A. 2017 06;114(23):E4648–57.

384. Martinez-Vicente M. Neuronal Mitophagy in Neurodegenerative Diseases. Front Mol Neurosci. 2017;10:64.

385. Shen J, Kelleher RJ. The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. Proc Natl Acad Sci U S A. 2007 Jan 9;104(2):403–9.

386. Rohn TT. The role of caspases in Alzheimer's disease; potential novel therapeutic opportunities. Apoptosis Int J Program Cell Death. 2010 Nov;15(11):1403–9.

387. Dursun E, Gezen-Ak D, Hanağası H, Bilgiç B, Lohmann E, Ertan S, et al. The interleukin 1 alpha, interleukin 1 beta, interleukin 6 and alpha-2-macroglobulin serum levels in patients with early or late onset Alzheimer's disease, mild cognitive impairment or Parkinson's disease. J Neuroimmunol. 2015 Jun 15;283:50–7.

388. Gaikwad S, Patel D, Agrawal-Rajput R. CD40 Negatively Regulates ATP-TLR4-Activated Inflammasome in Microglia. Cell Mol Neurobiol. 2017 Mar;37(2):351–9.

389. Zhao J, Chen C, Xiao J-R, Wei H-F, Zhou X, Mao X-X, et al. An Up-regulation of IRF-1 After a Spinal Cord Injury: Implications for Neuronal Apoptosis. J Mol Neurosci MN. 2015 Dec;57(4):595–604.

390. Tanaka T, Murakami K, Bando Y, Yoshida S. Interferon regulatory factor 7 participates in the M1-like microglial polarization switch. Glia. 2015 Apr;63(4):595–610.

391. Hagemeyer N, Prinz M. Burning down the house: IRF7 makes the difference for microglia. EMBO J. 2014 Dec 17;33(24):2885–6.

392. Strickland MR, Koller EJ, Deng DZ, Ceballos-Diaz C, Golde TE, Chakrabarty P. Ifngr1 and Stat1 mediated canonical Ifn-γ signaling drives nigrostriatal degeneration. Neurobiol Dis. 2018 Feb;110:133–41.

393. Wang S, Yuan Y-H, Chen N-H, Wang H-B. The mechanisms of NLRP3 inflammasome/pyroptosis activation and their role in Parkinson's disease. Int Immunopharmacol. 2019 Feb;67:458–64.

394. Gordon R, Albornoz EA, Christie DC, Langley MR, Kumar V, Mantovani S, et al. Inflammasome inhibition prevents α-synuclein pathology and dopaminergic neurodegeneration in mice. Sci Transl Med. 2018 Oct 31;10(465).

395. Mao Z, Liu C, Ji S, Yang Q, Ye H, Han H, et al. The NLRP3 Inflammasome is Involved in the Pathogenesis of Parkinson's Disease in Rats. Neurochem Res. 2017 Apr;42(4):1104–15.

396. Sarkar S, Malovic E, Harishchandra DS, Ghaisas S, Panicker N, Charli A, et al. Mitochondrial impairment in microglia amplifies NLRP3 inflammasome proinflammatory signaling in cell culture and animal models of Parkinson's disease. NPJ Park Dis. 2017;3:30.

397. Wen L, Zhang Q-S, Heng Y, Chen Y, Wang S, Yuan Y-H, et al. NLRP3 inflammasome activation in the thymus of MPTP-induced Parkinsonian mouse model. Toxicol Lett. 2018 May 15;288:1–8.

398. Luo Y, Reis C, Chen S. NLRP3 inflammasome in the pathophysiology of hemorrhagic stroke: A Review. Curr Neuropharmacol. 2018 Dec 27;

399. Burguillos MA, Deierborg T, Kavanagh E, Persson A, Hajji N, Garcia-Quintanilla A, et al. Caspase signalling controls microglia activation and neurotoxicity. Nature. 2011 Apr 21;472(7343):319–24.

400. Zhang C-J, Jiang M, Zhou H, Liu W, Wang C, Kang Z, et al. TLR-stimulated IRAKM activates caspase-8 inflammasome in microglia and promotes neuroinflammation. J Clin Invest. 2018 Dec 3;128(12):5399–412.

