Characterizing the mode of action of super long-acting β2-adrenergic receptor agonists: A more effective therapeutic agent for Parkinson's disease.

by

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Abstract:

Naik Bakht Arbabzada: Characterizing the mode of action of super long-acting β2-adrenergic receptor agonists: A more effective therapeutic agent for Parkinson's disease.

(Under the direction of Dr. Patrick Flood)

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a loss of dopaminergic neurons in the Substantia nigra (SN) resulting in hypokinetic motor movements. The long-acting β2-adrenergic receptor (β2-AR) agonist salmeterol has been shown to be neuroprotective in animal models of PD. Salmeterol was found to work by inhibiting the inflammatory response of microglial cells (Qian *et al.,* 2011), a key cell in the pathogenesis of PD. Recently, super long-acting β2-AR agonists vilanterol and indacaterol have been described; the objective of this study was to examine the effects of these super long-acting agonists on the inflammatory response of the microglial cell line BV-2, and compare their mode of action to that of the long-acting agonist salmeterol. We report that the super long-acting agonists were more effective in inhibiting TNF-α then the long-acting agonists, and that all three agonists enhanced IL-10 cytokine release from stimulated BV-2 cells. Furthermore, like salmeterol, indacaterol and vilanterol also exerted their inhibitory effect on TNF-α through the inhibition of NF-κB in a TAK-1 and β-arrestin2-dependent but PKA-independent manner. In contrast, the IL-10 effect was NF-κB, TAK1, and PKA-dependent but β-arrestin2-independent. Furthermore, inhibition of all three MAPKs (ERK1/2, JNK, and p38) consistently synergized with the long-acting agonist salmeterol to further inhibit TNF-α but not with the super long-acting agonists. As well, JNK activity is needed to reverse IL-10 enhancement by all three β2-AR agonists. Combined, our data suggests that β2-AR agonists mediate an anti-inflammatory conversion

in the cytokine phenotype of activated microglial cells. These findings provide insight into how β2-AR agonists' work to reverse the Central Nervous System (CNS) inflammation that occurs in PD. Characterization and validation of the reported data in *in vivo* models of PD will assist in selecting the more effective β2-AR agonist as an adjunct therapy for PD.

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I have been so blessed and privileged to have such a dedicated group of people to support me. I want to dedicate this thesis to all those affected by Parkinson's disease- I wish each and every one of you unconditional love and support.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
7TMR	7-Transmembrane Receptor
α-AR	Alpha-Adrenergic receptor
Akt	Protein Kinase B
AP-1	Activator Protein 1
APC	Antigen presentation cell
β-AR	Beta-Adrenergic receptor
β2-AR	β2-Adrenergic receptor
BBB	Blood brain barrier
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
COMT	catechol-*O*-methyltransferase
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal Fluid
DA	Dopamine
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
E2F	Eukaryotic Transcription 2

EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ETF	Eukaryotic Transcription Factor
GPCR	G-protein coupled receptor
$G_{\alpha s}$	G-protein- αs
G_i	G-protein-i
$G_{\alpha q11}$	G-protein-αq11
H2O2	Hydrogen peroxide
IκBK	IkappaB kinase
IκB-α	IkappaB-alpha
IKK	IkappaKinase
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IL-1R	IL-1 receptor-associated kinases
IP3	Inositol Trisphosphate
JNK	c-Jun N-terminal kinase
LRRK-2	Leucine Rich Repeat Kinase-2

L-DOPA	Leva-dopa
LPS	Lipopolysaccharide
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Min.	Minute/minutes
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP	1-Methyl-4-phenylpyridinium
MPO	Myeloperoxidase
MSN	Medium Spiny Neurons
MAO-B	Monoamine Oxidase B
NF-□B	Nuclear factor □B
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drugs
Pael-R	Parkin-associated endothelin-like receptor
PAMP	Pathogen associated molecular pattern
PD	Parkinson’s disease
PHOX	NADPH oxidase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	Protein kinase A

PNS	Peripheral Nervous System
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulate
SNS	Sympathetic Nervous System
SP-1	Specificity Protein-1
STAT	Signal transducer and activator of transcription
TH1	T-helper cell-1
TAK-1	Transforming growth factor beta-activated kinase 1
TCR	T cell receptor
TGF-α	Transforming Growth factor-alpha
TGF-β1	Transforming Growth factor-beta1
TH	Tyrosine Hydroxylase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor-alpha receptor
TRAF6	TNF-receptor associated factor 6

CHAPTER 1:

GENERAL OVERVIEW

Parkinson's disease (PD) is an incurable progressive neurodegenerative condition that affects 1% of the global population aged 50 and above (Thomas *et al.,* 2007). This disease is characterized by dopaminergic (DA) neuronal degeneration in the Substantia nigra (SN), resulting in the manifestation of cognitive and motor symptoms. The motor symptoms are by the far the most characteristic and debilitating aspect of the disease, and manifest early as resting tremors, rigidity, bradykinesia (slowness of movement) and postural instability. Parkinson's disease 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 (DA) in the SN pars compacta (SNpc) (Bernheimer *et al.,* 1973; Bezard *et al.,* 2001). These movement impairments and the associated muscular weakness add additional complexity to the management of the disease, as patients are prone to falling and/or choking on their food, and therefore require constant care. Furthermore, those afflicted with PD are likely to develop both dementia and depression during the late stages of the disease. Dementia is reported in 28% of PD cases with the prevalence rising to 65% in those aged 85 years and above. The range of cognitive symptoms varies and includes depression, emotional changes, and sleep abnormalities (Blonder *et al.,* 2011). As PD is mainly a movement disorder, muscle weakness and rigidity can lead to difficulty in swallowing, speech production, urinary problems, muscle cramps and associated pain as well as sexual dysfunctions. These symptoms further exacerbate the cognitive symptoms and also explain the increase in the prevalence of these cognitive symptoms with disease progression, as greater physical immobility can cause depression and other emotional imbalances. The associated costs, in terms of both treatments and indirect burdens such as security payments, lost income from inability to work, and caregiver expenses, are

estimated to be nearly $25 billion annually in the United States alone. In Canada, the *direct* cost for PD is $86.8 million annually. With longevity on the rise, the prevalence of PD is projected to increase significantly; consequently, the associated societal burdens will also increase. Currently, the gold-standard treatment for PD is the use of Levadopa (L-Dopa) therapy to replace the diminished dopamine levels in the brain. While L-Dopa improves certain clinical motor symptoms, it is not a permanent cure. These factors coupled with the projected increase in the incidence of PD render PD one of the major medical challenges of our time.

It is now understood that inflammatory processes are a major factor in the progression of the disease (McGeer *et al.,* 1988; Peterson *et al.,* 2014). Inflammation is defined as a localized response to injury and/or invading microbes characterized by redness, heat, pain, and swelling (Chiu *et al.,* 2012; Punchard *et al.,* 2004). After an injurious insult, the tissue homeostasis is disrupted; inflammation serves as a protective response to re-establish homeostasis and promote removal of the injurious insult as well as initiate tissue repair and recovery. Inflammation is a dynamic process involving immune cells, blood vessels, proteins and tissue-specific cells (Chiu *et al.,* 2012; Ousman *et al.,* 2012). Briefly, the immune cells play an important role in initiating inflammation by recognizing infiltrating pathogens and/or damage associated molecular patterns, PAMPs and DAMPs, respectively, through germline-encoded pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nod-like receptors (NLRs) (Chiu *et al.,* 2012). These activated immune cells then release various cytokines and chemokines that recruit additional immune cells through the vasculature (Chiu *et al.,* 2012). Acute inflammation is an immediate response that is normally resolved within a few days resulting in wound

healing and restoration of homeostasis. Acute inflammation can also lead to abscess formation and to chronic inflammation (Maskrey *et al.,* 2011). Conversely, chronic inflammation is characterized by a delayed response that can last anywhere from a few weeks to years and result in permanent tissue destruction and fibrosis (scar formation) (Maskrey *et al.,* 2011).

Evidence to support neuroinflammation—chronic inflammation in the Central Nervous System (CNS)— in the progression of PD originates from post-mortem *in vivo* studies in both patients and animal studies. Neuroinflammation is characterized by activation of microglia and astroglia that facilitate the expansion of an initial insult through secretion of pro-inflammatory cytokines and chemokines (Striet *et al.,* 2004; Hirsch *et al.,* 2009). Activation and proliferation of both microglia and astroglia have been reported in post-mortem PD brains (McGeer *et al.,* 1988; Damier *et al.,* 1993). Additionally, infiltrating peripheral lymphocytes such as cytotoxic CD8+ and CD4+ cells are localized to the SN in PD patients (Hirsch *et al.,* 2009), further supporting the role of inflammation in the disease. Analysis of post-mortem striatal tissue show increased expression of pro-inflammatory cytokines TNF-α, interleukins 1β and 6, transforming growth factor α (TGFα) and β1 (TGFβ1) (Mogi *et al.,* 1996). Likewise, these findings have been confirmed in patients diagnosed with PD through analysis of cerebrospinal fluid (CSF), blood and serum samples, and imaging techniques (McGeer *et al.,* 1988; Muller *et al.,* 1998; Hisanaga *et al.,* 2001; Gerhard *et al.,* 2006). The implication of these findings is that neuronal loss is accompanied, exacerbated and/or caused by inflammatory processes. While the role of astrocytes in the pathogenesis of PD remains uncharacterized and is unlikely to influence

cell death, the role of microglial activation in DA-neurotoxicity and neuroprotection is well characterized.

Astroglia and microglia are the two major cells that are known to participate in the inflammatory response of the CNS. 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 blood-brain-barrier (BBB), facilitating tissue repair and scar formation, and maintenance of extracellular ion homeostasis. The expression of receptors that are critical for innate immunity such as TLRs, nucleotide-binding oligomerization domains, double-stranded RNA-dependent protein kinase, scavenger receptors, mannose binding lectin receptor, and complement system components on astrocytes have implicated a role for astrocytes in innate immunity (Farina *et al.,* 2007). The role of astrocytes in PD is debatable and not well understood, as studies are inconclusive as to whether astrocytes have a neuroprotective effect and/or a neurotoxic effect in PD. However, activation of astrocytes has been reported in some cases of PD as demonstrated by an increase in the glial fibriallary acid proteins (GFAP) (Damier *et al.,* 1993; Mirza *et al.,* 2000; Wilhelmsson *et al.,* 2006). GFAP is an intermediate filament needed by astrocytes to synthesize cytoskeletal structures as well as a well-established biomarker for astrocytosis (Wilhelmsson *et al.,* 2006). Furthermore, activated astrocytes are reported in postmortem brain of PD patients; however, this activation is not confined to the SN and its function therefore still remains elusive (Knott *et al.,* 1999). In contrast, astrocyte activation is not only well documented in the MPTP and 6-OHDA models of PD, but it has been reported to precede neuronal cell death (Kohutnicka *et al.,* 1998; Nomura *et al.,* 2000; Teismann *et al.,* 2004). 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. There are also studies that contradict the presence of astrocytosis post 6-OHDA-injection (Depino *et al.,* 2003). Therefore, while astrocytes are an important cell type in the CNS, their role in PD is not yet well established, and will not be discussed further.

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 immune cells, microglia are responsible for scavenging the CNS milieu for potential infiltrating pathogens and/or endogenous insults. As such, phagocytic, cytotoxic and antigen presenting capabilities of these cells enable them to protect the CNS from various insults. Activated microglia target infiltrating pathogens and damaged cells by releasing toxic reactive oxygen species (ROS), free radicals, and pro-inflammatory cytokines, as well as by mediating phagocytosis. Evidence of microglial activation and its role in PD pathogenesis is indisputable. Knott and colleagues (1999) and others (McGeer *et al.,* 1988) have reported activated amoeboid-shaped microglia in postmortem brain of PD patients. These activated microglia are densely located and confined to the SN pars compacta (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 laboratory as well as other groups (Mount *et al.,* 2007; Qian *et al.,* 2009; Qian *et al.,* 2011) has shown that in various PD models, microglial cells are needed for the pathogenesis of PD. For example, our lab has shown that high doses of the LPS, DA-neurotoxin MPTP, and β2-adrenergic receptor agonist salmeterol can all induce dopaminergic cell toxicity. However, microglial cells are indispensible in the LPS, MPTP, and β2-AR-mediated

toxicity, as these neurotoxic agents have no effect on neuron-only cultures (Qian *et al.*, 2006; Arimoto *et al.*, 2007; Qian *et al.*, 2009; Qian *et al.*, 2011). In PD, elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are found in the basal ganglia as well as the CSF of PD patients (Mogi *et al.*, 1996; McGeer *et al.*, 2004; Hirsch *et al.*, 2009). Additionally, the microglial-derived reactive oxygen species as well as 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 SN in both patients and animal models (Hunot *et al.*, 1996; Gao *et al.*, 2003; Qin *et al.*, 2004; Qian *et al.*, 2006; Qin *et al.*, 2013). These pro-inflammatory mediators can cause neurocytotoxity in a direct receptor-mediated manner and/or in an indirect manner. 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; accordingly, TNFR knockout is resistant to DA-neurotoxicity in the MPTP model of PD (Sriram *et al.*, 2002). Indirectly, activated microglial cells releasing pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and reactive oxygen species can then activate additional microglial cells (Hunot *et al.*, 1996; Xing *et al.*, 2011) in a process called reactive microgliosis (Gao *et al.*, 2003; Qian *et al.*, 2008; Peterson *et al.*, 2014). This leads to a cyclical pathway whereby activation of a few cells can amplify the initial insult to a higher magnitude. Microgliosis is now a leading working model for understanding and targeting neuroinflammation in PD [Figure 1.1] (Peterson *et al.*, 2014).

Microglia are an enigmatic cell type; activation of microglial cells can be detrimental and/or beneficial during an inflammatory process to the surrounding environment. Similar to the activation of macrophages, microglial activation has also been characterized into

classical and alternate pathways (Orihuela *et al.,* 2015). Classical activation of microglial cells by TLRs and NLRs results in the release of pro-inflammatory mediators and can add to inflammation-induced cell death (Okun *et al.,* 2009). The alternate pathway involves activation by IL-4 and -13 leading to the suppression of the pro-inflammatory cytokines (Stein *et al.*, 1992; Tang *et al.,* 2014; Gong *et al.*, 2016). Accordingly, microglia has been termed as M1 (pro-inflammatory) and M2 (anti-inflammatory) based on the classical and alternate activation and phenotypic presentations. The M1 phenotype of microglial cells resulting from classical activation is characterized by tissue destruction, anti-microbial activity, and cell death (Okun *et al.,* 2009; Orihuela *et al.*, 2015). Tissue regeneration and wound healing generally characterize the M2 phenotype. M2 microglia can follow the classical M1 phase, result from direct activation of the M2 phenotype, and can revert to an M1 phenotype as well (Stein *et al.,* 1992; Gordon, 2003; Hu *et al.,* 2015). Consequently, subclasses of the M2 phenotype exist and are dependent on the activation signal: e.g. parasitic activation results in M2a polarization; signaling through Fcγ receptors, TLRs and immune complexes such as that seen in allergic responses result in M2b polarization; and lastly, anti-inflammatory mediators such as IL-10, TGF-β and glucocorticoids induce M2c polarization (Gordon, 2003; Lawrence and Natoli, 2011; Mills, 2012; Wynn *et al.,* 2013; Martinez and Gordon, 2014). M2a microglia primarily contributes to anti-inflammation whereas M2c microglia drives the would-healing and regeneration state (Varnum *et al.*, 2012). Additionally, these phenotypically distinct microglia are characterized by differing expression profile of surface markers (Tang *et al.,* 2014; Varnu *et al.,* 2015). While a great number of markers can be used to distinguish microglia and microglial-subtypes; M1-microglia are defined as I-A^{high}, $CD86^{high}$, $CD14^{high}$, and $CD206^{low}$, while M2-microglia

are defined as I-A^{low}, CD86^{low}, CD14^{low}, CD206^{high} and Arginase 1^{+} (Kigerl *et al.,* 2009; Liu *et al.,* 2013; Jablonski *et al.,* 2015). These surface proteins are essential in enabling the microglial cells to propagate wound deteriorating or healing effects. For example, the arginase enzymatic pathway contributes to tissue repair, as several of the by-products are important sources of collagen (Kigerl *et al.,* 2009). Another important surface marker is CD206—a transmembrane glycoprotein on the surface of microglial cells and a member of C-type lectin family. CD206 initiates immunosuppressive pathways and is important in CNS repair (Kigerl *et al.,* 2009).

While the differing microglial states are well characterized, how microglial cells adopt one state over another remains elusive. In a review, Italiani and colleagues (2014) highlight three hypotheses on the mechanism by which macrophages can adopt a particular state. Polarized populations of microglia and macrophage can be differentiated by protein expression and activity (Hu *et al.,* 2015); however, their polarization process is remarkably similar. Therefore, the mechanism by which macrophage alter their phenotype is applicable to microglia. One hypothesis is that different subtypes of macrophage populations adopt the different states; i.e. resident macrophages adopt an M2 while infiltrating macrophages adopt an M1 state. Another hypothesis highlights the role of the microenvironment; infiltrating macrophages encounter different microenvironments and therefore adopt M1 and/or M2 in a time-dependent manner. A third hypothesis posits that an M1 macrophage can switch into an M2 macrophage and vice-versa—a phenomenon of immune cell plasticity.

The plasticity of immune cells to alter their phenotype can serve as a marker for determining potential anti-inflammatory therapies for PD and other immune cell-driven

neuroinflammatory disorders. A number of therapeutic strategies have been proposed to limit disease progression by either down-regulating microglial activation and/or attenuating the production and release of pro-inflammatory cytokines. Strategies such as NSAIDS and COX-2 inhibitors to alleviate the inflammatory milieu, knockout studies in animal models for TNF-α, NO and several other factors have been tested (AlDakheel *et al.,* 2014). An emerging approach is to identify, at the molecular level, where, how, and which cell signaling molecules should be inhibited and/or stimulated to diminish inflammation and promote wound healing. For example, JNK and p38 pathways are crucial in the TNF-α-FasL mediated cell death (Wang *et al.,* 2004). Furthermore, TNF-α-mediated activation of microglia also stimulates the NF-κB as well as JNK and p38 pathways to phenotypically alter the activated microglia to an M1 (TNF-α secreting, pro-inflammation) state (Kroner *et al.,* 2014). While expanding on previous findings, in the study presented here we have found evidence to suggest that adrenergic receptors, specifically β2-AR, may have a promising effect in not only inhibiting but also possibly reversing the M1 inflammatory phenotype of microglial cells that is normally observed in PD.

Introduction to Adrenergic Receptors

Adrenergic receptors are a subtype of G-protein-coupled receptors located on the cell surface of neurons and immune cells. The type of G-protein the receptor couples to determines the receptor's downstream signaling effect [Figure 1.2]. The adrenergic receptor is divided into two main groups: α and β [Figure 1.3]. The α-group is further divided into two subtypes: α_1 coupled to G_q and α_2 coupled to G_i. The β-group is subdivided into β_1, β_2, and β_3 and these β-subtypes are all coupled to G_s with β_2 being coupled to G_i as well. The β_2-adrenergic receptor is found in most tissues and plays in important role in their

physiological functions. Of particular interest to our topic is the role that β_2-adrenergic receptor plays in the immune-nervous system interaction that occurs in PD. It has been found that stimulation of the β_2-adrenergic receptor inhibits the production and release of pro-inflammatory cytokines such as TNF-α, and IFN-γ and increases the production of anti-inflammatory cytokine such as IL-10 (Suberville *et al.,* 1996; Chou *et al.,* 1996; Elenkov *et al.,* 2000). Specifically, pretreatment with β_2-adrenergic receptor agonists primes microglial cells to release lower levels of TNF-α after being challenged with an inflammagen (Qian *et al.,* 2011; Johnson *et al.,* 2013)

The β2-AR agonists are functionally classified into short, long, and super long-acting agonists based on their *in vivo* half-life. In addition to their functional difference, the classes of β2-AR agonists differ in their molecular characteristics that dictate how they interact with the receptors. To specify, the short-acting agonists are hydrophilic so they are able to dissolve in the aqueous extracellular milieu and interact directly with the β2-AR on the cell membrane (Cazzola *et al.,* 2011). Such agonists include fast-acting bronchodilators used for managing asthma and other airway disorders. In contrast, the long-acting and super long-acting agonists are lipophilic (Cazzola *et al.,* 2011). Both long-acting and super long-acting agonists are currently being used for managing chronic bronchial diseases like chronic obstructive pulmonary disease (COPD: long-acting agonists include salmeterol (GSK, Serevent/Advair), arformoterol (Sunovion, Brovana) and formoterol (Astra-Zenica, Perforomist/Symbicort); and super long-acting agonists include indacaterol (Arcapta), vilanterol (Breo) and olodaterol (Striverdi Respimat). Pharmacologically, the super long-acting agonists have a higher affinity for the β2-ARs and a faster onset of action compared to long-acting agonists (Cazzola *et al.,* 2011). Two hypotheses aim to account for the

differences in *in vivo* half-lives of these compounds: exosite/exoreceptor and plasmalemma diffusion microkinetic (Anderson *et al.,* 1994). 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 (Anderson et al., 1994). The plasmalemma diffusion hypothesis posits that high concentrations of agonists are achieved in close proximity to the receptor and allows for a longer duration of action (Anderson et al, 1994). Both of these theories need further investigation and as well need to be studied within the CNS. Since our focus is on the effect of β2-AR agonists as anti-inflammatory agents, the pharmacology of β2-AR agonists will not be discussed further.

