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

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A B S T R A C T

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

List of abbreviations

PD	Parkinson's disease
DA	dopamine
SN	substantia nigra
β2-AR	beta2-adrenergic receptor
LPS	lipopolysaccharide
TNF-α	tumor necrosis factor-alpha
IL-1β	interleukin-1beta
NO	nitric oxide
ROS	reactive oxygen species
GPCR	G-protein coupled receptor
NE	norepinephrine
COPD	chronic obstructive pulmonary disorder
NF-κB	nuclear factor-kappa B
MAPK	mitogen activated protein kinase
TLR	toll-like receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
cAMP	cyclic-adenosine monophosphate
PKA	protein kinase A
CCL	chemokine (C—C) motif ligand
CXCL	chemokine (C-X—C) motif ligand
CREB	cAMP response element binding protein.

1. Introduction

Inflammation is a highly regulated process which involves multiple steps including the migration of immune cells, production of inflammatory mediators, a destructive phase that results in clearance of infection/debris and eventually the conversion into a regenerative stage that results in tissue repair. In chronic inflammation, this conversion to a regenerative stage is often delayed or absent, resulting in continuous tissue destruction and pathology. Similar to peripheral macrophages, microglial cells respond to endogenous stimuli in both a protective and pathogenic manner and functionally serve as the resident macrophages of the central nervous system (CNS). Activation of microglia and their subsequent production of inflammatory mediators have been shown in pathological studies of many chronic neurodegenerative diseases including Alzheimer's disease (AD) (Akiyama et al., 2000), Parkinson's disease (PD) (Wang et al., 2015), multiple sclerosis (Dendrou et al., 2015), stroke (Kanazawa et al., 2017), neuropathic pain (Zhao et al.,

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2017) and several others (Ransohoff, 2016a), with little to no evidence of conversion to a tissue regenerative state (Jin and Yamashita, 2016).

PD is a neurological movement disorder caused by degeneration of dopaminergic neurons which results into impaired motor basal ganglia circuitry in the mid brain region (Grayson, 2016). The pathological hallmarks of PD are the presence of Lewy bodies containing insoluble a-synuclein protein aggregates (Rocha et al., 2018), as well as neuroinflammation and activated glial cells (Hirsch and Hunot, 2009). Dopaminergic neurodegeneration and the accumulation of protein aggregates convert microglia into an activated state, and this process results in microglial priming (Perry and Holmes, 2014). These primed or activated microglia are then more susceptible to a secondary inflammatory stimulus which further leads to a pathological inflammatory response, characterized by the production of several inflammatory mediators which contribute to neuronal damage (Perry and Holmes, 2014). It has been suggested that the inhibition of the pro-inflammatory microglial response and an enhanced anti-inflammatory response during PD progression is required for halting and potentially reversing dopaminergic cell loss, and this immunologic conversion leads to resolved inflammation within the substantia nigra (SN) (Subramaniam and Federoff, 2017; Walker and Lue, 2015). An increased number of activated microglia in SN region is a hallmark of neuroinflammation and in the pathogenesis of PD (McGeer et al., 1988). Activated microglia in CNS milieu can perform a variety of functions with diverse phenotypes. These phenotypes broadly divided into two main states: a classically activated M1-like phenotype with cytotoxic/neurotoxic properties and an alternate activated M2-like phenotype with regenerative and neuroprotective properties (Subramaniam and Federoff, 2017). M1 or classically activated microglia produce pro-inflammatory mediators such as TNF-α (tumor necrosis factor-α), IL (interleukin)-6, IL-1β, and IL-18, CXCL10/IP-10 (C-X-C motif chemokine ligand 10/interferon gamma-induced protein 10), MCP-1/CCL2 (monocyte chemoattractant protein1/C-C motif chemokine ligand 2), NO (nitric oxide) and ROS (reactive oxygen species). In contrast, the protective phenotype of microglia characterized by the expression of an anti-inflammatory phenotype, including the expression of arginase-1, IL-10 and CXCL14 (Tang and Le, 2016).

Studies targeting neuroinflammation represent a novel therapeutic approach for neurodegenerative conditions such as PD (Esposito et al., 2007; Moore et al., 2010; Walker and Lue, 2015), and previously we have established several therapies targeting neuroinflammation and neurodegeneration in an animal model of PD. These therapies include D-morphinan-related compounds (Qian et al., 2007b), anti-inflammatory cytokines such as TGF-β (transforming growth factor-beta) (Qian et al., 2008) and IL-10 (Qian et al., 2006a; Qian et al., 2006b), and small molecule inhibitors targeting IKK (inhibitor of kappa B (IκB) kinase) (Zhang et al., 2010) and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Qian et al., 2007a).