401. Qian M, Liu J, Yao J, Wang W, Yang J, Wei L, et al. Caspase-8 Mediates Amyloid-β-induced Apoptosis in Differentiated PC12 Cells. J Mol Neurosci MN. 2015 Jun;56(2):491–9.

402. Dinter E, Saridaki T, Nippold M, Plum S, Diederichs L, Komnig D, et al. Rab7 induces clearance of α-synuclein aggregates. J Neurochem. 2016;138(5):758–74.

403. Atashrazm F, Hammond D, Perera G, Bolliger MF, Matar E, Halliday GM, et al. LRRK2-mediated Rab10 phosphorylation in immune cells from Parkinson's disease patients. Mov Disord Off J Mov Disord Soc. 2018 Dec 30;

404. Waschbüsch D, Hübel N, Ossendorf E, Lobbestael E, Baekelandt V, Lindsay AJ, et al. Rab32 interacts with SNX6 and affects retromer-dependent Golgi trafficking. PloS One. 2019;14(1):e0208889.

405. Jeong GR, Jang E-H, Bae JR, Jun S, Kang HC, Park C-H, et al. Dysregulated phosphorylation of Rab GTPases by LRRK2 induces neurodegeneration. Mol Neurodegener. 2018 13;13(1):8.

406. Alvarez-Castelao B, Castaño JG. Synphilin-1 inhibits alpha-synuclein degradation by the proteasome. Cell Mol Life Sci CMLS. 2011 Aug;68(15):2643–54.

407. Wong YC, Holzbaur ELF. Autophagosome dynamics in neurodegeneration at a glance. J Cell Sci. 2015 Apr 1;128(7):1259–67.

408. De Cecco E, Legname G. The role of the prion protein in the internalization of α-synuclein amyloids. Prion. 2018 02;12(1):23–7.

409. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet Lond Engl. 2005 Nov 19;366(9499):1809–20.

410. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. J Periodontol. 2005 Nov;76(11 Suppl):2033–41.

411. Yamaguchi Y, Kurita-Ochiai T, Kobayashi R, Suzuki T, Ando T. Activation of the NLRP3 inflammasome in Porphyromonas gingivalis-accelerated atherosclerosis. Pathog Dis. 2015 Jun;73(4).

412. Marchesan JT, Gerow EA, Schaff R, Taut AD, Shin S-Y, Sugai J, et al. Porphyromonas gingivalis oral infection exacerbates the development and severity of collagen-induced arthritis. Arthritis Res Ther. 2013 Nov 12;15(6):R186.

413. Maresz KJ, Hellvard A, Sroka A, Adamowicz K, Bielecka E, Koziel J, et al. Porphyromonas gingivalis facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD). PLoS Pathog. 2013 Sep;9(9):e1003627.

414. Ha NH, Woo BH, Kim DJ, Ha ES, Choi JI, Kim SJ, et al. Prolonged and repetitive exposure to Porphyromonas gingivalis increases aggressiveness of oral cancer cells by promoting acquisition of cancer stem cell properties. Tumour Biol J Int Soc Oncodevelopmental Biol Med. 2015 Dec;36(12):9947–60.

415. Nagy KN, Sonkodi I, Szöke I, Nagy E, Newman HN. The microflora associated with human oral carcinomas. Oral Oncol. 1998 Jul;34(4):304–8.

416. Chambrone L, Foz AM, Guglielmetti MR, Pannuti CM, Artese HPC, Feres M, et al. Periodontitis and chronic kidney disease: a systematic review of the association of diseases and the effect of periodontal treatment on estimated glomerular filtration rate. J Clin Periodontol. 2013 May;40(5):443–56.

417. Kimizuka R, Kato T, Ishihara K, Okuda K. Mixed infections with Porphyromonas gingivalis and Treponema denticola cause excessive inflammatory responses in a mouse pneumonia model compared with monoinfections. Microbes Infect. 2003 Dec;5(15):1357–62.