B2-ARs in Parkinson's disease

In Parkinson's disease, the major driver for neuronal death and deterioration is neuroinflammation. Consistent with this is that neurotoxin and inflammation-induced animal models of PD mimic clinical manifestation more so than genetic models of PD. Moreover, retroactive studies examining the effects of anti-inflammatory agents as well as knocking out inflammatory enzymes such as Phox are protective against PD. Consequently, inhibiting microglial cells – immune cells important in propagating inflammation in the CNS— activation would theoretically stop the progression of PD and be neuroprotective. β2-AR activation has been found to have anti-inflammatory effects (Farmer et al., 2000; Park et al., 2006; Qian et al., 2009). Accordingly, prophylactic treatment of salmeterol in animal models was found to be a highly effective therapeutic at inhibiting microglial cell activation and in preventing DA neurodegeneration in an animal model of PD (Qian *et al.,* 2011). The long-acting agonists were found to be more effective

than the short-acting agonists. The long-acting agonist salmeterol was reported to exercise neuroprotection by inhibiting NF-κB and Mitogen-associated protein kinases (MAPKs) in a β-arrestin2 and TAK1-dependent manner (Qian *et al.*, 2011; Peterson *et al.*, 2014). Super long-acting β2-AR agonists vilanterol and indacaterol are now commercially available. Therefore, the objective of this study was examine the anti-inflammatory effects of these super long-acting agonists and compare their mode of action to that the long-acting agonist salmeterol.

Thesis Statement

We hypothesize that these super long-acting agonists are more effective, i.e. lower effective dose with a prolonged duration of activity, than the long-acting agonists in inhibiting microglial activation.

To test this hypothesis, we sought to characterize the inhibitory effects of super long-acting β2-AR agonists vilanterol and indacaterol in activated murine BV-2 microglia; and compared their effectiveness and mode of action to that of salmeterol. In this process, levels of pro- and anti-inflammatory cytokines determined the effective dose and kinetics of action of vilanterol, indacaterol, and salmeterol. The effects of vilanterol and indacaterol on the function of microglial cells was also determined using various intracellular signaling molecules such as TAK1, β-arrrestin2, NF-κB, and MAPKs. Successful completion of these experiments has now identified these super long-acting agonists as the most effective β2-AR agonist currently available to be used for microglial inhibition. It also provides further insight into how β2-AR agonists work to reverse the CNS inflammation. Ultimately, it may provide a new and more effective therapeutic for the neuroinflammatory pathology seen in PD as well as other microglial-driven neurodegenerative disorders.

CHAPTER I FIGURES:

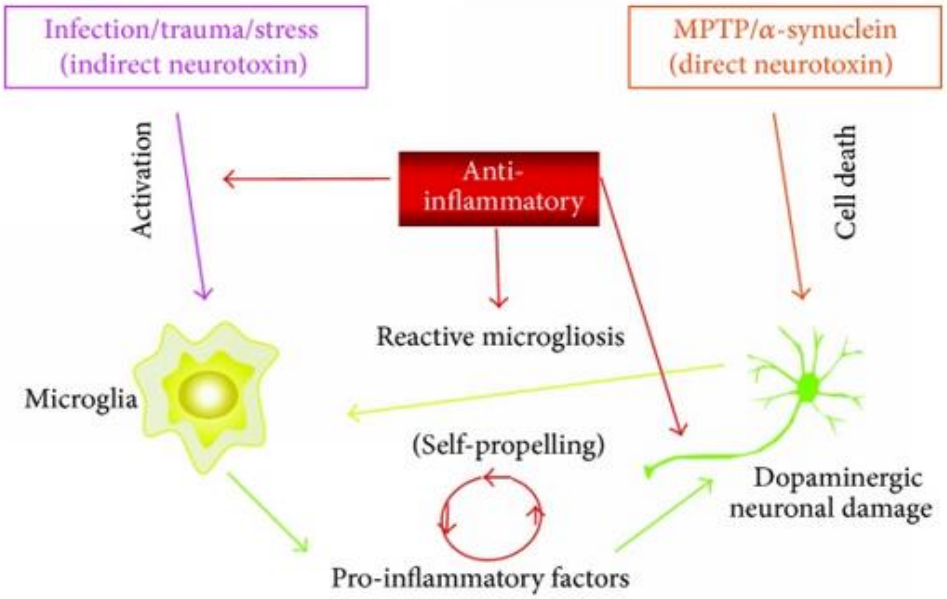

Figure 1.1 A schematic of the role of microglia in neurodegeneration seen in Parkinson's disease. Activated microglial cells release pro-inflammatory cytokines that can induce DA-neurotoxicity as well as reactive microgliosis. (Peterson et al., 2014)

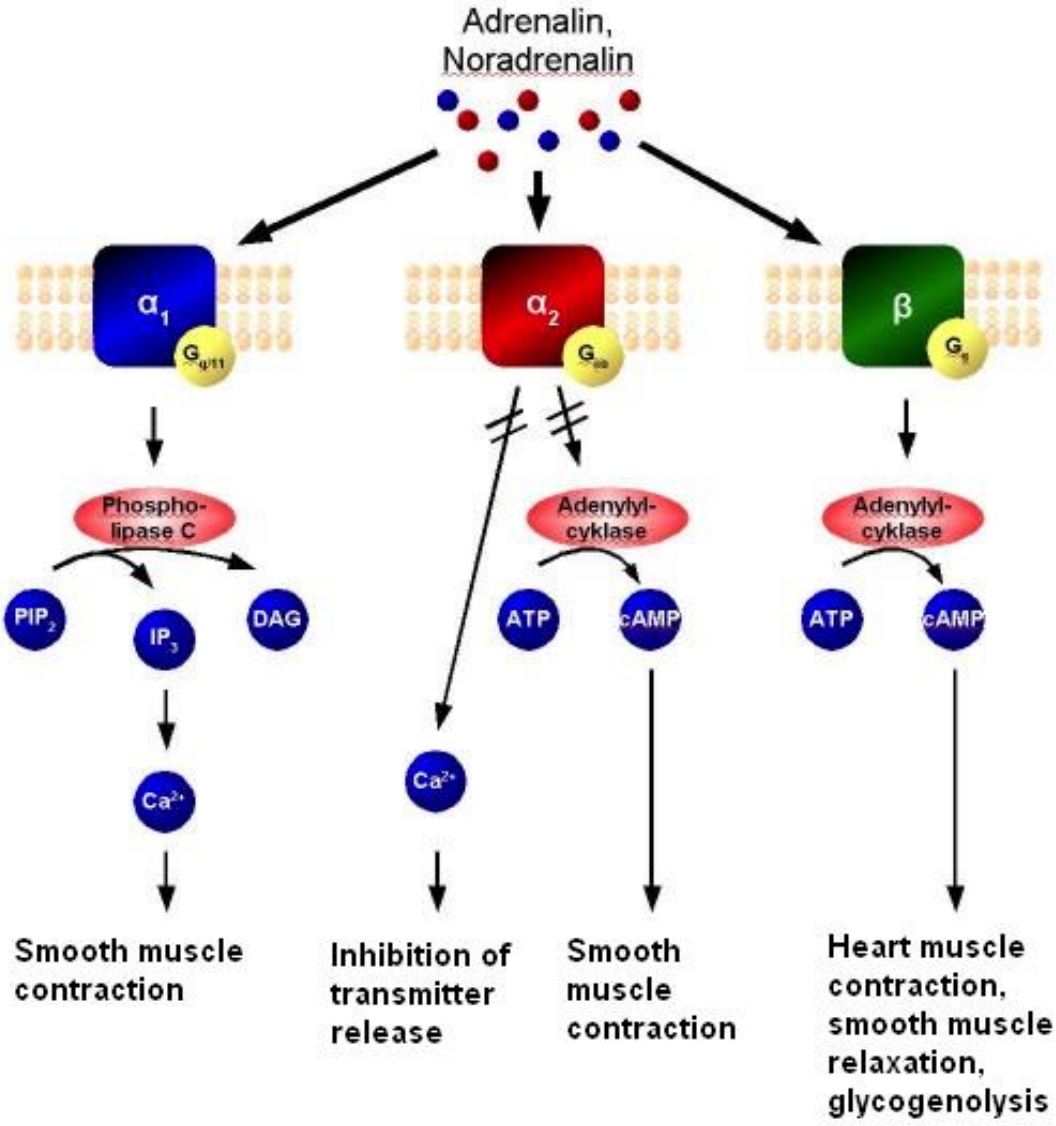

Figure 1.2 Adrenergic receptors are a class of G-protein-coupled receptors (GPCR) that regulate various activities. Receptor coupling to different isoforms of G-proteins gives rise to the diversity in adrenergic receptors signaling. Each G-protein transduces signals through its associated second messengers: cyclic AMP (cAMP), calcium, and inositol-phosphate (IP_3).

(Jähnichen, 2006)

A

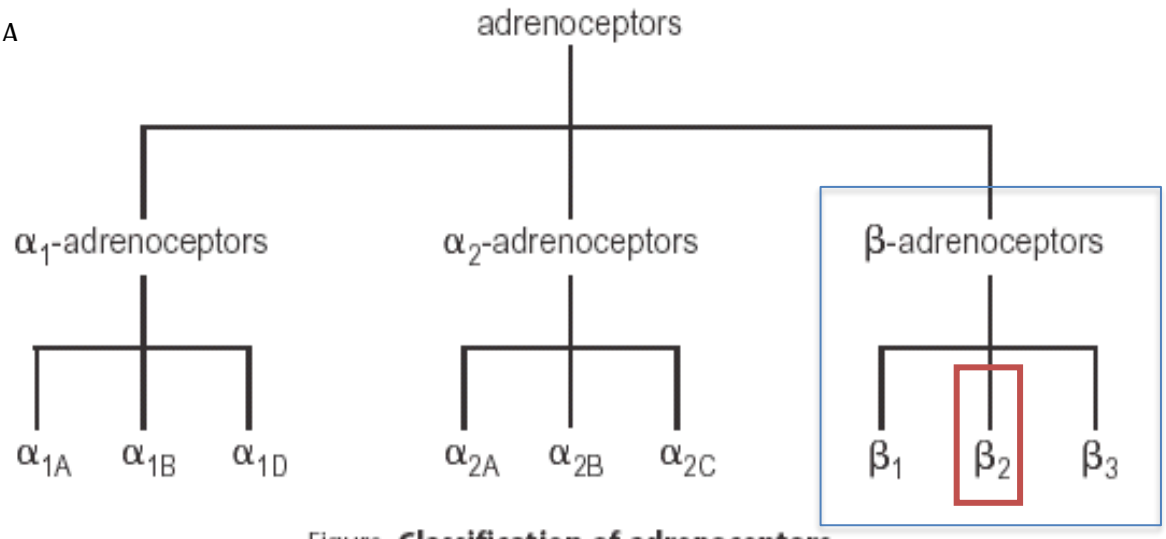

Figure. **Classification of adrenoceptors.**

B

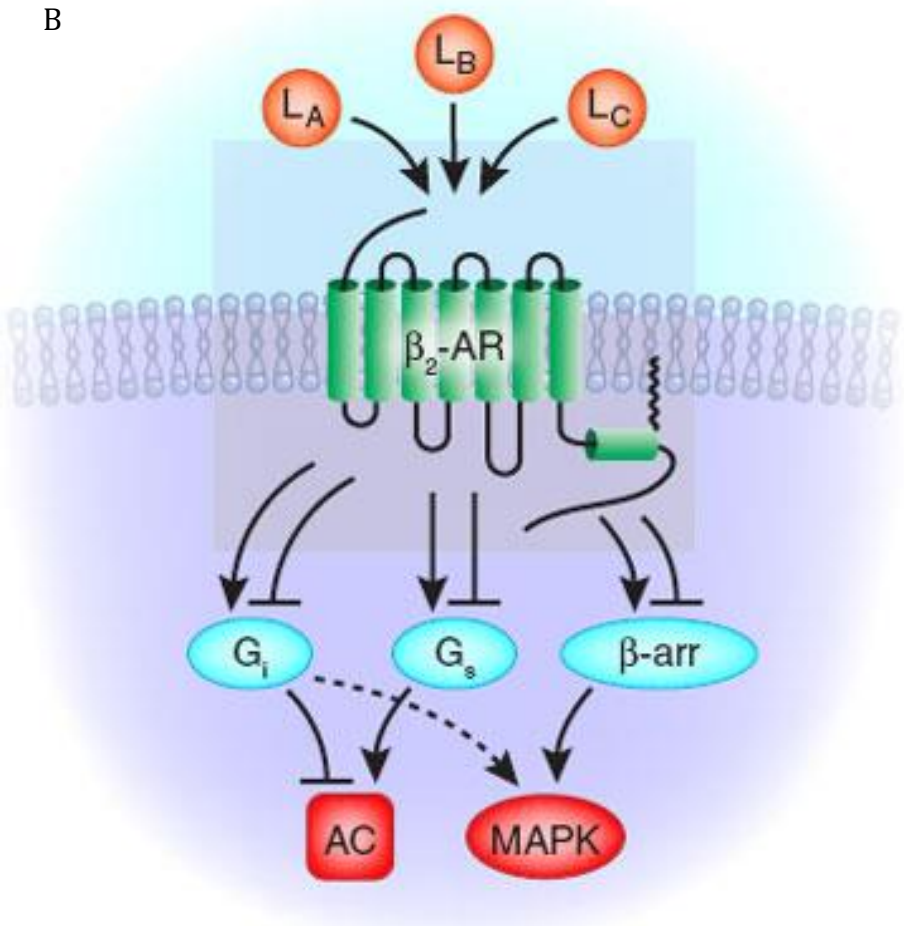

Figure 1.3 Signaling pathways downstream of β2-adrenergic receptor signaling. A. Adrenergic Receptors are divided into two major classes based on pharmacologic responses to adrenergic activating and blocking agents: Alpha (α) Adrenoceptors: The α-group is further divided into two subtypes: α_1 coupled to G_q and α_2 coupled to G_i. Beta (β) Adrenoceptors: The β-group is subdivided into β_1, β_2, and β_3 and these β-subtypes are all coupled to G_s with β_2 being coupled to G_i as well. B. β2-AR can regulate cellular response in a G-protein dependent manner (G_s and G_i) and/or β-arrestins. (Audet et al., 2008)

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CHAPTER 2

SUPER LONG-ACTING AND LONG-ACTING β2-ADRENERGIC RECEPTOR AGONISTS MEDIATE AN ANTI-INFLAMMATORY CONVERSION WITH SIMILAR KINETICS OF ACTION IN ACTIVATED MICROGLIAL CELLS.

.Introduction:

For centuries, the Central Nervous System (CNS) was thought to be immune-privileged because the blood-brain-barrier (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 (Chiu *et al.*, 2012; Ousman *et al.*, 2012). For example, receptors for cytokines, mediators of the immune system, have been found in the CNS. Likewise, receptors for neurotransmitters, hormones, and neuropeptides such as growth hormone, estradiol,

acetylcholine, and β-adrenergic agents have been identified on immune cells. As well, the bidirectional interaction is documented and supported by evidence demonstrating that the exogenous administration of cytokines result in physiological changes that are under neuronal control (Besdovsky *et al.*, 1997).

The innate immune response serves as the first line of defense to both infiltrating pathogens and/or endogenous insults. As the immediate responders, cells of the innate immune system play an important role in initiating an inflammatory response against various nonspecific components of endogenous damage-associated molecular patterns (DAMPs) and/or exogenous pathogen-associated molecular patterns (PAMPs). The innate cells, astroglia and microglia, have germline-encoded pattern recognition receptors (PRRs) such as Toll-Like Receptors (TLRs) against various DAMPs/PAMPs. Upon recognition, the immune cells become activated—releasing cytokines—and target the damaged cell or antigen for destruction and initiates an inflammatory response (Janeway *et al.*, 2002; Okun *et al.*, 2009). Therefore, TLRs are important for immunity against infectious pathogens. Since their discovery in 1985, TLRs have been studied in great depth and have been categorized by their respective activating antigens (O'Neill *et al.*, 2013).

The wide-variety of cytokines have been divided into two over-arching families: pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines, as the name suggests, are characterized by their ability to up-regulate the inflammatory process, while anti-inflammatory cytokines trigger the opposite effect. Pro-inflammatory cytokines are produced by activated immune cells to target infiltrating pathogens and/or damaged cells. Examples of pro-inflammatory cytokines include but are not limited to TNF-α, IL-6, and IL-1 (α and β) (Zhang *et al.*, 2007). Likewise, anti-inflammatory

cytokines are important in regulating the inflammatory response to avoid any excess pathological manifestation. Examples of anti-inflammatory mediators are IL-4, 10, and 13 and TGF-β (Ramesh *et al.,* 2013). IL-10 is a pleiotropic cytokine with important anti-inflammatory effects. Many immune cells including macrophages, microglia, dendritic cells (DC), B cells and CD4 and CD8 T cells can produce IL-10. Microglia were shown to be crucial for the anti-inflammatory effects of IL-10 (Qian *et al.,* 2006; Park *et al.,* 2007; Couper *et al.,* 2008). For example, IL-10 inhibits microglia from becoming activated to produce pro-inflammatory cytokines in an autocrine manner (Ledeboer *et al.,* 2002; Kremlev *et al.,* 2005). In this way, IL-10 can modulate the innate (microglia and dendritic cells) and adaptive arms (B and T cells) of immunity to limit inflammatory-mediated pathogenesis.

Parkinson's disease (PD) is a neurodegenerative disease characterized with activated microglial cells in the substania nigra (SN)—a group of dopaminergic cells (DA) in the midbrain that regulate motor response (McGeer *et al.,* 1988). Elevated levels of a number of pro-inflammatory cytokines such as TNF-α are consistently reported in brain and cerebrospinal fluid (CSF) of PD patients and in animal models (McGeer *et al.,* 1988; Hirsch *et al.,* 2009; Peterson *et al.,* 2014). Activated microglial cells, immune cells of the CNS, are the source of these elevated pro-inflammatory cytokines. These inflammatory cytokines play a critical role in microgliosis, a self-perpetuating cyclical activation of microglial cells (Goa *et al.,* 2003; Qian *et al.,* 2011; Peterson *et al.,* 2014). The synchrony in microgliosis and accompanying dopaminergic neuronal loss provide significant insight into the role of inflammatory mediators in the progression of the disease (Mount *et al.,* 2007; Qian *et al.,* 2009; Peterson *et al.,* 2014). In contrast, several groups (Qian *et al.,*

2006; Park *et al.,* 2007; Arimoto *et al.,* 2007) have reported on the ability of IL-10 to promote neuroprotection in animal models of PD. Specifically, IL-10 is reported to exert its anti-inflammatory effect by targeting the transcription of various pro-inflammatory cytokines (Ledeboer *et al.,* 2002; Murray, 2005; Qian *et al.,* 2006), enhancing dopamine uptake by dopaminergic cells, as well as increasing the number of tyrosine hydroxylase (TH) positive neurons- a marker for dopaminergic neurons (Qian *et al.,* 2006; Arimoto *et al.,* 2007). It is important to note that these anti-inflammatory effects of IL-10 are microglia-dependent within the CNS (Qian *et al.,* 2006; Arimoto *et al.,* 2007; Park *et al.,* 2007). Activated microglial cells release numerous cytokines, chemokines and other trophic factors that impact the health of the surrounding cells, especially neurons. The importance of this is that administration of exogenous IL-10 or agents that effectively enhance IL-10 while inhibiting TNF-α can serve as adjunct therapy for PD with the current dopamine-restoring therapy. Therefore, identifying agents that can modulate the repertoire and amount of production of various signaling mediators by activated microglial cells can provide significant therapeutic benefits for disorders such as PD.