One such highly effective anti-inflammatory approach is the use of therapeutic compounds that activate the beta 2-adrenergic receptor (β2-AR) (Qian et al., 2011; Sharma et al., 2017; Sharma and Flood, 2018a, 2018b). A number of different types of brain cells, including microglia, astroglia, and neurons express the β2-ARs (Tanaka et al., 2002), which is one of the main targets of the regulatory effects for noradrenaline or norepinephrine (NE) in PD (Delaville et al., 2011). Previously, we have found that the long-acting β2-AR agonist Salmeterol showed neuroprotective properties in both neurotoxin-based MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and inflammatory lipopolysaccharide (LPS) model of PD (Qian et al., 2011). Salmeterol is a long-acting β2-AR and an active ingredient in Advair[@] which is a FDA-approved drug and prescribed as a bronchodilator for the treatment of asthma and chronic obstructive pulmonary disorder (COPD) (Anwar et al., 2015). Further, we found that Salmeterol shows anti-inflammatory effects by enhancing the binding between β-arrestin2 and TAB1 (TAK1 (transforming growth factor-beta-activated-kinase1) binding protein) and reducing TAK1/TAB1 interaction, thereby suppressing the activation of NF-κB (Sharma and Flood, 2018a).

Previous results show that treatment with the anti-inflammatory cytokine IL-10 on neuron-glia culture suppressed LPS-induced degeneration of DA neurons which shows the role of IL-10 in regulating neuro-inflammation in PD (Qian et al., 2006a; Qian et al., 2006b). In addition, both the endogenous agonist (NE) and pharmacological agonist of β2-AR have been found to alter the LPS-activated M1-like phenotype of macrophages by enhancing the expression of Arg-1 and IL-10 (Bosmann et al., 2012; Grailer et al., 2014; Lamkin et al., 2016). Here, in current study we reveal that the long-acting β2-AR agonist Salmeterol has potent anti-inflammatory effects and suppresses the production of pro-inflammatory cytokines and chemokines which are the characteristic markers of classically activated M1 microglia, while concurrently induces the production of anti-inflammatory cytokine IL-10 in LPS-activated BV2 cells. In addition, we have found that β2-AR stimulation by Salmeterol enhances expression of arginase-1 (Arg-1) and CXCL14, thereby converting the inflammatory M1-like microglia to an M2-like microglial phenotype. Inhibition of classical signaling pathway of β2-AR via silencing *CREB* by siRNA and inhibiting protein kinase A (PKA) abrogates Salmeterol-mediated production of IL-10. In contrast, inhibition of β-arrestin2 via siRNA did not affect the Salmeterol-mediated production of IL-10. These immunomodulatory effects of Salmeterol may serve as a potential therapeutic avenue for neuroinflammatory and neurodegenerative diseases including PD.

2. Materials and methods

2.1. Reagents

Salmeterol and lipopolysaccharide from *E.coli* (0111:B4) were purchased from Sigma Aldrich and LKT laboratories, Inc., St. Paul, MN respectively. Ultra-long-acting β2-AR agonists Vilanterol and Indacaterol were purchased from MedChemExpress (M C E), NJ, USA and Selleckchem, TX, USA respectively. Antibodies against total-CREB, β-arrestin2, β-actin as well as horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were purchased from Cell Signaling Technology, Danvers, MA.

2.2. Cell culture and treatment

The murine microglial cell line BV2 was maintained in culture media RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 1% penicillin-streptomycin solution at 37 °C and 5% CO_2 in humidified incubator. BV2 cells were pre-treated with β2-AR agonists (Salmeterol, Vilanterol and Indacaterol; 10^{-9} M) for 45 min followed by stimulated with 1 μg/ml of LPS to activate TLR4-dependent inflammatory response. The concentration of β2-AR agonists was used on basis of our previous observations (Qian et al., 2011; Sharma and Flood, 2018a). Cells were also incubated for 1 h with various signaling pathways inhibitors (**U0126:** MEK1/2 inhibitor (10 μM; Tocris), **SP600125:** JNK inhibitor (10 μM; Selleckchem), **SB203580:** p38 MAPK inhibitor (5 μM; Tocris), **Wortmannin:** PI3K inhibitor (1 μM; Tocris), **KT5720:** PKA inhibitor (1 μM; Tocris), and **ICI 115855 hydrochloride:** β2-AR antagonist (1 nM; Tocris) prior to treatment with β2-AR agonist and LPS.