418. de Melo Neto JP, Melo MS a. E, dos Santos-Pereira SA, Martinez EF, Okajima LS, Saba-Chujfi E. Periodontal infections and community-acquired pneumonia: a case-control study. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol. 2013 Jan;32(1):27–32.

419. Ledić K, Marinković S, Puhar I, Spalj S, Popović-Grle S, Ivić-Kardum M, et al. Periodontal disease increases risk for chronic obstructive pulmonary disease. Coll Antropol. 2013 Sep;37(3):937–42.

420. Soroye M, Ayanbadejo P, Savage K, Oluwole A. Association between periodontal disease and pregnancy outcomes. Odonto-Stomatol Trop Trop Dent J. 2015 Dec;38(152):5–16.

421. Wang M, Krauss JL, Domon H, Hosur KB, Liang S, Magotti P, et al. Microbial hijacking of complement-toll-like receptor crosstalk. Sci Signal. 2010 Feb 16;3(109):ra11.

422. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol. 2005 Jan;17(1):1–14.

423. Asai Y, Ohyama Y, Gen K, Ogawa T. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. Infect Immun. 2001 Dec;69(12):7387–95.

424. Burns E, Bachrach G, Shapira L, Nussbaum G. Cutting Edge: TLR2 is required for the innate response to Porphyromonas gingivalis: activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone resorption. J Immunol Baltim Md 1950. 2006 Dec 15;177(12):8296–300.

425. Elimelech R, Mayer Y, Braun-Moscovici Y, Machtei EE, Balbir-Gurman A. Periodontal Conditions and Tumor Necrosis Factor-Alpha Level in Gingival Crevicular Fluid of Scleroderma Patients. Isr Med Assoc J IMAJ. 2015 Sep;17(9):549–53.

426. Chapple ILC, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000. 2007;43:160–232.

427. Kendall HK, Marshall RI, Bartold PM. Nitric oxide and tissue destruction. Oral Dis. 2001 Jan;7(1):2–10.

428. Moutsopoulos NM, Konkel J, Sarmadi M, Eskan MA, Wild T, Dutzan N, et al. Defective neutrophil recruitment in leukocyte adhesion deficiency type I disease causes local IL-17-driven inflammatory bone loss. Sci Transl Med. 2014 Mar 26;6(229):229ra40.

429. Chen LL, Yan J. Porphyromonas gingivalis lipopolysaccharide activated bone resorption of osteoclasts by inducing IL-1, TNF, and PGE. Acta Pharmacol Sin. 2001 Jul;22(7):614–8.

430. Garcia de Aquino S, Manzolli Leite FR, Stach-Machado DR, Francisco da Silva JA, Spolidorio LC, Rossa C. Signaling pathways associated with the expression of inflammatory mediators activated during the course of two models of experimental periodontitis. Life Sci. 2009 May 22;84(21–22):745–54.

431. Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. Periodontol 2000. 2015 Oct;69(1):7–17.

432. Zhang Y, Li X. Lipopolysaccharide-regulated production of bone sialoprotein and interleukin-8 in human periodontal ligament fibroblasts: the role of toll-like receptors 2 and 4 and the MAPK pathway. J Periodontal Res. 2015 Apr;50(2):141–51.

433. Schulze-Osthoff K, Bauer MK, Vogt M, Wesselborg S. Oxidative stress and signal transduction. Int J Vitam Nutr Res Int Z Vitam- Ernahrungsforschung J Int Vitaminol Nutr. 1997;67(5):336–42.

434. Sanders VM. The beta2-adrenergic receptor on T and B lymphocytes: do we understand it yet? Brain Behav Immun. 2012 Feb;26(2):195–200.

435. Kolmus K, Tavernier J, Gerlo S. β2-Adrenergic receptors in immunity and inflammation: stressing NF-κB. Brain Behav Immun. 2015 Mar;45:297–310.

436. Ezeamuzie CI, Shihab PK. Interactions between theophylline and salbutamol on cytokine release in human monocytes. J Pharmacol Exp Ther. 2010 Jul;334(1):302–9.