β-adrenergic agents, specifically β2-adrenergic receptor (β2-AR) agonists, possess the ability to modulate the activity of immune cells. The β2-ARs are a class of receptors found in most tissue and play an important role in the physiology of the various tissues. β2-ARs are expressed on the surface of both microglial cells and neurons (Tanaka *et al.,* 2002; Peterson *et al.,* 2014) and can play a critical role in modulating immune-nervous system interactions. Stimulation of the β_2-adrenergic receptor is reported to inhibit the release of pro-inflammatory cytokines and enhance the release of the anti-inflammatory cytokine IL-10 (Elenkov *et al.,* 2000); although their effect on both pro- and anti-

inflammatory cytokines have yet to be tested in a homogenous microglial cell population. Utilizing the anti-inflammatory property of β2-ARs to dampen production of pro-inflammatory cytokines and enhance production of anti-inflammatory cytokine, in theory, should suppress and reverse the inflammatory pathology seen in PD.

The ability of the β2-AR to modulate the phenotype of activated microglial cells is therefore an important therapeutic target. Clinical use of β2-AR agonists in management of asthma shows that the super long-acting agonists are more effective due to their longer half-life *in vivo*. Likewise, previous work from our laboratory (Qian *et al.,* 2009) has shown that β2-AR agonists, especially the long-acting agonist salmeterol, can modulate microglial cells in a concentration-dependent manner. As well, it was shown the long-acting agonists clenbuterol, bambuterol, formoterol, and salmeterol to be 100-1000-fold more potent than the short acting agonist salbutermol (Qian *et al.,* 2011). Based on these findings, we hypothesize that the super long-acting β2-AR would be more effective in inhibiting the inflammatory response of activated BV-2 murine microglial cells. In order to study the phenotypic modulation of these activated microglial cells by the β2-AR agonists, we chose to examine the effects of the β2-AR agonists on several important cytokines such as TNF-α, IL-6 and IL-10. We report that the super long-acting agonists indacaterol and vilanterol are more effective than the long-acting agonist salmeterol in inhibiting TNF-α release from activated BV-2 murine microglial cells. A salient feature of our report is that we report β2-AR agonists to inhibit TNF-α and up-regulate IL-10 within a homogenous BV-2 microglial cell population; which combined, we will refer to as the β2-AR-mediated anti-inflammatory conversion. Lastly, the anti-inflammatory conversion due to the β2-AR agonists was solely due to the β2-adrenergic receptor.

Materials and Methods:

Cell culture and treatment: The murine microglial cell line BV-2 were cultured in complete Roswell Park Memorial Institute (RPMI)-1640 (1X, Gibco, FisherScientic Inc., Burlington, ON) containing 10% fetal bovine serum (Hyclone), and 5% antibiotics (10000 IU/ml penicillin with 10000 μg/ml Streptomycin; Hyclone). The cells were incubated in a humidified incubator with 5% CO_2 at 37°C. BV-2 cells were trypsinized (0.125% trypsin, Hyclone), then centrifuged at 3500 rpm for 5 minutes, and re-suspended in complete RPMI-1640 media. Cells were counted with a hemocytometer using trypan blue staining (0.4%, Gibco, FisherScientic Inc.); cells showing more than 98% cell viability were used. Cells were seeded in 24-well plates ($3X10^5$ cells) 24 hours prior to treatment. Salmeterol (LKT Laboratories, Cedarlane Laboratories Corp., Burlington, ON), indacaterol maleate (Selleckchem, Cedarlane Laboratories Corp.) and vilanterol trifenatate (MCE MedChem Express, Cedarlane Laboratories Corp.) were dissolved in 1% DMSO (Sigma Aldrich Canada, Oakville, ON) in complete RPMI and in DMSO, respectively, to a stock solution of 1mg/ml. The drugs were further diluted to working concentrations in complete RPMI-1640 media and a serial dilution was performed. Cells were pretreated with these agonists from 10^{-5}M to 10^{-15}M for 45 minutes, and then fresh media containing *Escherichia coli (E.coli)* LPS (0111:B4; Sigma Aldrich, 1μg/ml) was added to the cells and incubated for 4 hours. The LPS 0111:B4 was dissolved in DMSO to a stock solution of 1mg/ml. As BV-2 cells are an adherent cell line, their adherence to the plate was used as a determinant of viability after the experiments. However, trypan blue staining was used to confirm viability in a few wells chosen at random.

A time course study was conducted: pretreatment with β2-AR agonists then LPS administration, pretreatment with LPS then β2-AR agonist administration, and simultaneous treatments. All test paradigms were conducted for 4, 24, and 48 hours. We chose to study the 4-hours period, as it showed similar cytokine modulation across the three paradigms: pretreatment with β2-AR, pretreatment with LPS and simultaneous treatment (data not shown). Furthermore, we were confident in the chosen model as pretreatment of cells with β2-AR agonists for 45 minutes was established to be an effective time point for activating β2-ARs (Tan *et al.*, 2007).

The BV-2 murine microglia was a generous gift from Dr. Chris Power, University of Alberta.

Kinetic Experiments: The cells were seeded at a confluence of $3X10^5$ cells/well 24 hours prior to the experiment. The cells were treated with the β2-AR agonists at 10^{-9}M for 5, 10, 15, 30 and 45 minutes. The media was removed and fresh media containing LPS (1μg/ml) was added for 4 hours. Adherence to the plate was used as a determinant of viability after the experiments. However, trypan blue staining was used to confirm viability in a few wells chosen at random.

Recovery Experiment: The cells were seeded at a confluence of $2X10^5$cells/well (for the 12 hours) and $2.5X10^5$ cells/well for the rest for 24 hours prior to the experiment. The cells were treated with the β2-AR agonists (10^{-9}M) for 45 minutes, and then fresh media was added for 30 minutes, 3 hours, 6 hours and 12 hours followed by LPS (1μg/ml) administration for 4 hours. In one group, the LPS (1μg/ml) was added right after the β2-AR agonists (0 minutes). Adherence to the plate was used as a determinant of viability

after the experiments and trypan blue staining was used to confirm viability in a few wells chosen at random.

TLR activation: BV-2 murine microglial cells were seeded at a confluence of $3X10^5$ cells 24 hours prior to the experiment. The TLR1-9 kit (InvivoGene, Cedarlane Laboratories) consisted of the following TLR agonist: Pam3CSK4, HKLM, Poly IC: HMW, Poly IC: LMW, LPS-EK, FSL-I, FLA-ST, ssRNA, and ODN40 [Table 1]. These agonists were diluted in endotoxin-free water provided by the manufacturer. For the experiment, the concentration of TLR agonists used was the mid-way point of the recommended working concentration of each TLR agonist, as per the manufacturer's protocol. The cells were pretreated with the β2-AR agonists salmeterol, indacaterol and vilanterol (10^{-9}M) for 45 minutes followed by activation of the cells with these TLR agonists and LPS 0111:B4 for 4 hours. An ELISA was performed on the collected supernatants for IL-6, TNF-α and IL-10. Adherence to the plate was used as a determinant of viability after the experiments and trypan blue staining was used to confirm viability in a few wells chosen at random.

Enzyme-linked Immunosorbent Assay (ELISA): TNF-α, IL-6 and IL-10 proteins were determined in the supernatant of stimulated BV-2 cells by ELISA kits (R and D Systems, Minneapolis, MN). For ELISA, cells were cultured as described above. ELISA was performed according to the manufacturer's instructions.

Analysis: A one-way ANOVA with Bonferroni posthoc test was used for statistical analysis. A p value of <0.05 was deemed as statistically significant. The ANOVA results were further verified using a two-tailed student's t-test with $p<0.05$ as significant.

Results:

Long-acting and super long-acting β2-AR agonists inhibit microglial TNF-α and IL-6 responses when challenged with the TLR2/4 agonist lipopolysaccharide (LPS).

In identifying an appropriate inflammagen, an agent that induces inflammation, for the murine BV-2 microglia cell line, various TLRs were activated with their respective agonist and the level of secreted cytokines TNF-α and IL-10 were measured. We found that a high level of TNF-α was released only with agonists for TLR2 and TLR4 (Pam3CSK3, LPS-EK, LPS 0111:B4, FSL-I) [Figure 2.1A]. In contrast, BV-2 cells released very small but measureable amounts of IL-10 when activated with all of the TLRs except TLR7, indicating that BV-2 cells can produce IL-10 but do not produce it in significant quantities in response to inflammagenic signals [Figure 2.2A]. These data indicate that *Escherichia coli* (*E.coli*) lipopolysaccharide (LPS) strain 0111:B4 is a robust *in vitro* stimulant of inflammation in BV-2 cells, $p<0.0001$ compared to all other TLR agonists, as indicated by the TNF-α response [Figure 2.1A].

Previous work (Qian *et al.*, 2011) showed long-acting β2-AR agonist salmeterol to inhibit the production of pro-inflammatory mediators such as superoxide, iROS, TNF-α, and nitrite in LPS-induced microglia-enriched cultures. The objective of the next study was to assess whether both long-acting agonist salmeterol and super long-acting β2-AR agonists indacaterol and vilanterol inhibit release of pro-inflammatory mediators from activated microglial cells. For this, BV-2 murine microglial cells were pretreated with β2-AR agonists at a concentration of 10^{-9}M then activated with agonists to TLRs 1-9 for 4 hours [Table 1]. This dose for β2-AR agonists was chosen based on the previous work of Qian and colleagues' (2011), whose study demonstrated that low-dose salmeterol was the most effective in inhibiting inflammation. While, β2-AR agonist pretreatment was done

for all the TLRs (data not shown), we found that β2-AR agonist pretreatment inhibited TNF-α response only against the TLR2/4 agonists, since they were the only TLR agonists that produced an inflammatory response in the BV-2 cells [Figure 2.1B-D]. Conversely, salmeterol, indacaterol and vilanterol did *not* synergize with the other TLR agonists to *increase* TNF response, suggesting that these β2-AR agonists did not enhance a pro-inflammatory response in BV-2 cells as measured by TNF-α production [Figure 1.1A]. Furthermore, as the majority of the compounds used in this study were dissolved in DMSO, we sought to examine the effect of DMSO on activated microglial cells. We found that DMSO and LPS *without* any β2-AR agonist has no effect on our model, in that the modulation of cytokines previously reported were not affected by the vehicle DMSO [Figure 2.3]. Therefore, TLR2/4-induced microglial inflammation with LPS 0111:B4 is an excellent model to study the comparative anti-inflammatory effects of long-acting and super long-acting β2-AR agonists.

Long-acting and super long-acting β2-AR agonists cause an anti-inflammatory conversion in activated BV-2 cells.

Previously our laboratory demonstrated that salmeterol was effective against both MPTP and LPS-induced dopaminergic neurodegeneration in animal models of PD (Qian *et al.*, 2011). Having established that LPS 0111:B4 is an appropriate inducer of inflammation in the murine BV-2 cells, we sought to examine the effects of super long-acting β2-AR agonists indacaterol and vilanterol on the LPS-induced inflammatory response, and compare that effect to the effect of long-acting agonist salmeterol. Previous literature has focused on the anti-inflammatory effects of these β2-AR agonists with regard to inhibition of the production of pro-inflammatory cytokines such TNF-α, IL-1β, and IL-

6 (Dello Russo *et al.*, 2004; Strandberg *et al.*, 2007; Qian *et al.*, 2009) and reactive oxygen species (Dello Russo *et al.*, 2004; Qian *et al.*, 2006), and enhancement of IL-10 (Suberville *et al.*, 1996). However, the effect of β2-AR agonists on the production of both pro- and anti-inflammatory cytokines in a homogenous microglial cell line such as BV-2 has not yet been examined. Like previously observed with salmeterol, pretreatment of BV-2 cells with super long-acting β2-AR agonists vilanterol and indacaterol *significantly inhibited* the levels of the pro-inflammatory cytokine TNF-α [Figure 2.1B-D]. Interestingly, both the long-acting agonist salmeterol and the super long-acting agonists vilanterol and indacaterol *significantly up-regulated* the production of IL-10, a prototypical anti-inflammatory cytokine [Figure 2.2B] following LPS stimulation. Therefore, the data suggests that β2-AR agonists can *switch* the phenotype of activated microglial cells from a pro-inflammatory state to one that is anti-inflammatory, which we will now refer to as the anti-inflammatory conversion mediated by β2-AR agonists. As well, cell surface expression markers for M1 and M2-microglia were examined. We found no difference in surface marker expression; therefore the anti-inflammatory conversion reported is restricted to TNF-α and IL-10 as output measures (data not shown). Previous work (Tan et al., 2007) and preliminary data (data not shown) show that β2-AR agonists by themselves up-regulate mRNA and protein levels of pro-inflammatory cytokines, suggesting that the LPS signal is required for the anti-inflammatory effects of β2-AR agonists. The data further verifies our chosen model of LPS 0111:B4-induced BV-2 microglial activation as a good model to study the anti-inflammatory converting effects of β2-AR agonists—i.e. its ability to *switch* the cytokine phenotype of activated microglial cells from an M1 (TNF-α-secreting) to an M2 (IL-10 secretion).

Super long-acting β2-AR agonists are more effective than the long-acting agonist in inhibiting TNF-α and up-regulating IL-10.

To determine at what effective dose(s) the β2-AR agonists indacaterol and vilanterol inhibit the release of TNF-α production, BV-2 cells were pretreated with each β2-AR agonist over a range of concentrations (10μM to 10fM) for 45 minutes, and then challenged with LPS. Our results indicate that salmeterol inhibits TNF-α release from 10^{-5}M to 10^{-12}M and the effect titrates out at 10^{-13}M, at which point we no longer observe the inhibition of TNF-α production [Figure 2.4A]. In contrast to salmeterol, indacaterol and vilanterol were both able to exert a significant inhibitory effect on TNF-α production to a concentration of 10^{-14}M but not at 10^{-15}M [Figure 2.4B-C], thereby suggesting that the super long-acting β2-AR agonists are 100-fold more potent than the long-acting agonist salmeterol. Furthermore, the β2-AR-mediated TNF-α down-regulation was reversed with the β2-AR selective antagonist ICI 118 551 hydrochloride (1μM) [Figure 2.5], indicating that the inhibitory effects were solely mediated through the β2-ARs.

We also measured the effect of β2-AR agonists on the production of anti-inflammatory cytokines in BV-2 cells by measuring the production of the prototypical anti-inflammatory cytokine IL-10. In contrast to the pro-inflammatory cytokine TNF-α, the β2-AR agonists significantly *up-regulated* IL-10 levels in stimulated microglial cells. Salmeterol was able to up-regulate IL-10 at 10^{-6}M, 10^{-7}M, 10^{-9}M, and 10^{-13}M [Figure 2.6A]. Indacaterol was the most effective at up-regulating IL-10, showing significant up-regulation of IL-10 production at every dose from 10^{-6} to 10^{-13}M, while vilanterol showed a similar response to salmeterol in that it only up-regulated IL-10 at 10^{-6}M and 10^{-8} to 10^{-10}M [Figure 2.6B-C]. The effect on the IL-10 response was concentration-dependent as the

effects titrated out at 10^{-11}M for salmeterol and vilanterol and at 10^{-14}M for indacaterol [Figure 2.6A-C]. The combined data suggests that indacaterol is more effective in up-regulating IL-10 consistently throughout a concentration gradient. Furthermore, the β2-AR antagonist ICI 118 551 [Figure 2.7] inhibited the β2-AR agonist-mediated IL-10 enhancement for all of the β2-AR agonists, indicating that the enhancement of IL-10 release was mediated solely by the β2-ARs. This verifies that the β2-AR agonists do indeed signal solely through the β2-ARs in mediating the inhibition of TNF-α and enhancement of IL-10 production. These results suggest that signaling through the β2-ARs not only inhibits the LPS-induced production of the pro-inflammatory cytokine TNF-α, but also converts this LPS-mediated signal to one that actually enhances the production of the anti-inflammatory cytokine IL-10 in activated microglial cells.

Both long-acting and super long-acting β2-AR agonists mediate the anti-inflammatory conversion within 5 minutes of receptor engagement.

Next, we sought to examine the time-course needed for these β2-AR agonists to exert their anti-inflammatory conversion effect. To do this, we conducted a time course experiment. BV-2 cells were pretreated with β2-AR agonist salmeterol, indacaterol or vilanterol (10^{-9}M) for 5, 10, 15, 30 and 45 minutes prior to activating the cells with LPS for 4 hours. We report that all three β2-AR agonists can inhibit the TNF-α response effectively at all time points tested [Figure 2.8]. The ability of the β2-AR agonists to enhance IL-10 release also occurred at all time points tested: i.e. 5, 10, 15, 20, 30 and 45 minutes of pretreatment with these β2-AR agonists [Figure 2.9]. Combined, the data suggests that receptor activation occurs as quickly as 5 minutes upon exposure to these β2-

AR agonists, and the signal is transduced into intracellular signals responsible for mediating the anti-inflammatory conversion in BV-2 cells.

The β2-ARs have a similar kinetics of recovery when stimulated with either the long-acting agonist or the super long-acting agonists.

We speculated that exposure to the super long-acting β2-AR agonists vilanterol or indacaterol, because of their increased potency in activating BV-2 cells, would result in a longer duration of activation of the β2-ARs compared to salmeterol. To determine this, we conducted a time-course study where we pretreated the cells with the β2-AR agonists for 45 minutes and then exposed the cells to fresh media for 0 (immediate addition of the LPS), 30 minutes, 3 hours, 6 hours and 12 hours before activating the cells with LPS. The ability of the β2-ARs to suppress TNF-α release diminished 30 minutes post β2-AR treatment, as the secreted TNF-α levels returned to levels comparable to LPS-only treated conditions by 30 minutes [Figure 2.10A-C]. Likewise, their ability to enhance IL-10 production diminished within 30 minutes as well [Figure 2.11]. Collectively, the data suggests that the β2-ARs are activated relatively quickly upon exposure to the agonists; and verifies that constant presence of the agonists is needed for the β2-ARs to mediate the anti-inflammatory conversion of microglial cells. More importantly, this observation suggests that the super-long acting agonists have a comparable functional effect on the receptor as the long-acting agonist at the time-points tested. It is possible that shorter time intervals may help distinguish between these classes of agonists, or that any differences may be due to their stability and ability to maintain an effective concentration *in vivo*, rather than a differential effect on the receptor or the cell, i.e. β2-adrenergic receptor has a similar

sensitivity to both super long-acting and long-acting agonists. Further experimentation would be needed to distinguish these two possibilities.

Summary:

We have previously reported that low doses of the long-acting β2-AR agonist salmeterol can inhibit the production of the pro-inflammatory cytokine TNF-α, and have speculated that this inhibition is at least one mechanism by which it protects DA-neurons from degeneration in PD (Qian *et al.,* 2009; Qian *et al.,* 2011). We now find that two super long-acting agonists indacaterol and vilanterol are 100-fold more potent in inhibiting TNF-α release in the activated microglial cell line BV-2. We have also now observed that all three of these agonists can not only inhibit the release of the pro-inflammatory cytokine TNF-α, but also enhance the release of the anti-inflammatory cytokine IL-10; the combined effect that we have referred to as the anti-inflammatory conversion mediated by these β2-AR agonists. All three of these agonists have a similar profile of activity in that continuous activation of the receptor with the agonists is required for the anti-inflammatory conversion. Interestingly, these β2-AR agonists show a differential dose-response in inhibiting TNF-α and in enhancing IL-10, which suggests that perhaps the β2-AR agonists mediate their anti-inflammatory effect on TNF-α and IL-10 via different signaling mechanisms. Furthermore, the shift in phenotype of microglial cells from a pro-inflammatory to an anti-inflammatory response was coupled to TLR2/4-mediated activation of microglial cells.

Previously, our group has shown that salmeterol has neuroprotective capabilities in animal models of PD (Qian *et al.,* 2009). Since our data indicate that indacaterol and vilanterol are 100-fold more potent, despite sharing a similar effect on the β2-AR, it will

be important to examine whether indacaterol and vilanterol have the same protective activity in an animal model of the disease. Furthermore, while we find using kinetic approaches that there are significant differences in the potency between salmeterol, vilanterol and indacaterol, we report no difference in the effectiveness of the super long-acting agonists in maintaining the anti-inflammatory conversion compared to the long-acting agonist. However, these data suggest that these two super-long acting agonists might have a significantly increased efficiency *in vivo*. The β2-AR agonists are primarily defined as long-acting or super-long acting based on their *in vivo* half-lives [Cazolla et al., 2011]; and this fact, coupled with the 100-fold higher efficiency of the super long-acting agonists *in vitro*, suggest that these compounds may maintain an effective concentration for a longer period of time *in vivo* than the long-acting agonist salmeterol. However, our *in vitro* model is too limited in scope to determine if clearance rate of compounds from the body are the major factor differentiating between these long and super-long acting agonists. The super-long agonists are 100-fold more potent than the long-acting agonists; therefore even without an increase in duration of activity as found in our experimental design, these super-long acting agonists may be a more effective therapeutic for neurodegenerative disorders with microglial activation such as PD.