2.3. Total RNA extraction and quantitative RT-PCR

Total cellular RNA was extracted from BV2 cells by Trizol reagent (Invitrogen) according to manufacturer's protocol. The quality of RNA was assessed by Nanodrop (Thermo Scientific) for purity (OD260/OD280), and Agarose gel electrophoresis for RNA integrity and potential contamination. cDNA was synthesized from 1 μg of total RNA by using QuantiTect Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's instructions. The qPCR was performed by using

Table 1
List of primers used for gene expression analysis.

Target gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Accession number
TNFA	ACCATGAGCACTGAAAGCAT	AGATGAGGTACAGGCCCTCT	NM_013693.3
IL6	GCTGGAGTCACAGAAGGAG	GAGAACAACATAAGTCAGATACC	NM_031168.2
IL18	TCCAACTGCAGACTGGCAC	GTCTGGTCTGGGGTTCACTG	NM_001357221.1
NOS2	ACATCAGGTGGTCGGCCATCACT	CGTACCGGATGAGCTGTGAATT	NM_010927.4
CCL2	GATGCAGTTAACGCCCCACT	ACCCATTCCTTCTTGGGGTC	NM_011333.3
CCL3	GCAACCAAGTCTTCTCAGCG	AGCAAAGGCTGCTGGTTTCA	NM_011337.2
CCL4	TGTGCAAACCTAACCCCGAG	TGGAGCAAAGACTGCTGGTC	NM_013652.2
CXCL10	TCCCCATCAGCACCATGAAC	GGACCATGGCTTGACCATCA	NM_021274.2
ARG1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC	NM_007482.3
IL10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG	NM_010548.2
CXCL14	GAGTCACCGAGTGGTTCTGCAT	CTTCGTAGACCCTGCGCTTC	NM_019568.2
GAPDH	GCCTTCCGTGTTCCTACC	CTTCACCACCTTCTTGATGTC	NM_008084.3

300 ng of cDNA template with SYBR Green and the volume of reaction was made up to 20 µl by nucleases free water. The PCR reaction condition as follows denaturation at 95 °C for 30s, primer annealing at 55 °C for 30s followed by the final extension at 72 °C for 60s. Primer pairs used for amplification are listed in Table 1.

2.4. Preparation of cell lysates and Western Blotting

Protein samples were prepared as whole-cell lysate. Cells were lysed in NP-40 lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40 and freshly added 10^{-6} M Protease inhibitor) and then centrifuge. Supernatant containing cell lysate was collected. Protein concentration was determined by standard procedure of Pierce BCA protein assay kit (Thermo Scientific). Cell lysates were separated on 10% polyacrylamide gels and blotted onto a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat skimmed milk (in Tris-buffer saline containing 0.1% Tween20; TBST). After blocking, blots were probed with following primary antibodies: total CREB, β-arrestin-2, and β-actin in 5% milk overnight at 4 °C. After 3 washes with TBST, blots were probed with respective HRP conjugated secondary antibodies. Later, blots were washed and developed with enhanced chemiluminescence reagent (Amersham, GE healthcare). Blots were further visualized using Bio-Rad ChemiDoc gel Imaging system and band intensities was quantitatively determined by Image J Software (NIH).

2.5. RNA interference

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

2.6. Enzyme-linked immunosorbent assay (ELISA)

BV2 cells were seeded in 24-well tissue culture plates (5×10^4 cells per well). Cells were pre-incubated for 45 min with Salmeterol (10^{-9} M). After 45 min culture media replaced with fresh media and cells were stimulated with LPS (1 µg/ml) and culture supernatant was collected after 6 h. In separate experiments cells were pre-incubated with pharmacological inhibitors (mentioned above in section 2.2) for 1 h and then subjected to treatment with Salmeterol and LPS. The concentration of cytokines TNFα and IL-10 in culture medium was measured by commercially available ELISA kits (R&D systems, Minneapolis, MN) according to manufacturer's protocol.