437. Bostanci N, Belibasakis GN. Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. FEMS Microbiol Lett. 2012 Aug;333(1):1–9.

438. Darveau RP, Arbabi S, Garcia I, Bainbridge B, Maier RV. Porphyromonas gingivalis lipopolysaccharide is both agonist and antagonist for p38 mitogen-activated protein kinase activation. Infect Immun. 2002 Apr;70(4):1867–73.

439. Bainbridge BW, Darveau RP. Porphyromonas gingivalis lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. Acta Odontol Scand. 2001 Jun;59(3):131–8.

440. Hajishengallis G, Tapping RI, Harokopakis E, Nishiyama S, Ratti P, Schifferle RE, et al. Differential interactions of fimbriae and lipopolysaccharide from Porphyromonas gingivalis with the Toll-like receptor 2-centred pattern recognition apparatus. Cell Microbiol. 2006 Oct;8(10):1557–70.

441. Coats SR, Reife RA, Bainbridge BW, Pham TT-T, Darveau RP. Porphyromonas gingivalis lipopolysaccharide antagonizes Escherichia coli lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infect Immun. 2003 Dec;71(12):6799–807.

442. Wendell KJ, Stein SH. Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. J Periodontol. 2001 Aug;72(8):1038–44.

443. Hajishengallis G, Sojar H, Genco RJ, DeNardin E. Intracellular signaling and cytokine induction upon interactions of Porphyromonas gingivalis fimbriae with pattern-recognition receptors. Immunol Invest. 2004 May;33(2):157–72.

444. Zhang P, Fan Y, Li Q, Chen J, Zhou W, Luo Y, et al. Macrophage activating factor: A potential biomarker of periodontal health status. Arch Oral Biol. 2016 Oct;70:94–9.

445. Silva MJB, Sousa LMA, Lara VPL, Cardoso FP, Júnior GM, Totola AH, et al. The role of iNOS and PHOX in periapical bone resorption. J Dent Res. 2011 Apr;90(4):495–500.

446. Cintra LTA, Samuel RO, Azuma MM, de Queiróz AOS, Ervolino E, Sumida DH, et al. Multiple Apical Periodontitis Influences Serum Levels of Cytokines and Nitric Oxide. J Endod. 2016 May;42(5):747–51.

447. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001 Feb;13(2):85–94.

448. Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol. 2004 Oct 1;500(1–3):255–66.

449. Kabashima H, Nagata K, Maeda K, Iijima T. Interferon-gamma-producing cells and inducible nitric oxide synthase-producing cells in periapical granulomas. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 1998 Mar;27(3):95–100.

450. Liu B, Wang J, Cheng L, Liang J. Role of JNK and NF-κB pathways in Porphyromonas gingivalis LPS-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. Mol Med Rep. 2013 Nov;8(5):1594–600.

451. Fujita Y, Nakayama M, Naito M, Yamachika E, Inoue T, Nakayama K, et al. Hemoglobin receptor protein from Porphyromonas gingivalis induces interleukin-8 production in human gingival epithelial cells through stimulation of the mitogen-activated protein kinase and NF-κB signal transduction pathways. Infect Immun. 2014 Jan;82(1):202–11.

452. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem. 1994 Feb 18;269(7):4705–8.

453. Kopp EB, Ghosh S. NF-kappa B and rel proteins in innate immunity. Adv Immunol. 1995;58:1–27.

454. Tukhovskaya EA, Turovsky EA, Turovskaya MV, Levin SG, Murashev AN, Zinchenko VP, et al. Anti-inflammatory cytokine interleukin-10 increases resistance to brain ischemia through modulation of ischemia-induced intracellular Ca^{2+} response. Neurosci Lett. 2014 Jun 13;571:55–60.

455. Henn A, Lund S, Hedtjärn M, Schrattenholz A, Pörzgen P, Leist M. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX. 2009;26(2):83–94.

456. Das A, Kim SH, Arifuzzaman S, Yoon T, Chai JC, Lee YS, et al. Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia. J Neuroinflammation. 2016 11;13(1):182.