Table 1

TLR Ligands	TLR	Concentration	Comments
Pam3CSK4	TLR1/2	150ng/ml	Synthetic molecule that mimicks the acylated amino terminal of bacterial lipoproteins
HKLM	TRL2	10^7 cells	Heat-killed *Listeria monocytogenes*
Poly(I:C) High Molecular Weight (HMW)	TLR3	1 μg/ml	Synthetic analog of double-stranded RNA
Poly(I:C) Low Molecular Weight (LMW)	TLR3	1 μg/ml	Synthetic analog of double-stranded RNA

LPS-EK	TLR4	1 μg/ml	LPS from *E.coli* K12
FLA-ST	TLR5	1 μg/ml	Flagellin from *S. typhimurium*
FSL-I	TLR6/2	50ng/ml	Synthetic lipoprotein mimicking lipoprotein LP44 of *Mycoplasma salivarium*
ssRNA40	TLR7	0.25μg/ml	ssRNA
ODN1826	TLR9	5μM	Synthetic oligonucleotide containing unmethylated CpG dinucleotides
LPS	TLR4	1 μg/ml	LPS from Gram-negative *E.coli* bacteria.

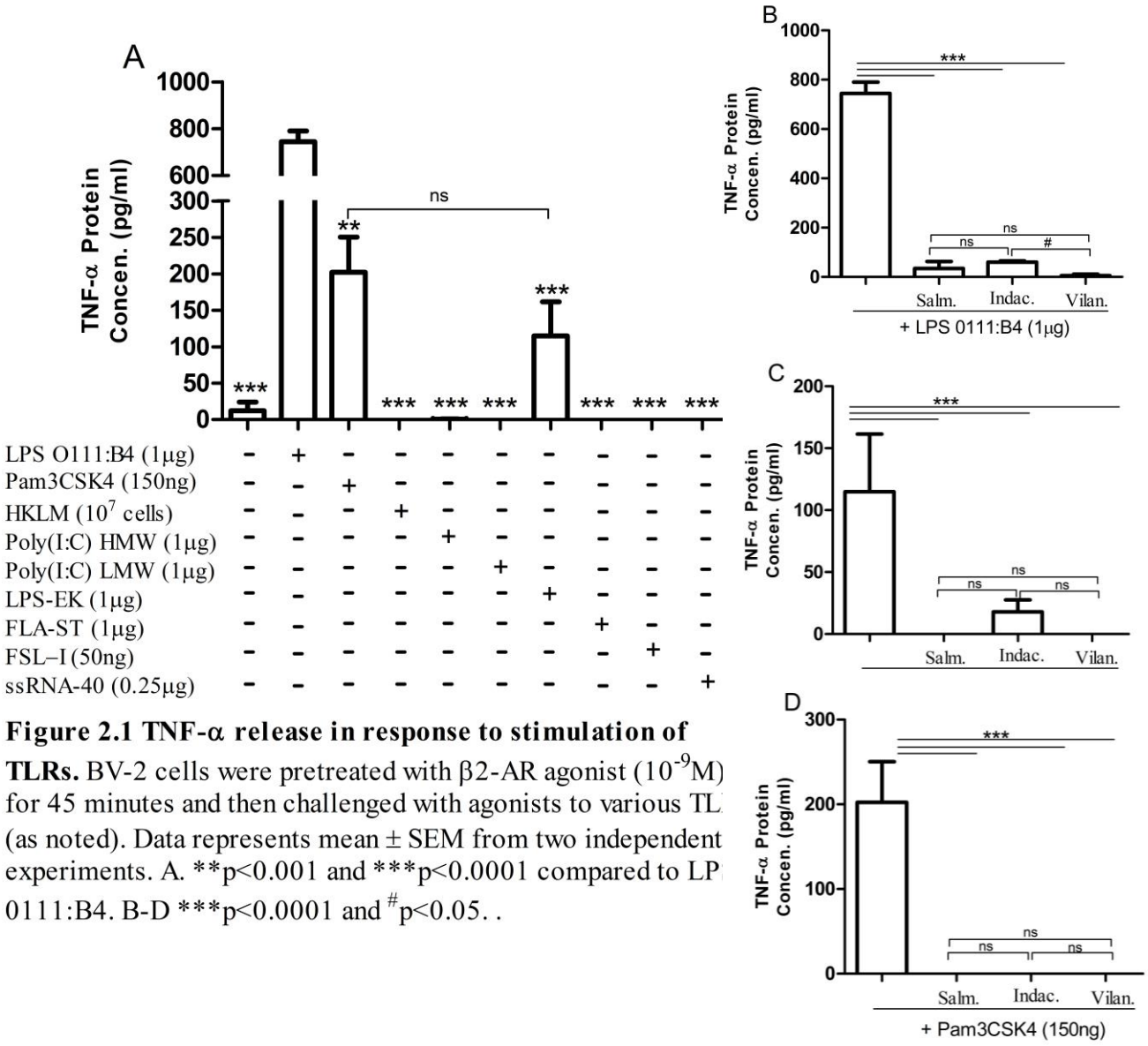


Figure 2.1 TNF-α release in response to stimulation of TLRs. BV-2 cells were pretreated with β2-AR agonist (10^{-9}M) for 45 minutes and then challenged with agonists to various TL (as noted). Data represents mean ± SEM from two independent experiments. A. **p<0.001 and ***p<0.0001 compared to LP 0111:B4. B-D ***p<0.0001 and $^{\#}$p<0.05. .

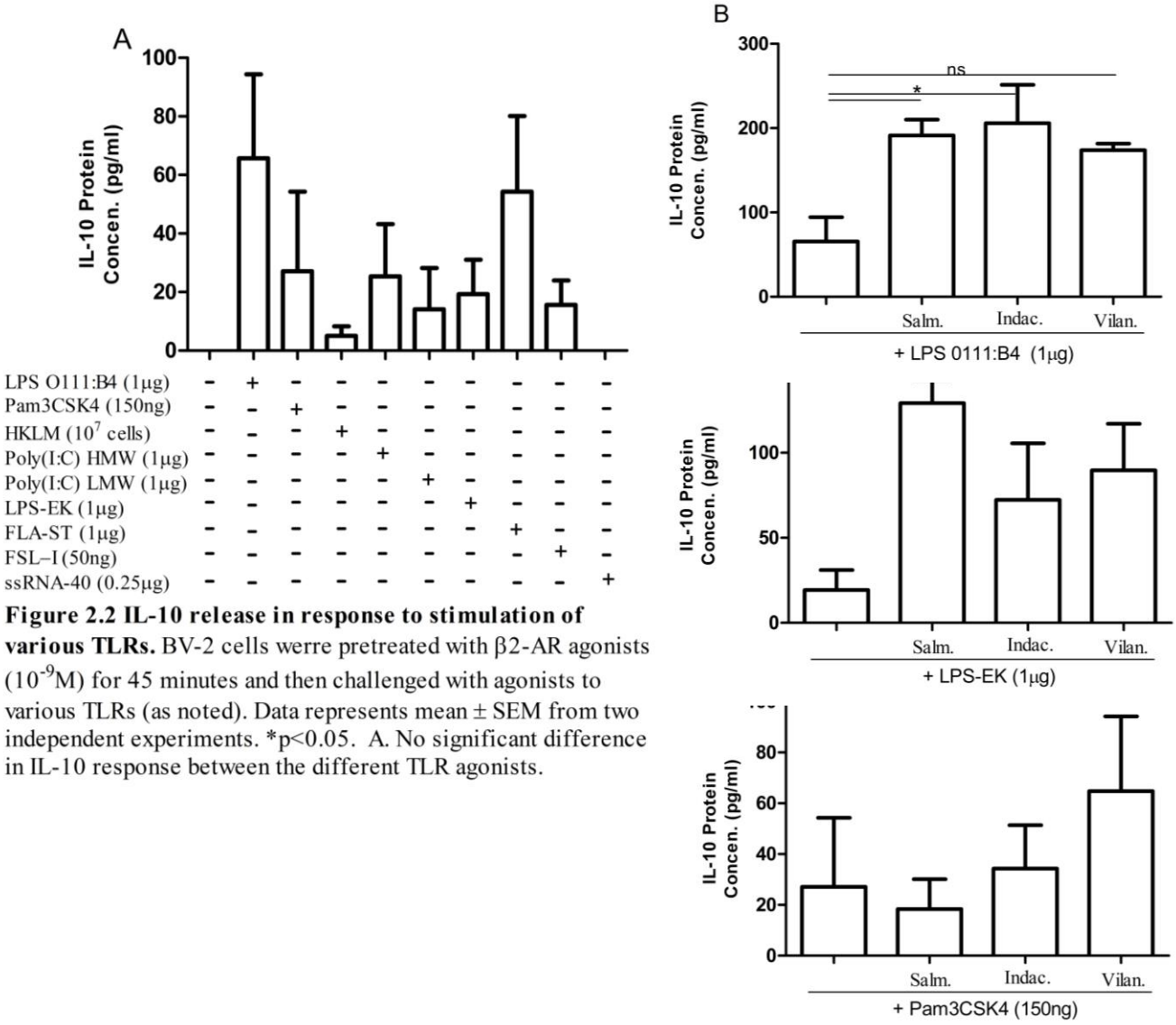


Figure 2.2 IL-10 release in response to stimulation of various TLRs. BV-2 cells werre pretreated with β2-AR agonists (10^{-9}M) for 45 minutes and then challenged with agonists to various TLRs (as noted). Data represents mean ± SEM from two independent experiments. *$p<0.05$. A. No significant difference in IL-10 response between the different TLR agonists.

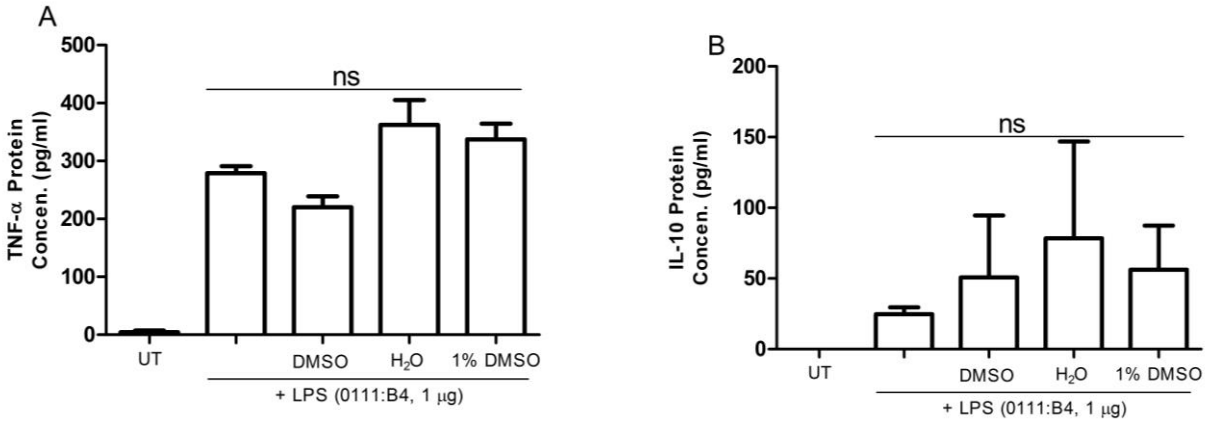


Figure 2.3 Vehicle Controls. DMSO and endotoxin free water were used as vehicles. BV-2 cells pretreated with DMSO and H_2O (10μl) for 45 minutes and challenged with LPS have no effect on the LPS-mediated cytokine response.

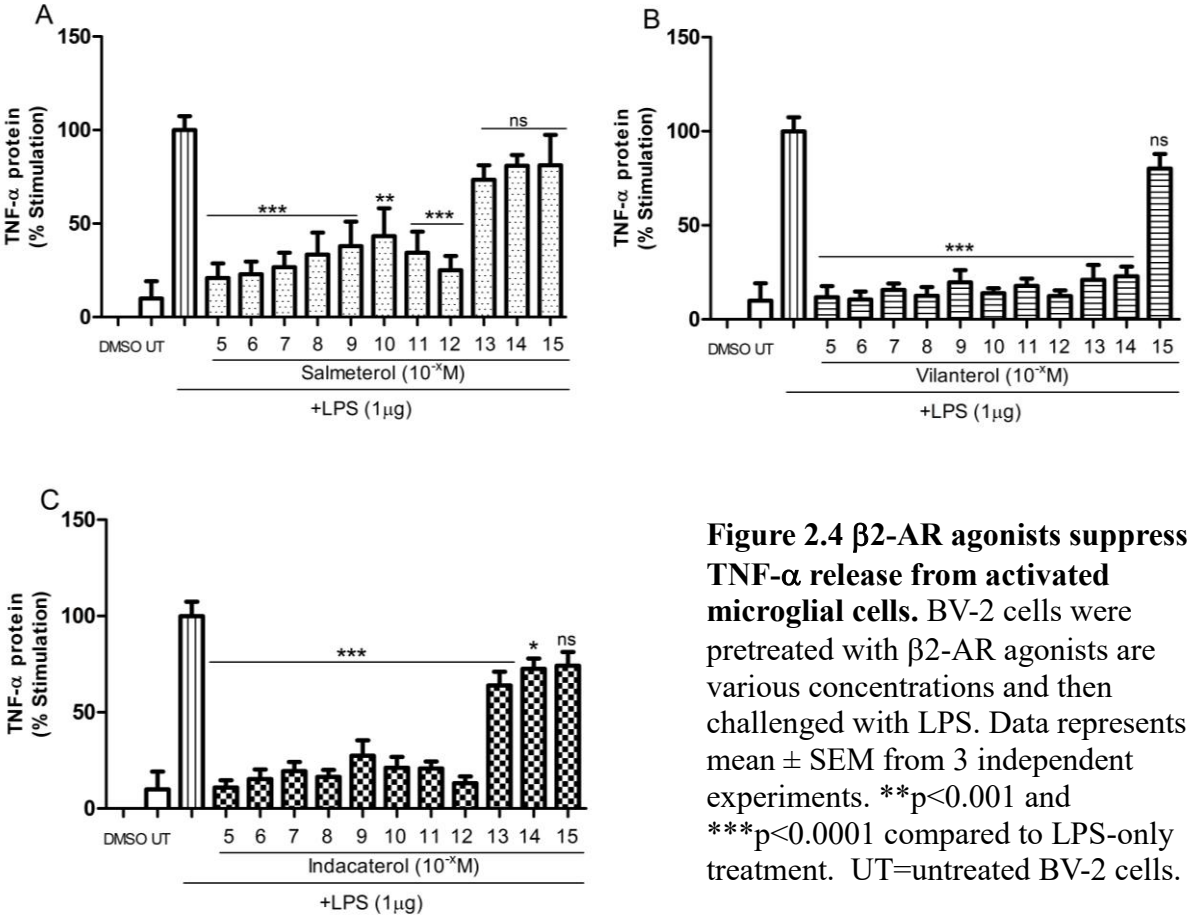


Figure 2.4 β2-AR agonists suppress TNF-α release from activated microglial cells. BV-2 cells were pretreated with β2-AR agonists are various concentrations and then challenged with LPS. Data represents mean ± SEM from 3 independent experiments. **p<0.001 and ***p<0.0001 compared to LPS-only treatment. UT=untreated BV-2 cells.

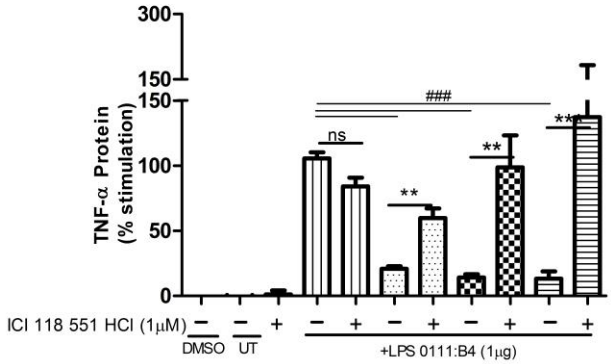


Figure 2.5 Salmeterol, indacaterol and vilanterol exert their anti-inflammatory effect through the β2-ARs. BV-2 cells were treated with the β2-AR antagonist ICI 118 551 hydrochloride (1μM) for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ± SEM from three independent experiments. UT= untreated BV-2 cells. **p<0.001, ***p<0.0001, $^{\#\#}$p<0.001 and $^{\#\#\#}$p<0.0001.

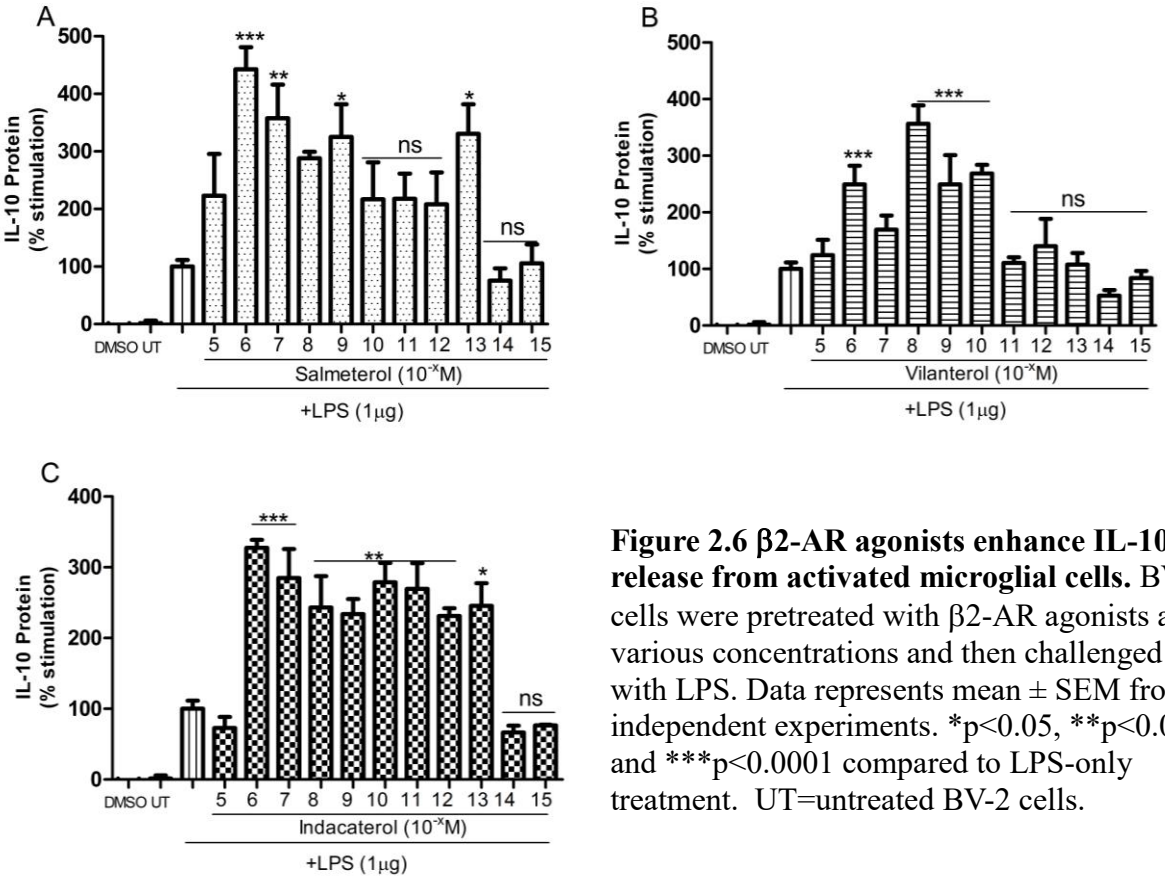


Figure 2.6 β2-AR agonists enhance IL-10 release from activated microglial cells. BV-2 cells were pretreated with β2-AR agonists at various concentrations and then challenged with LPS. Data represents mean ± SEM from 2 independent experiments. *p<0.05, **p<0.001 and ***p<0.0001 compared to LPS-only treatment. UT=untreated BV-2 cells.

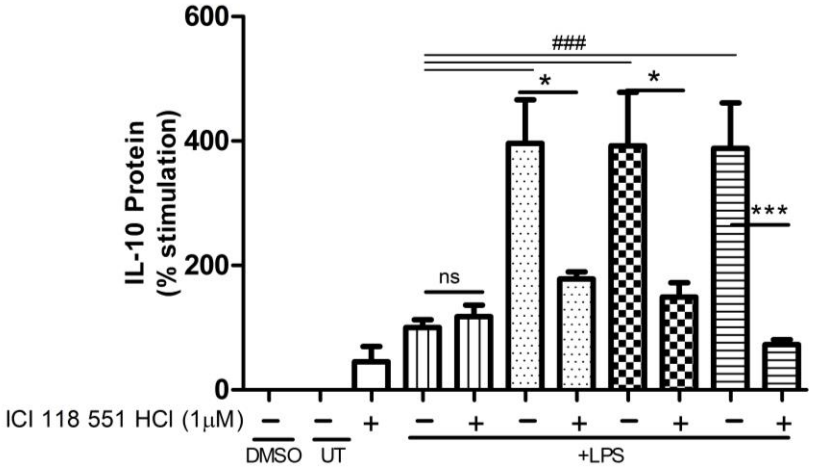


Figure 2.7 Salmeterol, indacaterol and vilanterol exert their anti-inflammatory effect through the β2-ARs. BV-2 cells were treated with the β2-AR antagonist ICI 118 551 hydrochloride (1μM) for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents means ± SEM from two independent experiments. UT= untreated BV-2 cells. $^{*}p<0.05$, $^{***}p<0.0001$ and $^{###}p<0.0001$.

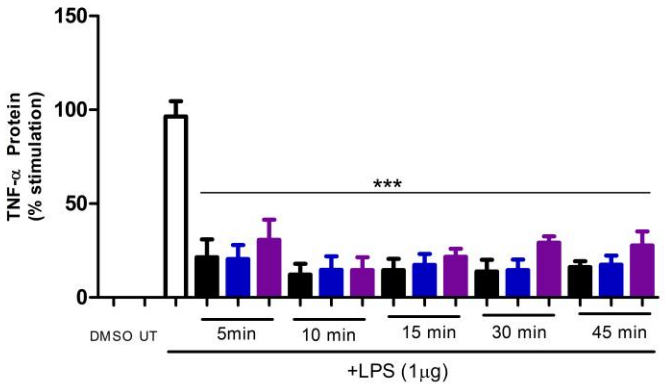


Figure 2.8 β2-AR agonists can modulate cytokine response within 5 minutes of receptor engagement. BV-2 cells were treated with β2-AR agonists (10^{-9}M) for various time points and then challenged with LPS. Data expressed as mean ± SEM of two independent experiments. ***p<0.0001. UT=untreated BV-2 cells.

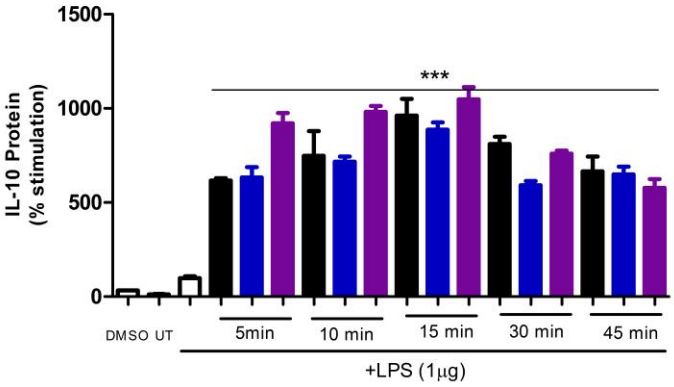
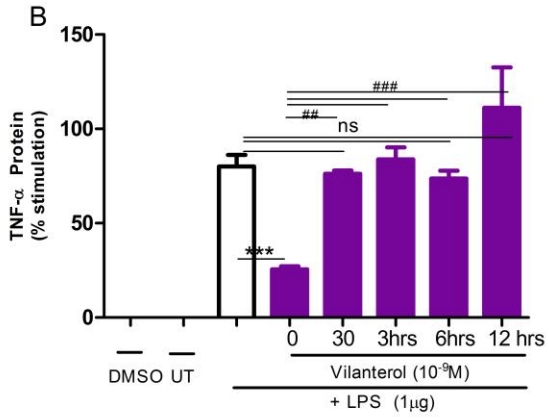


Figure 2.9 β2-AR agonists can modulate cytokine response within 5 minutes of receptor engagement. BV-2 cells were treated with β2-AR agonists (10^{-9}M) for various time points and then challenged with LPS. Data expressed as mean ± SEM. ***p<0.0001. UT=untreated BV-2 cells.



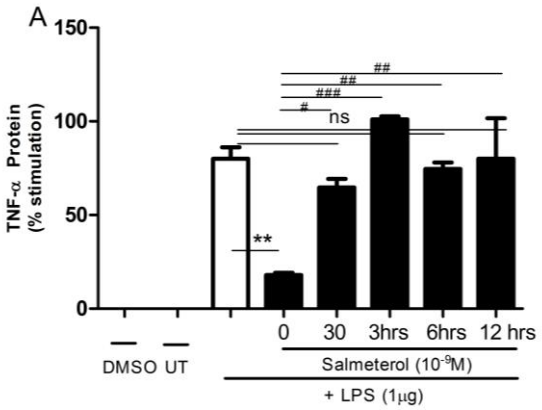
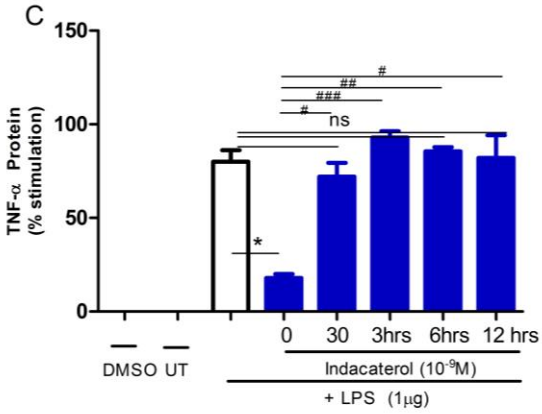


Figure 2.10 Recovery post β2-AR stimulation occurs within 30 minutes. BV-2 cells were pretreated with β2-AR agonists for 45 minutes, exposed to fresh media for various time points, and then challenged with LPS. Data expressed as mean ± SEM of two independent experiments. ***p<0.0001 and ###p<0.0001. UT=untreated BV-2 cells.



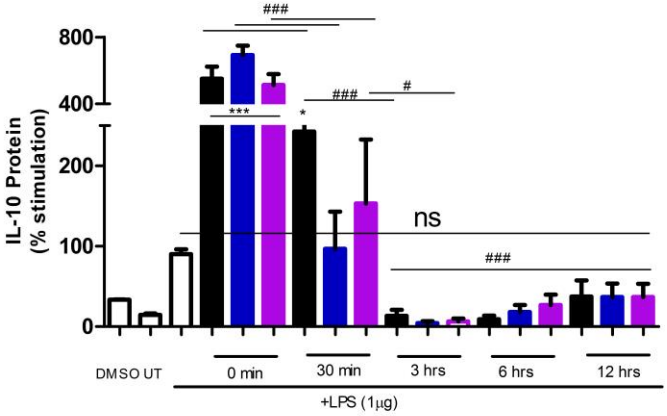


Figure 2.11 Recovery post β2-AR stimulation occurs within 30 minutes. BV-2 cells were treated with β2-AR agonists (10^{-9}M) for 45 minutes, then exposed to fresh media for various time points, and then challenged with LPS. Data expressed as mean ± SEM. ***p<0.0001 and ###p<0.0001. UT=untreated BV-2 cells.

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CHAPTER 3

LONG-ACTING AND SUPER LONG-ACTING β2-ADRENERGIC RECEPTORS AGONISTS USE MULTIPLE SIGNALING PATHWAYS TO MEDIATE THEIR ANTI-INFLAMMATORY CONVERSION IN BV-2 MURINE MICROGLIAL CELLS.

Introduction:

Several studies have shown long-acting β2-AR-agonists to be neuroprotective in *in vivo* models of neuronal damage (Culmsee *et al.,* 1999; Junker *et al.,* 2002; Culmsee *et al.,* 2007; Peterson *et al.,* 2014). Microglia, the resident macrophages of the Central Nervous System (CNS), have been implicated to have a significant role in the pathogenesis of several neurodegenerative diseases (Esiri, 2007). In Parkinson's disease (PD), activated microglial cells are reported to exacerbate neurotoxicity because of its propensity to activate additional microglia in a cyclical manner known as microgliosis (Qian *et al.,* 2011; Peterson *et al.,* 2014). Therefore, inhibiting microglial cell activation would theoretically stop the progression of PD, and would therefore be neuroprotective. Qian and colleagues (2009 and 2011) studied the mechanism of action of the β2-AR agonist salmeterol as a therapeutic agent against the inflammatory process driven by activated microglial cells in PD. A novel mechanism of action for salmeterol—one that is independent of the classical G-protein signaling but dependent on two important upstream scaffolding proteins was demonstrated: β-arrestin2 and transforming growth factor β activated kinase-1 (TAK1) (Qian *et al.,* 2011). β-arrestins function as scaffolding proteins to recruit and couple signaling complexes to the G-protein coupled receptor (GPCR), thereby having an important regulatory function downstream of the β2-AR signaling pathway. There are two variants of the β-arrestin protein: β-arrestin1 and β-arrestin2 (Attramadal *et al.,* 1992; DeWire *et al.,* 2007). It is now determined that β2-ARs predominately couple to β-arrestin2 (Attramadal *et al.,* 1992). As β-arrestin2 is an upstream regulator of the G-protein signaling pathway, TAK1 is an important upstream regulatory protein in the Tumor Necrosis Factor (TNF), interleukin-1 (IL-1) and the TLR-induced inflammatory cascade (Wang *et al.,*

2001; Adhikari *et al.,* 2007); consequently, it plays a critical role in the regulation of inflammation.

The inflammatory response induced by Lipopolysaccharide (LPS) involves activation of NF-κB as well as MAPKs (c-Jun N-terminal kinase (JNK), p38, and Extracellular signal-regulated kinase 1/2 (ERK1/2)) that then work in a concerted manner to elevate the transcription, translation and release of various pro-inflammatory cytokines and cell surface markers (Gong *et al.,* 2016). Earlier, we had reported that the super long-acting agonists indacaterol and vilanterol cause an anti-inflammatory conversion similar to that of the long-acting agonist salmeterol in that they inhibit the release of TNF-α and up-regulate IL-10 production. Previous work from our laboratory (Qian *et al.,* 2011) reported that the anti-inflammatory ability of salmeterol to inhibit pro-inflammatory mediator release was TAK1 and β-arrestin2-dependent but G-protein-independent. However, the mechanism by which β2-AR agonists salmeterol, indacaterol and vilanterol mediate the anti-inflammatory conversion to enhance IL-10 production is unknown. Therefore, we hypothesize that indacaterol and vilanterol will also inhibit TNF-α production in a TAK1 and β-arrestin2-dependent but G-protein-independent manner similar to salmeterol; and may use the same pathways to enhance IL-10 production.

To do this, we used specific inhibitors of NF-κB and MAPKs to characterize and compare the mechanism of action of the super long-acting agonists indacaterol and vilanterol to that of the long-acting agonist salmeterol. Here, we report that similar to salmeterol, indacaterol and vilanterol inhibited activated microglial TNF-α response through the inhibition of NF-κB in a TAK1 and β-arrestin2-dependent but G-protein or PKA-independent manner. Additionally, we found that the activation of the anti-

inflammatory conversion leading to enhanced IL-10 production functions in a TAK1 and PKA-dependent but β-arrestin2-*independent* manner. These findings provide further insight into how β2-AR agonists work to reverse the CNS inflammation that occurs in PD, and may provide a new and more effective therapeutic for PD.

Materials/Methods:

Cell Culture and treatments: The murine microglial cell line BV-2 was cultured in complete Roswell Park Memorial Institute (RPMI)-1640 (1X, Gibco, FisherScientific Inc. Mississauga, ON) containing 10% fetal bovine serum (Hyclone, FisherScientific), and 5% antibiotics (10,000 IU/ml penicillin with 10,000μg/ml Streptomycin; Hyclone). The cells were incubated in a humidified incubator with 5% CO_2 at 37°C. BV-2 cells were trypsinized (0.125% trypsin, Hyclone), then centrifuged at 3500rpm for 5 minutes, and re-suspended in complete RPMI-1640 media. Cells were counted with a hemocytometer using trypan blue staining (0.4%, Gibco, FisherScientific). Cell cultures showing more than 98% cell viability were used. Cells were seeded in 24-well plates ($3X10^5$ cells), 12-well plates ($5X10^5$), and 6-well plates (Cornstar) ($8X10^5$) 24 hours prior to treatment. Salmeterol (LKT Laboratories, Cedarlane Laboratories Corp., Burlington, ON), indacaterol maleate (Selleckchem, Cedarlane Laboratories Corp.), vilanterol trifenatate (MCE MedChem Express, Cedarlane Laboratories Corp.) were dissolved in 1% DMSO (Sigma Aldrich Canada, Oakville, ON) in complete RPMI, and in DMSO, respectively, for a stock solution of 1mg/ml. The drugs were diluted to working concentrations in complete RPMI-1640 media. The *Escherichia coli (E.coli)* LPS (0111:B4; Sigma Aldrich Canada) was dissolved in DMSO to a stock solution of 1mg/ml.

KT5720, SB203580 hydrochloride, 5-(Z)-7-Oxozeaenol, c-Jun Peptide, ICI 118551 hydrochloride, U0126 were purchased from Tocris Bioscience (Cedarlane Laboratories Corp.). SP600125 and H89 2HCl were purchased from Selleckchem (Cedarlane Laboratories Corp.) and Bay 43-9006 from Cayman Chemical Company (Cedarlane Laboratories Corp.) [Table 2]. The inhibitors were all dissolved in DMSO and a vehicle control containing the same amount of DMSO was used in cell culture experiments with the exception of ICI 118 551, which was dissolved in endotoxin free water provided by the manufacturerr. Cells were treated with the inhibitors at the respective concentrations [Table 2] for 30 minutes, then treated with β2-AR agonists at 10^{-9}M for 45 minutes, and challenged with LPS (1μg) for 4 hours. This study paradigm was selected based on work by Tan and colleagues (2007). Since, a vehicle control with LPS was not conducted during these experiments, an independent experiment was conducted to test for the effect of DMSO and LPS. The BV-2 murine microglia was a generous gift from Dr. Chris Power, University of Alberta.

Western Blot: Cells were washed with ice-cold PBS (1X), lysed using cell lysis buffer containing protease inhibitors (Cell Signaling Technologies, Beverley, MA), and homogenized using Sonic Dismembrator (Model 100, FisherScientific). Protein concentration was quantified using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Protein, 50μg, was resolved on 12% sodium dodecyl sulfate (SDS) gels. Following gel electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham, Piscatawy, NJ), and bands were detected with Ponceau (FisherScientific). Membranes were washed with 1XTBST and blocked with 5% non-fat milk in 1X-TBST at room temperature for 1 hour. The membranes were probed with β-arrestin2 antibody (Cell

Signaling Technologies, FishcerScientific) overnight at 4° with 1:1000 dilution in 5% milk. Antibodies were detected with corresponding horseradish peroxidase-linked secondary antibodies (Cell Signaling Technologies) at 1:1000 dilution incubated at room temperature for 1 hour. Blots were developed using Enhanced Chemiluminescent (ECL) reagent (Perkin Elmer). Signals were captured using the Biorad ImageDoc. The blots were stripped for 20 minutes, blocked in 5% non-fat milk for 30 minutes, and re-probed for β-actin overnight at 4° with 1:1000 dilution in 5% non-fat milk. Again, the blots were developed and image captured using the Biorad ImageDoc. Western bands were quantified using ImageJ, and measured level of β-actin was used to normalize the level of β-arrestin2 protein.

Enzyme-linked Immunosorbent Assay (ELISA): TNF-α and IL-10 proteins were determined in the supernatant of BV-2 cells by ELISA. For ELISA, cells were cultured as described above. Cytokine proteins were determined using ELISA kits (R and D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer's instructions.

Silencing β-arrestin2: BV-2 murine microglial cells were seeded at a confluence of $2X10^5$ cells/well in a 12-well plate 24 hours prior. β-arrestin2 siRNA (ambion life technologies, Burlington, ON) and scrambled siRNA (ambion life technologies, Burlington, ON) were dissolved in nuclease-free water provided by the manufacturer. β-arrestin2 siRNA (5mmoles/well), scrambled siRNA (5mmoles/well), and lipofectamine 2000 (2μl/well; FisherScientific Inc.) were diluted in incomplete RPMI-1640 (Gibco) media. The diluted lipofectamine and β-arrestin2 siRNA solution and the lipofectamine and scrambled siRNA were mixed and incubated at room temperature for 20 minutes. The siRNA complex was added to the cells (1ml/well) and incubated for 20 hours. The siRNA complex was removed

and fresh complete media was added for another 8 hours. The cells were then subjected to β2-AR agonists (10^{-9}M) for 45 minutes followed by activation with LPS for 4 hours. The supernatant was collected for ELISA, and the cells were washed with ice-cold PBS (1X, Gibco) and lysed and collected for western blot analysis for β-arrestin2. Adherence to the plate was used as a determinant of viability after the experiments.

Analysis: A one-way ANOVA with Bonferroni posthoc test was used for statistical analysis. A p value of 0.05 was deemed as statistically significant. The ANOVA results were further verified using a two-tailed student's t-test with $p<0.05$ as significant.

Results:

Long-acting and super long-acting β2-AR agonists exert an inhibitory effect on TNF-α in an β-arrestin2-dependent manner, but the anti-inflammatory conversion is β-arrestin2-independent.

We had previously found that inhibition of TNF-α by salmeterol in activated microglial cells was mediated through β-arrestin2 (Qian *et al.,* 2011). As such, the objective of the next set of experiments was to determine if the (i) super long-acting β2-AR agonists indacaterol and vilanterol inhibit TNF-α in a β-arrestin2-dependent manner, and (ii) whether β-arrestin2 was involved in the anti-inflammatory conversion leading to the up-regulation of IL-10 production by these β2-AR agonists. To do this, we used silencing RNA (siRNA) to selectively silence β-arrestin2 in the BV-2 murine microglial cells. Following silencing of the β-arrestin2, the BV-2 cells were then treated with the β2-AR agonists salmeterol, vilanterol or indacaterol (10^{-9}M) for 45 minutes followed by activation with LPS for 4 hours. We report that silencing β-arrestin2 [Figure 3.1A-B] blocked the β2-AR agonist-mediated inhibition in TNF-α production [Figure 3.1C]. In

contrast, we found no effect of silenced β-arrestin2 on the β2-AR-mediated IL-10 enhancement [Figure 3.1D]. Collectively, the data suggests that while β2-ARs signals through β-arrestin2 to inhibit TNF-α, it is able to induce the anti-inflammatory conversion response, i.e. up-regulation of IL-10, in an β-arrestin2-independent manner.

Both long-acting and super long-acting agonists inhibit TNF-α in a PKA-independent manner, but up-regulate IL-10 in a PKA-dependent manner.

Classical activation of adrenergic receptors involves Gs-mediated activation of adenylyl cyclase resulting in increased intracellular levels of cyclic adenosine monophosphate (cAMP). Increased levels of intracellular cAMP lead to catalytic activation of PKA. Previous work from our laboratory showed that salmeterol exerts its anti-inflammatory effect independently of PKA (Qian *et al.,* 2011). In order to determine whether indacaterol and vilanterol function in a similar mechanism, i.e., independently of PKA, we inhibited PKA with the PKA inhibitor H89 hydrochloride, and examined the levels of TNF-α and IL-10 as output measures. We report that inhibition of PKA with H-89 hydrochloride actually enhanced the LPS-mediated TNF-α level [Figure 3.2]. The H89 inhibitor had no effect on the β2-AR-mediated inhibition of TNF-α release [Figure 3.2]. In contrast, inhibition of PKA with H89 hydrochloride inhibited the baseline LPS-induced IL-10 response [Figure 3.3], and inhibited the β2-AR-mediated IL-10 enhancement [Figure 3.3]. The data suggests that β2-AR agonists inhibit TNF-α in a PKA-independent manner, but the anti-inflammatory conversion leading to enhanced IL-10 production is PKA-dependent.

Transforming growth factor β activated kinase-1 (TAK1) activity is important for both pro-inflammatory cytokine TNF-α and anti-inflammatory conversion leading to IL-10 production.

Previous work from laboratory has shown salmeterol to inhibit the phosphorylation of TAK1, rendering TAK1 and the downstream TAK1-dependent signaling pathways dysfunctional (Qian *et al.,* 2011). To determine if inhibiting TAK1 would have any effect on the anti-inflammatory conversion mediated by indacaterol and vilanterol, i.e. inhibition of the TNF-α and enhancement of IL-10, TAK1 was inhibited with the TAK1-selective inhibitor 5-(Z)-7-Oxozeaenol (10μM). Using 5-(Z)-7-Oxozeaenol, we report that both TNF-α and IL-10 levels were completely abrogated [Figure 3.4-3.5]. This suggests that TAK1 is critical in the production of both of these cytokines; which is congruent with TAK1 being an upstream regulator of several signaling pathways (Lu *et al.*, 2008).

Both long-acting and super long-acting β2-AR agonists modulate transcription factor(s) downstream of IKK to achieve their anti-inflammatory conversion.

In order to better understand and compare the mechanism by which salmeterol, indacaterol and vilanterol utilize the NF-κB pathway to mediate an anti-inflammatory conversion, NF-κB was inhibited using the inhibitor for the NF-κB regulatory kinase IKKβ, Compound A (Ziegelbauer *et al.,* 2005). As expected, inhibition of NF-κB with Compound A inhibits the LPS-mediated TNF-α response [Figure 3.6]. However, we find that inhibiting NF-κB with Compound A had no additional effect on the β2-AR-mediated inhibition of TNF-α response. As such, the data suggests that β2-AR agonists may be as efficient in inhibiting NF-κB, and consequently, no further effect was achieved with an upstream NF-κB inhibitor. Such a finding is in accordance with previous studies (Witherow *et al.,* 2004;

Qian *et al.,* 2011; Farmer *et al.,* 2000). In contrast, inhibition of NF-κB with Compound A inhibited the β2-AR-mediated IL-10 enhancement [Figure 3.7]. Collectively, the data posits that NF-κB (and/or downstream IKK) activity is important for the anti-inflammatory conversion in that it modulates cytokine production.

Long-acting and super long-acting β2-AR agonists require JNK kinase activity to mediate their anti-inflammatory conversion.

To determine if JNK activity was needed in the anti-inflammatory conversion mediated by salmeterol, indacaterol and vilanterol, SP600125 and c-Jun peptide were used to inhibit JNK activity. SP600125 inhibits the kinase activity of JNK while c-Jun Peptide interrupts the JNK/c-Jun interaction. Inhibition of JNK with SP600125 [Figure 2.8] inhibited the LPS-mediated TNF-α response as expected with no additional effects on the β2-AR-mediated inhibition of TNF-α. Interestingly, inhibition of JNK with another inhibitor c-Jun peptide [Figure 3.9] did not inhibit the LPS-mediated TNF-α response and had no additional effect on β2-AR-mediated TNF-α inhibition. Inhibition of JNK with SP600125 [Figure 3.10] had no effect on the baseline (LPS-induced) IL-10 release but inhibited the β2-AR-mediated IL-10 enhancement. In contrast, c-Jun peptide inhibited the baseline IL-10 response [Figure 3.11] and had no effect on the β2-AR-mediated IL-10 enhancement. Together, the data indicates that JNK kinase activity that is independent of c-Jun peptide is necessary for the β2-AR agonists to convert the LPS-induced inflammatory response to an anti-inflammatory response.

Long-acting agonist salmeterol mediates TNF-α inhibition through p38; and the super long-acting agonist vilanterol requires p38 to up-regulate IL-10.

To determine the role of p38 in the anti-inflammatory conversion activity of salmeterol, indacaterol and vilanterol, the p38 inhibitor SB203580 was used. SB203580 is a specific inhibitor of the p38 pathway that functions by interrupting its catalytic activity. Inhibition of p38 with SB203580 [Figure 3.12] directly inhibited the LPS-mediated TNF-α response, and synergized with salmeterol to further inhibit TNF-α production [Figure 3.12]. However, the addition of indacaterol or vilanterol did not show this synergistic effect on the inhibition of TNF-α production. In contrast, SB203580 had no effect on the LPS-induced IL-10 response, but significantly inhibited the vilanterol-mediated enhancement of the IL-10 production, but not that of salmeterol and indacaterol [Figure 3.13]. Taken together, this data shows that p38 is required for the LPS-mediated TNF-α response, but its role in the anti-inflammatory conversion response is not as clear. Therefore, further experiments are required to demonstrate the role of p38 in the β2-AR-mediated anti-inflammatory conversion.

Long-acting β2-AR agonist salmeterol mediates its anti-inflammatory conversion through ERK1/2.

To determine if ERK1/2 was needed for the anti-inflammatory conversion mediated by salmeterol, indacaterol and vilanterol, inhibitors of kinases responsible for activating ERK1/2 such as U0126 and PD98580 were used. Inhibition of ERK1/2 with U0126 (10μM) alone has no effect on the LPS-mediated TNF-α response [Figure 3.14]. ERK1/2 inhibition by U0126 showed some synergy with salmeterol but not with indacaterol and vilanterol to further inhibit TNF-α [Figure 3.14]. Similarly, the ERK1/2 inhibitor PD 98059 alone did not inhibit the LPS-mediated TNF-α response, but also showed some synergy with both salmeterol and vilanterol in inhibiting TNF-α [Figure 3.15]. Conversely, both ERK1/2

inhibitors had no effect on the baseline IL-10 response. However, U0126 [Figure 3.16] reversed the enhancement of IL-10 response mediated by salmeterol, while PD98059 [Figure 3.17] blocked the enhancement of IL-10 response mediated by both salmeterol and indacaterol. Surprisingly, both U0126 and PD98059 had no significant effect on the enhancement of IL-10 response mediated by vilanterol. These results demonstrate that ERK1/2 inhibitors synergize with some but not all β2-AR agonists to further inhibit TNF-α and reverse the β2-AR-mediated IL-10 enhancement. Long-acting and super long-acting β2-AR agonists show differential responses in using ERK1/2 to achieve its anti-inflammatory conversion. While, it is clear that the long-acting β2-AR agonists salmeterol is consistent in using ERK1/2 to mediate its anti-inflammatory conversion; the role ERK1/2 plays in the anti-inflammatory conversion of the super long-acting β2-AR agonists is inconsistent and requires further examination.

Summary

Salmeterol, a well-studied long-acting β2-AR agonist, is reported to inhibit microglial inflammatory response—as measured by elevated levels of TNF-α, iROS, nitrite and superoxide—in a TAK1 and β-arrestin2-dependent but PKA-independent manner (Qian *et al.*, 2011). In characterizing the mechanism of action of the super long-acting agonists to compare to previously reported mechanism of action of salmeterol, these same pathways were inhibited to examine the resulting effect on the β2-AR-mediated TNF-α and IL-10 responses. In our study, we found that β2-AR-mediated inhibition of TNF-α was rescued by silencing β-arrestin2, but that β-arrestin2 is not involved in the β2-AR-mediated up-regulation of the IL-10 response. Furthermore, inhibition of TAK1 resulted in

an abrogation of both cytokine responses confirming the role of TAK1 as an upstream regulator of transcription of both pro- and anti-inflammatory cytokines.

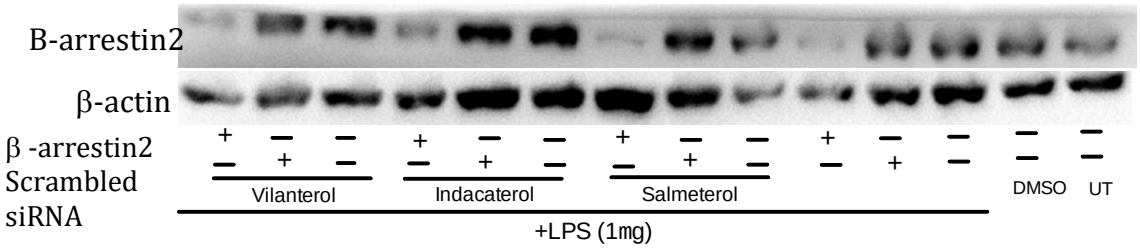
Examining signaling pathways downstream of TAK1, we found that inhibition of NF-κB inhibits both the LPS-induced production of TNF as well as the β2-AR agonist-mediated enhancement of IL-10 production. With regard to MAPKs, inhibition of ERK1/2 synergized with salmeterol and vilanterol in inhibiting TNF-α; and rescued the IL-10 response due to salmeterol and indacaterol. Similarly, p38 inhibition synergized with salmeterol to inhibit TNF-α and reversed the vilanterol-mediated enhancement of IL-10. JNK is required for the inflammation-induced TNF-α response and inhibition of the JNK kinase activity reversed the β2-AR-mediated IL-10 enhancement, suggesting that β2-AR agonists augment JNK kinase activity to enhance IL-10 production. In summary, the super long-acting agonists differ from each other as well as compared to the long-acting agonist salmeterol in utilizing MAPKs, but appear to use similar upstream mediators to cause an anti-inflammatory conversion i.e. inhibition TNF-α and up-regulation IL-10.

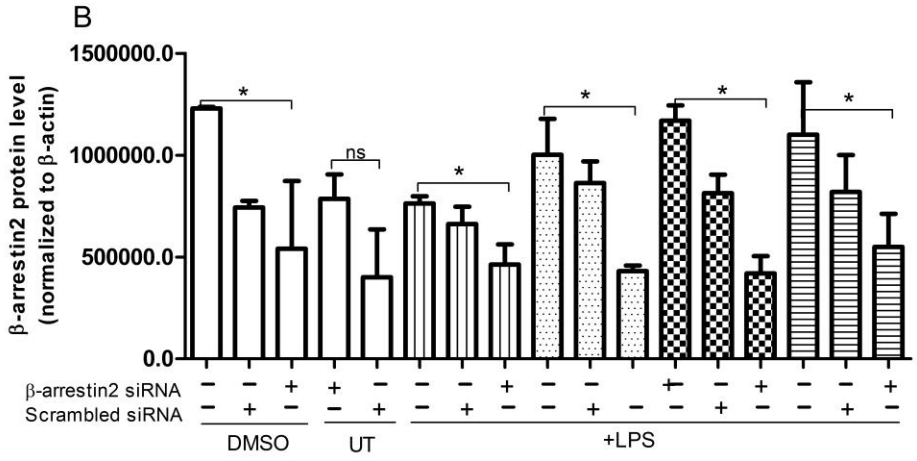
TABLE 2

Inhibitor	Effector	Specificity	Concentration used
5(Z7)-Oxazoneal	Transforming growth factor β activated kinase-1 (TAK1)	ATP-competitive irreversible inhibitor of TAK1	10 μM
β-arrestin2 siRNA	β-arrestin2	β-arrestin2	2 mM
Compound A	NF-κB	IKKβ Inhibitor	10 μM
c-Jun Peptide	c-Jun N-terminal kinase (JNK)	JNK/c-Jun Interaction	10 μM
H89 dihydrochloride	Protein Kinase A (PKA)	PKA	48 nM
ICI 115 855 hydrochloride	β2-adrenergic receptor	β2-AR	10 μM
PD98059	Extracellular signal-regulated kinase 1/2	MEK1 Inhibitor	10 μM
SB203580	p38	p38	10 μM
U0126	Extracellular signal-regulated kinase 1/2	MEK1/2	10 μM
SP600125	c-Jun N-terminal kinase	JNK Kinase activiy	10 μM

FIGURES:

A


B-arrestin2
β-actin
β -arrestin2 Scrambled siRNA
Vilanterol
Indacaterol
Salmeterol
DMSO
UT
+LPS (1mg)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UT
+LPS

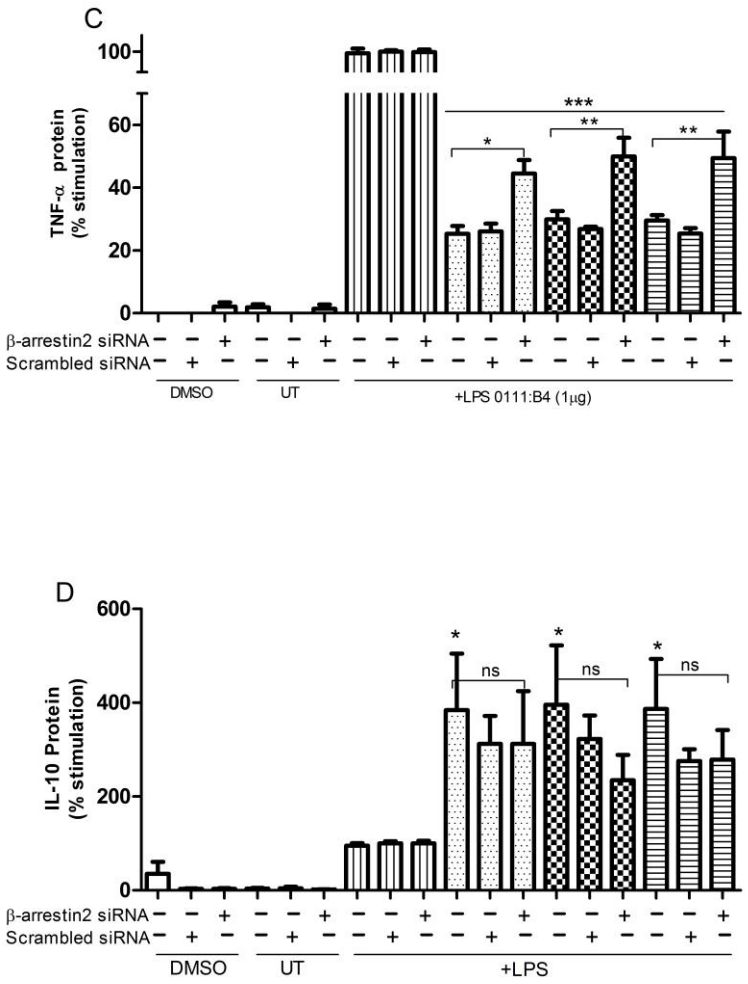

Figure 3.1 β-arrestin2 is important in the β2-AR agonist-mediated inhibition of TNF-α, but not in the β2-AR agonist-mediated IL-10 enhancement. β-arrestin2 was silenced (see Methods for siRNA protocol). A. Western blot showing protein levels of β-arrestin2. B. Quantification of protein level. BV-2 cells were then treated with β2-AR agonists (10^{-9}M) for 45 minutes and then challenged with LPS. Levels TNF-α (C) and IL-10 (D) in the supernatent were measured. Data is expressed as mean ± SEM from four independent experiments. UT=untreated BV-2 cells.*$p<0.05$ and **$p<0.001$ as indicated. C. ***$p<0.0001$ compared to LPS groups D. *$p<0.05$ compared to LPS group.

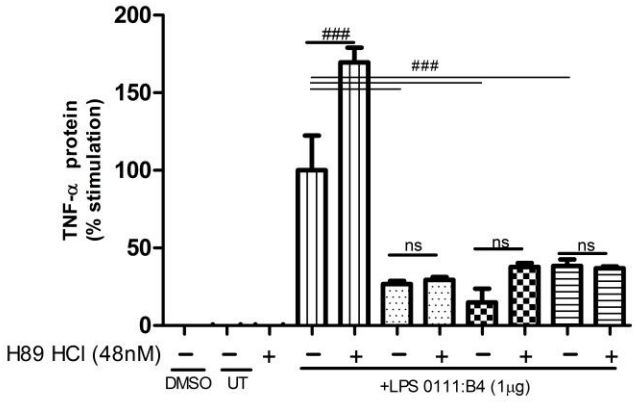

Figure 3.2 β2-AR agonist-mediated inhibition of TNF-α is PKA-independent. BV-2 cells were treated with the PKA inhibitor H89 hydrochloride for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as ±SEM of 2-3 independent experiments with $^{###}p<0.0001$. UT= untreated BV-2 cells.

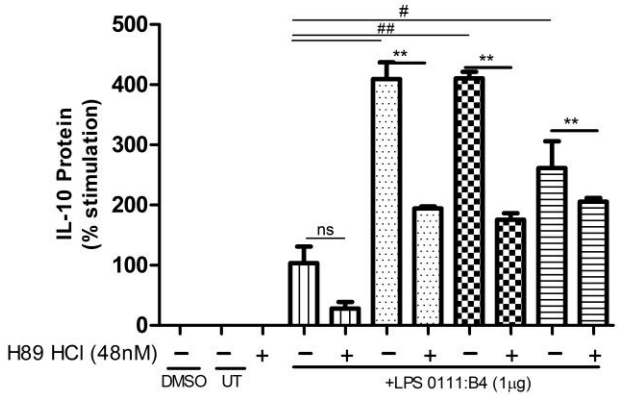

Figure 3.3 The β2-AR agonist-mediated enhancement of IL-10 is PKA-dependent. BV-2 cells were treated with the PKA inhibitor H89 hydrochloride for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean as ±SEM of 2-3 independent experiments with $^{#}p<0.05$, $^{##}p<0.001$ and $^{**}p<0.001$. UT= untreated BV-2 cells.

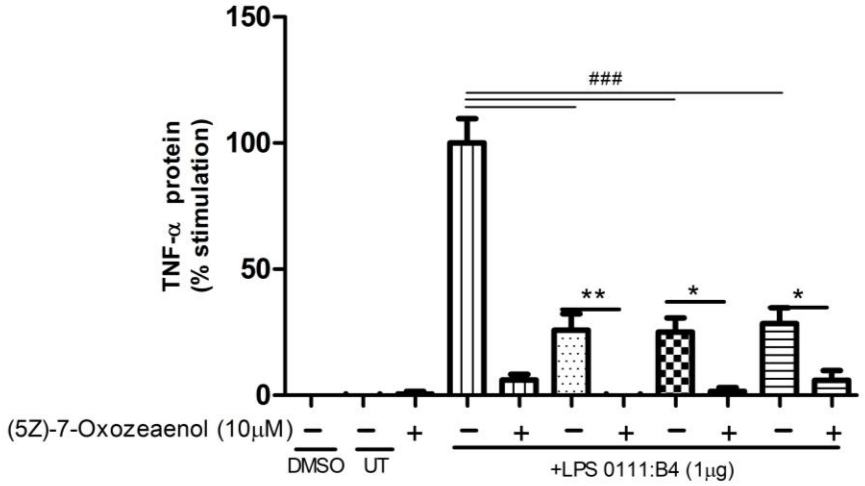

Figure 3.4 Transforming Growth factor β activated kinase-1 (TAK1) is needed for production of TNF-α. BV-2 cells were treated with the TAK1 inhibitor (5Z)-7-Oxozeanol for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from 3 independent experiments. *p<0.05, **p<0.001, and ###p<0.0001. UT=untreated BV-2 cells.

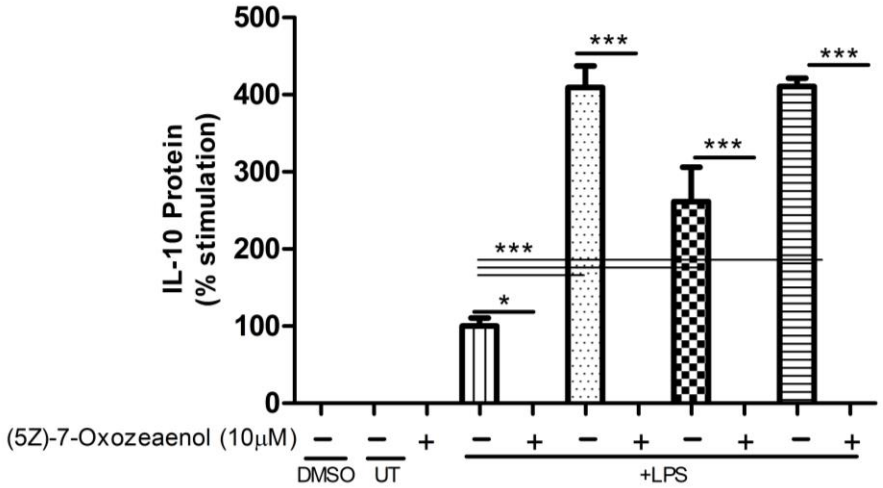

Figure 3.5 Transforming Growth factor β activated kinase-1 (TAK1) is needed for production of IL-10. BV-2 cells were treated with the TAK1 inhibitor (5Z)-7-Oxozeanol for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM. *p<0.05 and ***p<0.0001. UT=untreated BV-2 cells.

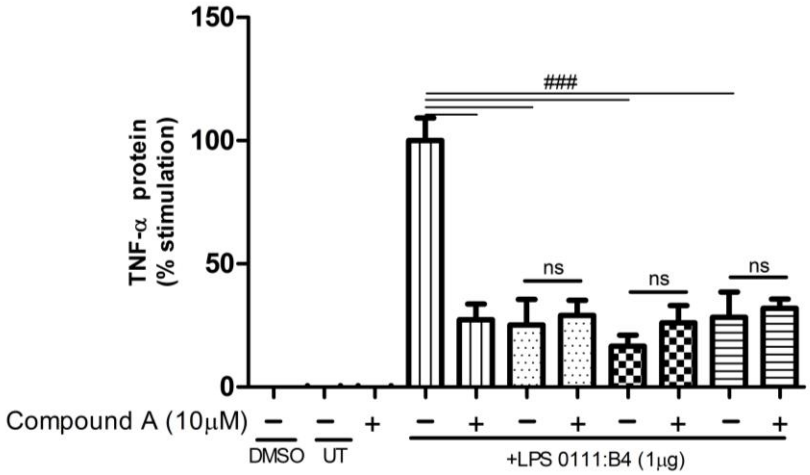

Figure 3.6 TNF-α production is IKK-dependent. BV-2 cells were treated with the IKKβ inhibitor Compound A for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed mean ±SEM from three independent experiements. $^{###}p<0.0001$. UT=untreated BV-2 cells.

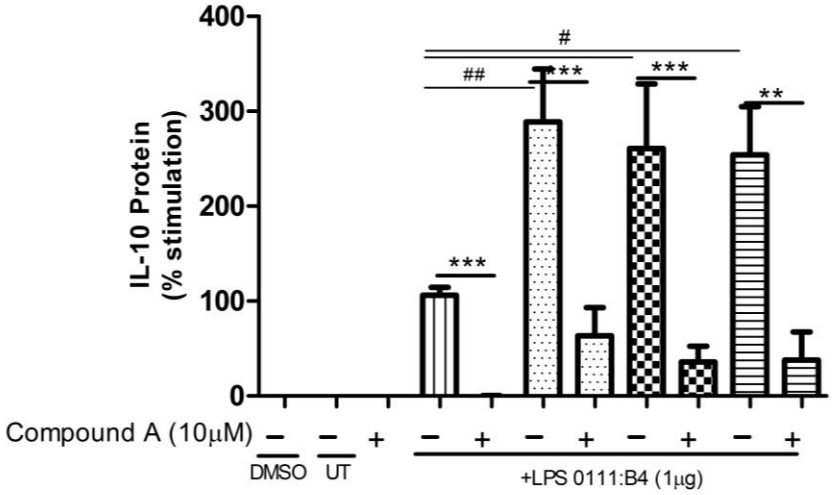

Figure 3.7 IL-10 production is IKK-dependent. BV-2 cells were treated with the IKKβ inhibitor Compound A for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from two independent experiments. $^{**}p<0.001$, $^{***}p<0.0001$, $^{#}p<0.05$ and $^{##}p<0.001$. UT=untreated BV-2 cells.

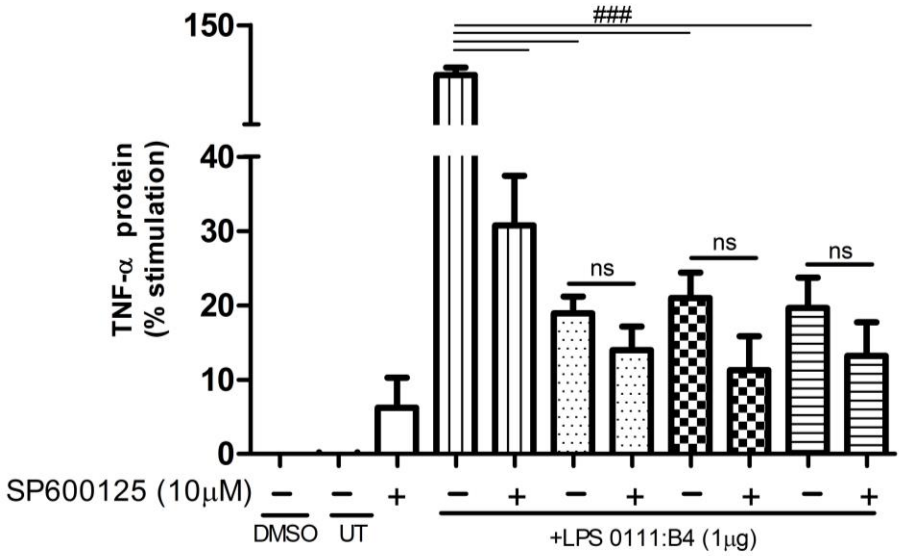

Figure 3.8 LPS-mediated TNF-α response is JNK-dependent. BV-2 cells were treated with the JNK inhibitor SP600125 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM from 3 independent experiments. $^{###}p<0.0001$. UT=untreated BV-2 cells.

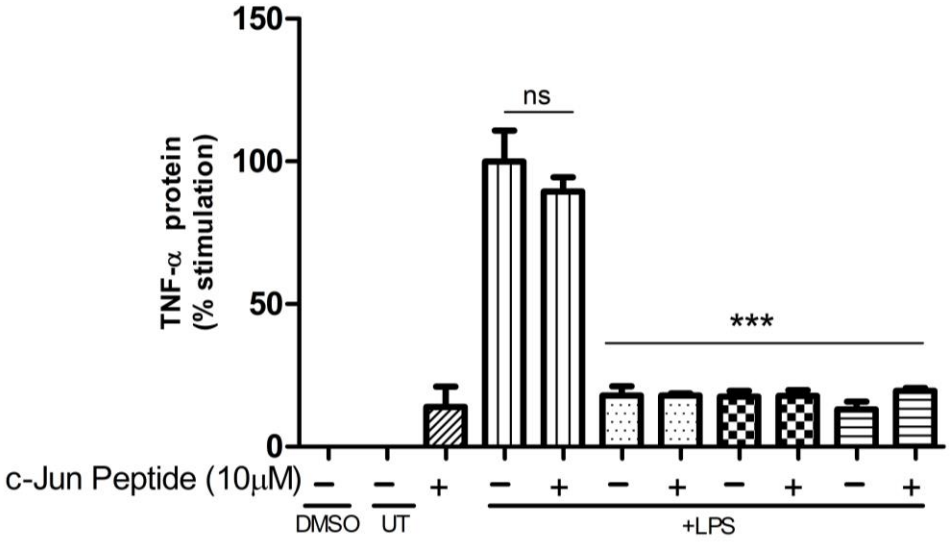

Figure 3.9 β2-AR agonist-mediated inhibition of TNF-α secretion is independent of the JNK/c-Jun interaction. BV-2 cells were treated with the JNK inhibitor c-Jun Peptide for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM. ***p<0.0001 compared to LPS treated conditions. UT=untreated BV-2 cells.

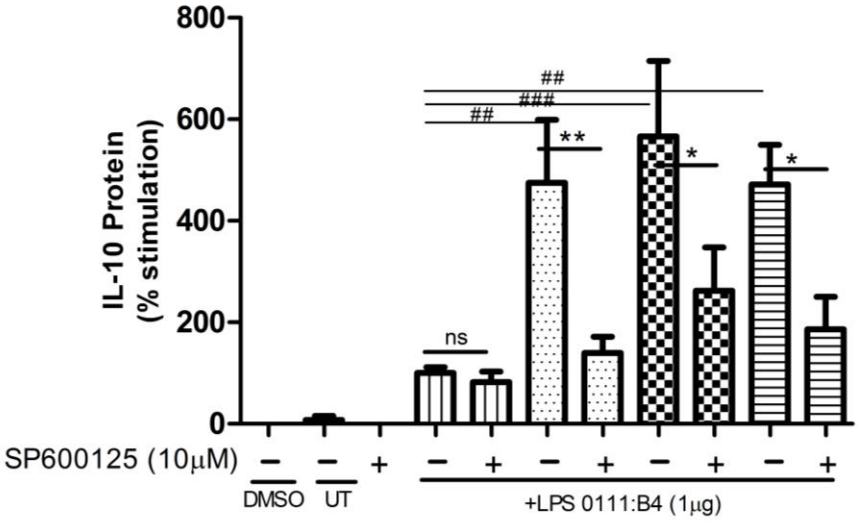

Figure 3.10 β2-AR agonist-mediated enhancement of IL-10 is JNK-dependent. BV-2 cells were treated with the JNK inhibitor SP600125 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM from 3 independent experiments. *p<0.05, **p<0.001, $^{\#\#}$p<0.001 and $^{\#\#\#}$p<0.0001. UT=untreated BV-2 cells.

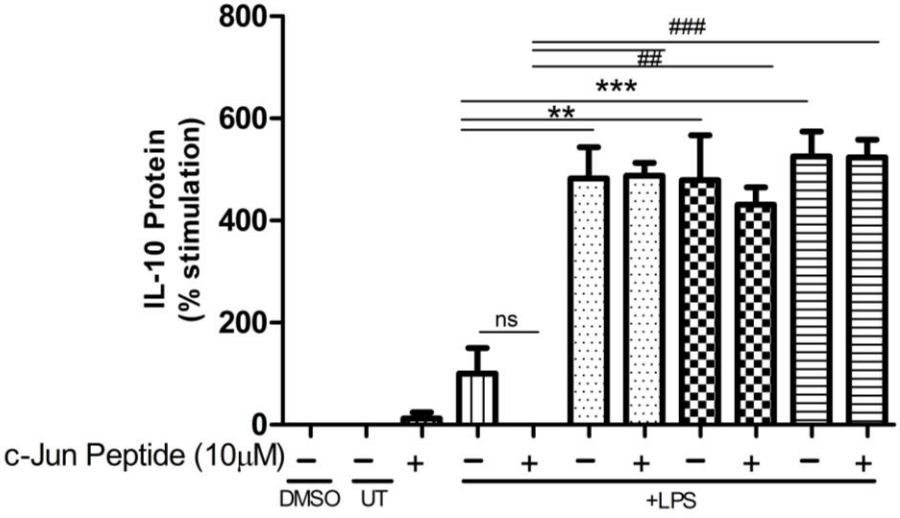

Figure 3.11 β2-AR agonist-mediated IL-10 enhancement is independent of JNK/c-Jun interaction. BV-2 cells were treated with the JNK inhibitor c-Jun Peptide for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM. $^{\#\#}$p<0.001, $^{\#\#\#}$p<0.0001, **p<0.001 and ***p<0.0001. UT=untreated BV-2 cells.

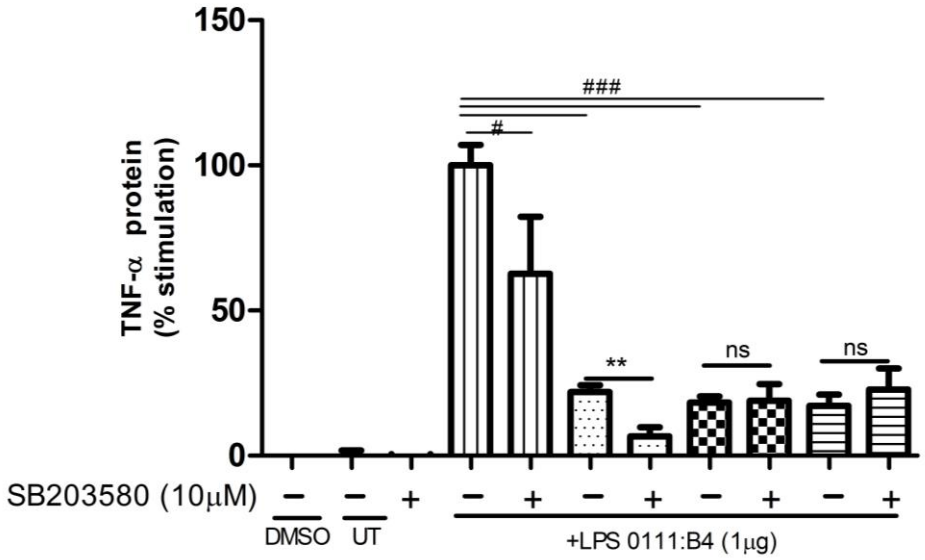

Figure 3.12 LPS-mediated TNF-α response is p38-dependent. BV-2 cells were treated with the p38 inhibitor SB203580 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM from 3 independent experiments. **p<0.001, $^{\#}$p<0.05 and $^{\#\#\#}$p<0.0001.

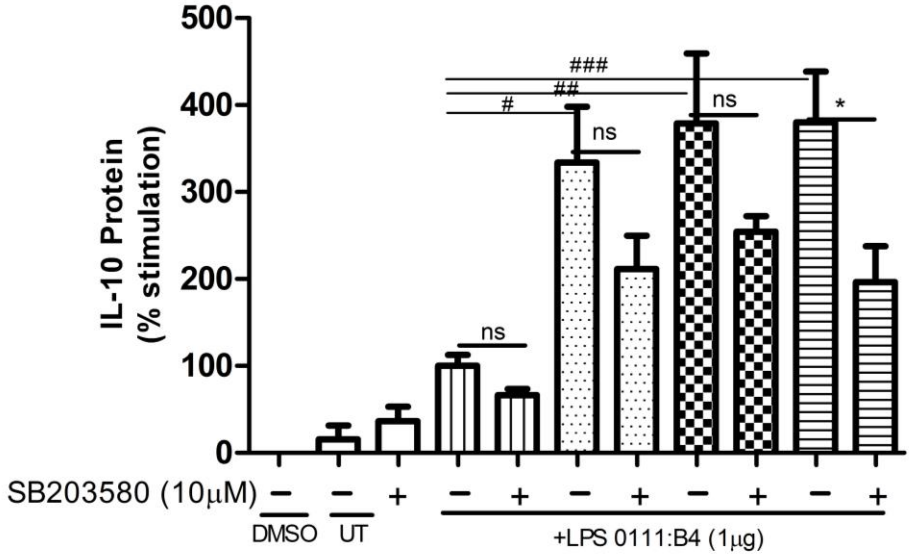

Figure 3.13 Vilanterol-mediated enhancement of IL-10 secretion is p38-dependent. BV-2 cells were treated with the p38 inhibitor SB203580 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data expressed as mean ±SEM from 3 independent experiements.*p<0.05, $^{\#}$p<0.05, $^{\#\#}$p<0.001, and $^{\#\#\#}$p<0.0001. UT=untreated BV-2 cells.

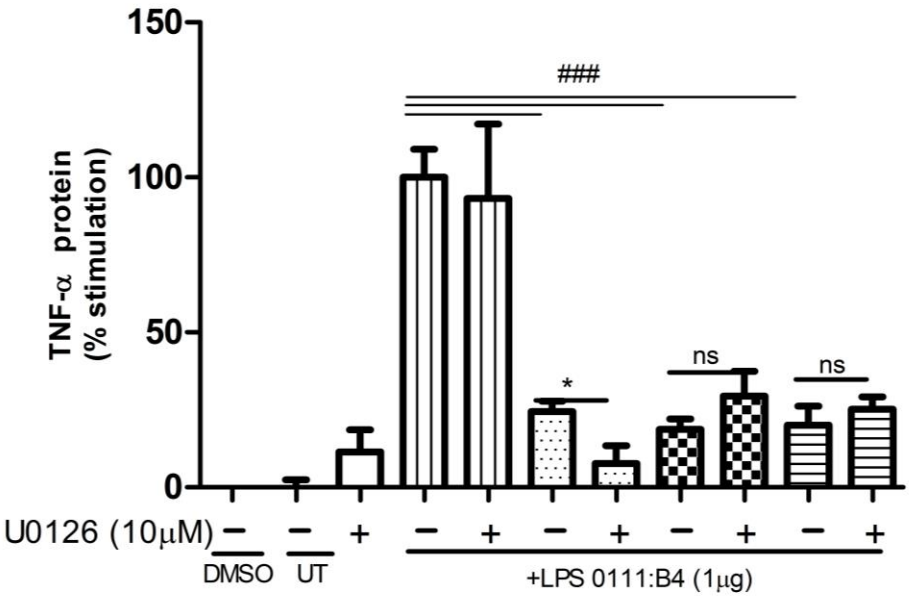

Figure 3.14 Inhibition of ERK1/2 with U0126 synergizes with salmeterol to further inhibit TNF-α. BV-2 cells were treated with the ERK1/2 inhibitor U0126 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from three independent experiments.*p<0.05 and $^{###}$p<0.0001. UT= untreatd BV-2 cells.

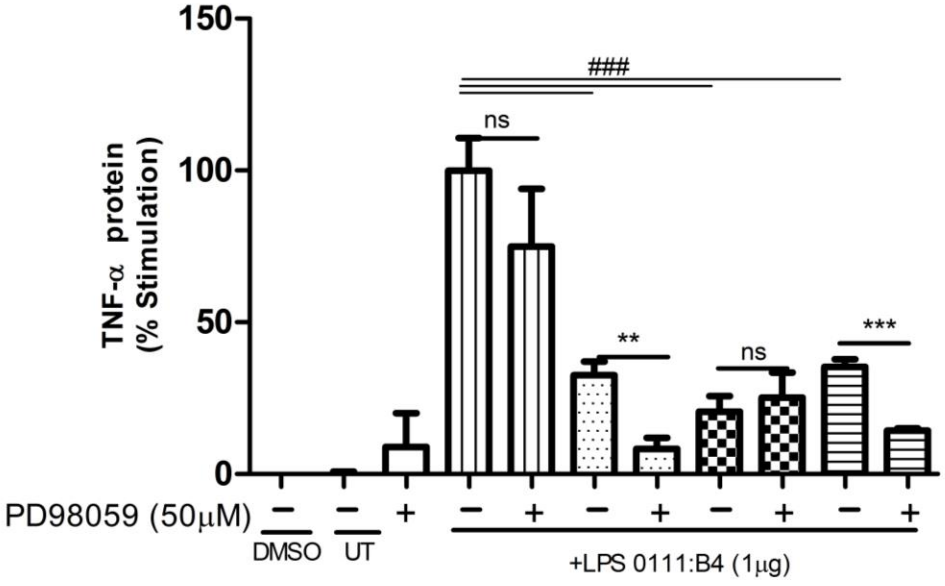

Figure 3.15 EKR1/2 inhibition synergizes with salmeterol and vilanterol to inhibit TNF-α. BV-2 cells were treated with the ERK1/2 inhibitor PD98059 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from two independent experiments. $^{###}$p<0.0001, **p<0.001 and ***p<0.0001. UT= untreatd BV-2 cells.

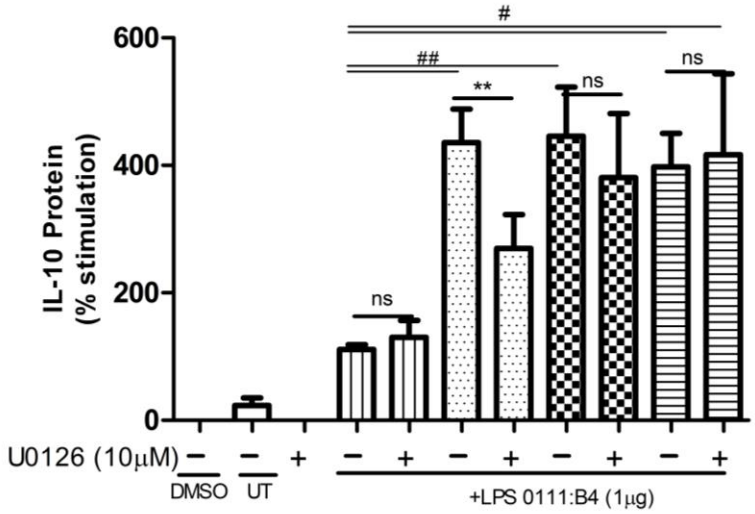

Figure 3.16 Salmeterol-mediated up-regulation of IL-10 response is ERK1/2-dependent. BV-2 cells were treated with the ERK1/2 inhibitor U0126 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from three independent experiments. $^{\#}p<0.05$ and $^{\#\#}p<0.001$ and $^{**}p<0.001$. UT= untreatd BV-2 cells.

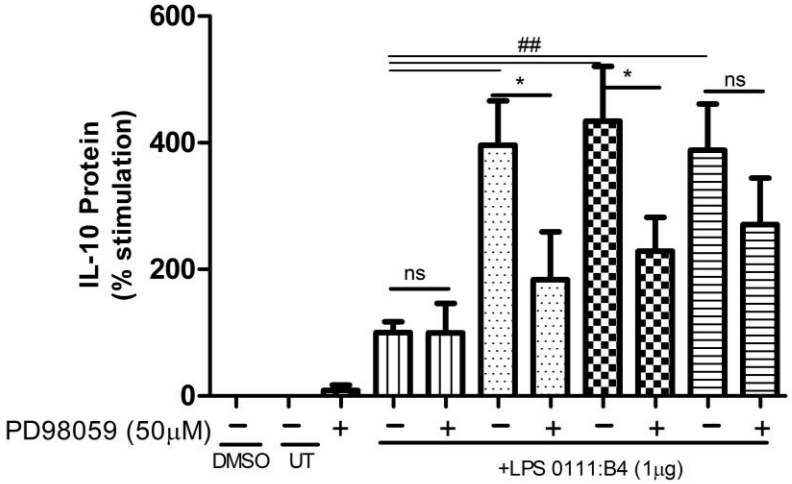

Figure 3.17 Salmeterol and vilanterol-mediated enhancement of IL-10 response is ERK1/2-dependent. BV-2 cells were treated with the ERK1/2 inhibitor PD98059 for 30 minutes, then treated with β2-AR agonist (10^{-9}M) for 45 minutes, and then stimulated with LPS for 4 hours. Data represents mean ±SEM from two independent experiments. $^{*}p<0.05$ and $^{\#\#}p<0.001$. UT= untreatd BV-2 cells.

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CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Neuroinflammation has been proposed as a major contributing factor in the progression of the dopaminergic neuronal loss in the substantia nigra (SN)—a hallmark of Parkinson's disease (PD). Specifically, activated microglial cells were demonstrated to be the principle drivers of the neuroinflammatory pathology (Farmer *et al.,* 2000; Qian *et al.*, 2009). However, activated microglial cells can exhibit an M1 (pro-inflammatory) or an M2 (anti-inflammatory) phenotype depending on the activation stimuli (Stein *et al.,* 1992). While the efficacies of many anti-inflammatory agents are measured by their ability to inhibit a mixed or M1-characterized microglial population; their ability to alter the phenotype of a homogenous population of microglial cells, from an M1 to an M2, is yet to be tested. Clearly, in a disease such as PD where an M1 microglial population predominates, identifying anti-inflammatory agent(s) that can modify the phenotype of microglial cells holds great therapeutic value.

β2-adrenergic receptor (β2-AR) agonists are a class of drugs with promising anti-inflammatory effects. Microglial cells are demonstrated to be indispensable for the neuroprotective effects mediated by these β2-AR agonists. As such, our laboratory (Qian *et al.,* 2009; and 2011) examined selected short and long-acting β2-AR agonists in animal models of PD. While all β2-AR agonists tested demonstrated neuroprotective effects, the long-acting agonists, salmeterol, formoterol and clenbuterol, were shown to facilitate this effect at lower doses compared to the short-acting agonist salbuterol (Qian *et al.,* 2011). Extrapolating based on these results, the recent super long-acting β2-AR agonists, indacaterol maleate and vilanterol trifenatate, should in theory be more potent compared to the long-acting agonists. As indacaterol (Aracapta) and vilanterol (Breo) have been recently introduced for management of asthma and chronic obstructive pulmonary disease

(COPD), their anti-inflammatory effects in PD are not yet tested. As well, the ability of β2-ARs to mediate the anti-inflammatory response i.e. enabling microglial cells to switch from an M1 to an M2, of both the long-acting and the super-long agonist remain elusive. As such, based on the data we collected, we will discuss (i) the ability of β2-AR agonists to modify the phenotype of activated microglial cells in regard to secretion of pro- and anti-inflammatory cytokines; compare and contrast the (ii) efficacy and kinetics, and (iii) mode of action of the super long-acting β2-AR agonists indacaterol maleate and vilanterol trifenatate to that of the long-acting agonist salmeterol in the activated murine BV-2 microglial cell line.

An important feature of our study was that we demonstrated β2-AR agonists, both long and super long-acting, to not only inhibit TNF-α production but also enhance IL-10 production in activated microglial cells—a phenomenon we termed anti-inflammatory conversion. Many studies have looked at the anti-inflammatory effect(s) of various drugs, including β2-AR agonists, with regard to their effect on pro- and anti-inflammatory cytokines as well as the effect of IL-10 on TNF-α (Platzer *et al.,* 1995; Armstrong *et al.,* 1996; Brennan *et al.,* 2008; Qian *et al.,* 2006; Park *et al.,* 2007). However, the efficacy of anti-inflammatory drugs in inhibiting pro-inflammatory cytokine release and up-regulating anti-inflammatory cytokine release in a homogeneous microglial cell population has never been tested. Consequently, our finding has important implications for therapeutic efficacy as well as in reducing unnecessary use of pharmaceuticals to inhibit pro-inflammatory and enhance anti-inflammatory mediator release. More importantly, our data demonstrates that β2-AR agonists do not simply dampen microglial activity, but rather modulates its phenotype by altering intracellular signaling pathways.

Therefore, it supports the plasticity hypothesis of microglial cells: i.e. the ability of individual microglia to convert their phenotype from an M1 (TNF-α-secreting pro-inflammatory) to an M2 (IL-10 secreting anti-inflammatory) active state (Stout *et al.*, 2005; Italiani *et al.*, 2014) in the presence of appropriate stimulants. Further studies would need to be conducted to determine the extent of this conversion, since it appears the production of IL-6 is not affected by β2-AR (data not shown). In addition, it is not clear how many other markers of the M1 to M2 conversion are regulated by β2-AR exposure, as we were unable to find any difference in the expression profile in activated BV-2 cells. Therefore, the extent to which β2-AR agonists switch the phenotype of activated microglial cells will need to be further examined.

Regardless, these finding are readily applicable to a disease such as PD where the neuroinflammatory pathology is already robust and characterized by the presence of a 'toxic' M1 microglial population. The hypothesis that different populations, resident vs. infiltrating, of monocytes adopt M1 and M2 in a time-dependent manner (Italiani *et al.*, 2014) provides minimal therapeutic potential for PD. However, demonstrating that microglial cells can alter their phenotype provides a therapeutic opportunity to utilize the M1 population of microglial cells already present within the Substantia nigra pars compacta (SNpc) for wound-healing purposes.

Secondly, the data gathered indicates that the super long-acting β2-AR agonists indacaterol and vilanterol are 100-fold more potent in inhibiting TNF-α release from activated microglial cells than the long-acting agonist salmeterol. In addition, indacaterol was more consistent in enhancing IL-10 release compared to salmeterol and even vilanterol. Our finding is congruent with previous work (Qian *et al.*, 2011) which

demonstrated that compared to short-acting agonists, long-acting agonists were 10-1000-fold more potent. This suggests that the differences between short-acting, long-acting, and super long-acting agonists may not simply be their *in vivo* half-life, which is how they are functionally defined, but also their effectiveness at stimulating cell surface receptors to mediate an anti-inflammatory response and/or conversion.

Previous work from our laboratory (Qian *et al.,* 2009) has also demonstrated salmeterol to have a dose-dependent effect on the microglial cell: neuroprotective at lower concentrations and neurotoxic at higher concentrations. The reported dose-response can potentially be due to the activation of other adrenergic receptor as well as β2-ARs, despite the higher affinity for β2-ARs. As well, at higher concentrations the agonists maybe be able to activate a plethora of signaling cascades allowing for the reported difference in the response between high dose and low-dose. Due to their higher affinity for the β2-ARs, it is reasonable to assume that β2-ARs are selectively activated at these lower concentrations and that the anti-inflammatory effect seen at low doses is mediated through the β2-ARs. In examining whether the super long-acting agonists have a concentration-dependent effect; we report salmeterol as well as the super long-acting agonists to demonstrate anti-inflammatory capabilities from 1μM to 1pM with the effects titrating out at 1-10fM concentrations. Therefore, we report no neurotoxic effects, i.e. ability to enhance pro-inflammatory cytokine production by microglial cells in our study. The difference in our current study and the previous study is that the previous study (Qian *et al.,* 2009) used salmeterol by itself without an inflammatory stimulant. Consequently, our data posits that β2-AR agonists require both an LPS-induced TLR2/4 and an agonist-induced β2-AR activation in mediating an anti-inflammatory conversion. This is an area that will require

further research as LPS does produce IL-10 (Park *et al.*, 2007), but β2-ARs have yet to be shown to induce an IL-10 response by themselves. Furthermore, many studies suggest that IL-10 inhibits TNF-α (Platzer *et al.*, 1995; Armstrong *et al.*, 1996) as well as the LPS-induced pro-inflammatory response (Qian et al. 2006; Park *et al.*, 2007). Therefore, our finding showing that β2-AR agonists enhance the IL-10 response while simultaneously inhibiting the TNF-α provides a good foundation for further research to identify the type of feedback or feed-forward mechanism responsible for such a conversion activity.

In examining the kinetics of action of β2-AR agonists, we found that the β2-AR agonists exerted their anti-inflammatory effect relatively quickly as indicated by the shift in the cytokine profile as early as 5 minutes of pretreatment with the agonists. This suggests that both the super long-acting and long-acting β2-AR agonists have a comparable functional effect on the β2-ARs *in vitro*. The super long-acting agonists are reported to have a higher intrinsic activity (bind with a higher affinity for β2-AR), efficacy (rapid onset) and longer duration of activity compared to long-acting agonist salmeterol (Cazzola *et al.*, 2011) in the human lung. For example, vilanterol has a faster onset of activity, 3.1 minutes, compared to salmeterol's 8.3 minutes (Cazzola *et al.*, 2011). Therefore, while we report no difference in the kinetics of activity between these super long-acting and long-acting agonists, shorter time intervals could be tested prior to concluding that no difference exists in their kinetics of activity. However, at the range of intervals tested, 5 to 45-minutes, we report no difference in onset of activity between the long-acting and the super long-acting agonists.

We also tested whether that the super long-acting β2-AR agonists would have a longer duration of activity on the β2-AR compared to the long-acting agonist *in vitro*.

Results of the recovery experiments indicate that the anti-inflammatory effect of both long-acting and super long-acting agonists regress after 30 minutes indicating that there is no difference between the two classes of agonists at the time points tested: 30 minutes, 3 hours, 6 hours, and 12 hours. To account for this, GPCR-desensitization may provide an explanation. GPRC are desensitized and inactivated immediately after receptor-activation (Lefkowitz *et al.*, 2005; Ozawa *et al.*, 2008). In this case, desensitization involves internalization and recycling of the β2-ARs—interrupting receptor-agonist interactions as a result. The entire process of receptor-mediated desensitization takes approximately 5 minutes (Lefkowitz *et al.*, 2005). However, both vilanterol and indacaterol are reported to have β2-AR-mediated activity in the lung after 22-24 hours which was not the case for salmeterol (Cazzola *et al.*, 2011). GPCR-desensitization should, in theory, be consistent throughout the body; therefore, based on our data, we speculate clearance rates from the body of the β2-AR agonists will have an impact on the ability of these agonists to stay in close proximity to β2-ARs at an effective concentration.

Interestingly, the β2-AR agonists are effective in inhibiting the inflammatory response when the agonists are removed and LPS is added immediately. However, intermittent exposure of the cells to fresh media (without any active reagent) for a period of even 30 minutes prior to adding LPS abolishes the ability of these agonists to counter the inflammatory response. This suggests that the inflammatory effects of β2-AR agonists are coupled to LPS-induced inflammatory response in a time-sensitive manner, as a delay between the removal of the agonists and addition of the LPS resulted in the abrogation of the β2-AR agonist-mediated anti-inflammatory conversion. This further verifies our conclusion that an LPS-induced TLR2/4 and an agonist-induced β2-AR activation are both

needed for the β2-AR agonists to exert their anti-inflammatory activity. In order to test the possibility that *in vivo* factors may play a role in differentiating a long-acting agonist from a super long-acting agonist, *in vivo* experiments should to be conducted. However, prior to *in vivo* experiments, basic research demonstrating a similar mechanism of action will strengthen the hypothesis that *in vivo* half-life and/or other factor(s) differentiate the efficacy in the duration of action of the super long-acting agonists compared to the long-acting agonist.

Thirdly, mechanistic studies did reveal that both long-acting and super long-acting agonists do indeed share a similar signaling mechanism. For example, for both the long-acting and super long-acting agonists, the inhibition of TNF-α production was β-arrestin2-dependent but PKA-independent, while the enhancement of IL-10 production was PKA-dependent but β-arrestin2-independent. This suggests that independent signaling pathways through the β2-ARs must both be activated to mediate an overall anti-inflammatory conversion in activated microglial cells. Most importantly, it appears that the LPS signal is needed for both aspects of the anti-inflammatory conversion mediated by the β2-AR agonists, as cytokine production is dependent on TAK1 and IKK. In the next section, we will discuss in detail the mechanism of action of these β2-AR agonists with regard to PKA, TAK1, β-arrestin2 and NF-κB, and finally MAPKs.

β2-ARs are a class of G-protein coupled receptors (GPCR). Classical activation of a GPCR results in intracellular changes using cyclic AMP (cAMP) as the second messenger. Elevated cAMP activates PKA, and theoretically results in an increased transcription of cytokines with a cAMP-response-element binding (CREB) domain. TNF-α and IL-10 both have a CREB domain (Tsai *et al.*, 2000; Saraiva *et al.*, 2010); therefore,

increased cAMP-PKA activity should, theoretically, elevate both IL-10 and TNF-α levels. Examining each cytokine separately, we report that PKA inhibition resulted in a reversal of the β2-AR-mediated IL-10 enhancement indicating that PKA activity is required for the β2-AR agonist-mediated enhancement of IL-10. In contrast, PKA inhibition had no reversal or synergistic effect on the β2-AR-mediated TNF-α inhibition. Previous work by Suberville and colleagues (1996) also found the β-adrenergic agonist isoproterenol to enhance IL-10 in a PKA-dependent mechanism. In contrast to Il-10, our data as well others (Farmer *et al.*, 2000; Qian *et al.*, 2011) indicate that the anti-inflammatory effect on TNF-α by β2-AR agonists is independent of the classical GPCR activation. Combined, the data supports the notion that while β2-ARs induce an anti-inflammatory effect on TNF-α in a G-protein-independent mechanism, the conventional G-protein signaling pathway mediates the IL-10 enhancement.

In identifying other upstream regulators of the β2-AR-mediated anti-inflammatory conversion, TAK1 was found to be involved. TAK1 is a scaffolding protein that works in complex with other proteins to initiate a cascade of concerted downstream signaling effects. Inhibiting TAK1 resulted in an abolition of all three cytokines tested: TNF-α, IL-6 (data not presented in this report) and IL-10. Previously, Qian and colleagues (2011) reported that salmeterol inhibits the phosphorylation of TAK1 and therefore would inhibit the activation of signaling pathways downstream of TAK1 such as NF-κB and MAPKs. Competitive irreversible inhibition of TAK1 with 5-(Z)-7-Oxozeaenol eliminated all cytokine production, suggesting that while β2-AR agonists inhibit the phosphorylation of TAK1, it does not completely render TAK1 dysfunctional because cytokine production is

TAK1-dependent. Furthermore, it suggests that the transcription factor(s) needed to enhance IL-10 is downstream of TAK1.

Silencing β-arrestin2, the upstream scaffolding protein in the GPCR signaling pathway, resulted in a reversal of the inhibitory effect of β2-AR agonists on TNF-α with no effect on the IL-10 enhancement. Similarly, several groups have reported β-arrestin2 to inhibit NF-κB activation (Lefkowitz *et al.,* 2005; Ma *et al.,* 2007). β-arrestin2 is shown to bind and stabilize the IκBα (Ma *et al.,* 2007). Inhibition of degradation of IκBα effectively renders NF-κB inactive as it sequesters NF-κB in the cytoplasm. Our data supports this finding as silencing β-arrestin2 reverses the β2-AR agonist-mediated inhibition of TNF-α production. Furthermore, inhibiting NF-κB with the IKKβ inhibitor, Compound A, resulted in an inhibition of the LPS-mediated TNF-α response to a level comparable to β2-AR agonist-induced TNF-α response. This suggests that perhaps β2-AR agonists inhibit NF-κB resulting in low levels of TNF-α. Interestingly, using the IKKβ inhibitor, the β2-AR agonist-mediated IL-10 enhancement was also inhibited. It is generally understood that NF-κB has a very minimal role in the transcription of IL-10, and in fact may actually lead to IL-10 inhibition (Wang *et al.*, 1995). Therefore, based on the effect of Compound A on IL-10, concluding that β2-AR agonists inhibit NF-κB becomes problematic. While NF-κB is an important transcription factor for various pro-and anti-inflammatory cytokines such as TNF-α; other transcription factors such as C/EBPβ, SP1, CREB and transcription factor myocyte enhancer factor 2D (MEF2D) are known to be important for IL-10 (Yoshidoma *et al.,* 1999; Chanteux *et al.*, 2007; Csoka *et al.*, 2007; Avni *et al.,* 2010; and Yang *et al.*, 2015). Since Compound A is an inhibitor of IKKβ, we can deduce that inhibiting IKKβ

inhibits another transcription factor that is needed to up-regulate IL-10. Alternatively, it is possible that inhibition of NF-κB by these β2-AR agonists positively affects other signaling pathway that is responsible for the up-regulation of IL-10. To confirm the role of NF-κB in the β2-AR-mediated inhibition of TNF-α and up-regulation of IL-10, specific inhibitors to NF-κB can be used. Specifically, p65 phosphorylation and translocation to the nucleus can be halted and/or an IκB-α specific inhibitor could be used. Furthermore, the above transcription factors important for IL-10 are not known to interact with IKK. Consequently, these contradictory results further suggest that more experimentation is needed to determine the exact role of NF-κB in the inflammatory conversion of microglial cells by β2-AR agonists.

The MAPKs have a three-tiered activation loop consisting of a MAPK kinase kinase (MAPKKK) that activates a MAPK kinase (MAPKK) that then activates a MAPK. TAK1 serves as a MAPKKK. Like TAK1, β-arrestin2 is also reported to recruit these kinases and enable interaction and the activation of the MAPKs: ERK1/2, JNK and p38 (Lefkowitz *et al.*, 2005; DeWire *et al.*, 2007). Previous data (Qian *et al.*, 2011) show salmeterol to inhibit the phosphorylation of TAK1 and the downstream MAPKs, suggest that β2-AR agonists inhibit TAK1 and the downstream MAPKs to inhibit pro-inflammatory cytokine production. We report that only inhibition of p38 and JNK but not ERK1/2 resulted in an inhibition of the inflammagen-induced TNF-α production. As β-arrestin2 is shown to activate these MAPKs, our data suggests that β2-AR agonists utilize β-arrestin2 in a mechanism not involving MAPKs to inhibit TNF-α. The data strongly suggests that β-arrestin2 inhibits TNF-α production by acting on NF-κB and not on MAPKs.

In contrast to the effect of inhibiting MAPKs on the inflammagen-induced TNF-α response; inhibition of JNK reversed enhancement of IL-10 due to all three β2-AR agonists, inhibition of p38 resulted in reversal of only vilanterol-mediated IL-10 enhancement, and ERK1/2 inhibition reversed IL-10 response due to salmeterol and indacaterol. As we have established that TAK1 inhibition results in an abrogation of the IL-10 response, it can be deduced that the β2-AR agonists activate MAPKs in a TAK1-independent mechanism. Some studies have shown that PKA can result in the activation of MAPKs, specifically p38 and ERK1/2 (Chiu *et al.,* 2004). Since β2-AR agonist-mediated enhancement of IL-10 is PKA-dependent, it is possible that PKA-dependent activation of MAPKs can result in the up-regulation of IL-10. This is also consistent with findings that GPCRs can activate MAPKs in a G-protein-dependent but β-arrestin2-independent mechanism (Lefkowitz *et al.,* 2005). Since, we have established that IL-10 enhancement is mediated through the conventional GPCR pathway, the role of G-proteins in activating MAPKs to enhance IL-10 will need to be further examined. As well, the differences found in the role the various MAPKs plays in the mechanism of action of salmeterol, indacaterol, and vilanterol is also potential evidence that these agonists differ in their mechanism of action. Specifically, it is possible that the differences may be as a result of differing concentration and/or kinetic-dependent mechanism of action of these β2-AR agonists that we have not yet identified.

Furthermore, while our data indicates that β-arrestin2 is not needed for the IL-10 response; it may be possible that transient activation of MAPKs by β-arrestin2 can result in the increased IL-10 response. Since, we were not able to completely silence β-arrestin2, it is possible that low levels of β-arrestin2 is sufficient for the β2-AR agonists to activate

MAPKs. Previous results have shown that peritoneal macrophages deficient in β-arrestin2 had impaired IL-10 production (Li *et al.*, 2014); therefore further experiments will be needed to verify the role of β-arrestin2 in enhancing IL-10 production downstream of β2-AR agonists.

In comparing and contrasting long-acting and super long-acting β2-AR agonists, we have found that (i) both classes of agonists induce an anti-inflammatory conversion and that the (ii) super long-acting β2-AR agonists vilanterol and indacaterol are more potent in inhibiting TNF-α than long-acting β2-AR agonist salmeterol. This is an important finding because several anti-inflammatory agents have been found to be protective against PD or at least able to delay in the progression of the disease. Such findings support neuroinflammatory pathology as the primary mechanism by which PD progresses. This can be further confirmed with knockout studies showing that compared to knockouts of implicated PD-genes, knocking out important inflammatory regulators such as Phox is protective against neurotoxin and inflammation-induced animal models of PD. Based on these and our data, super long-acting agonists can be a more potent anti-inflammatory adjunct therapy to inhibit and reverse the CNS inflammation seen in PD.

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