2.7. Flow cytometry analysis

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

2.8. Reactive oxygen species (ROS) detection assay

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

2.9. Statistical analysis

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

3. Results

3.1. β2-AR agonist stimulation suppresses TLR-induced M1 markers of microglial activation

Many studies have shown that pharmacological and endogenous β2-AR agonists can suppress the LPS-induced production of TNF-α (Ağaç et al., 2018; Keränen et al., 2017). Previously, we have shown that β2-AR agonist Salmeterol inhibited the activation of the LPS-induced NF-κB signaling pathway, which is a key transcription factor for the

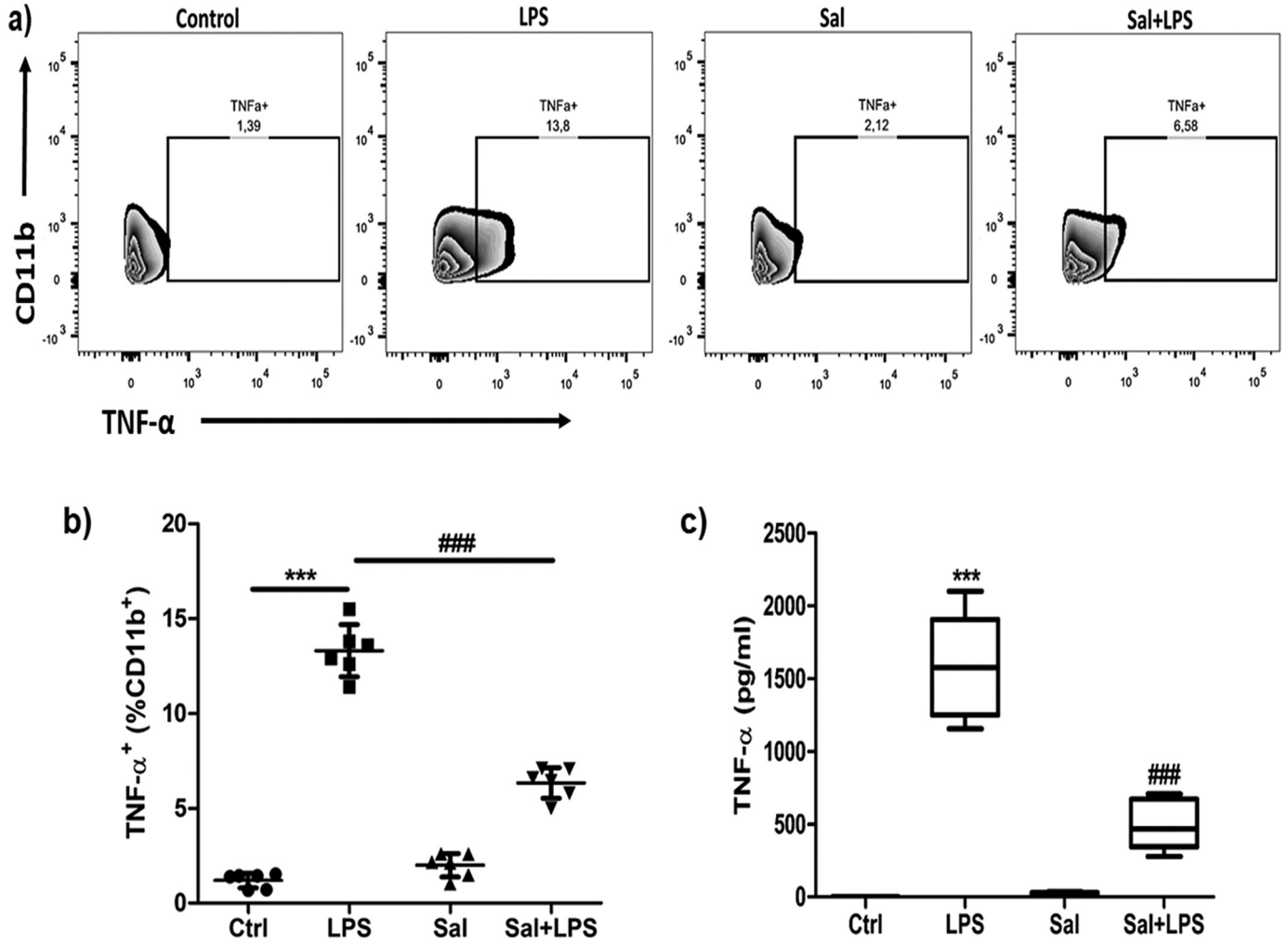

Fig. 1. β2-AR agonist Salmeterol suppresses LPS-induced TNF-α production in BV2 cells. BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min then stimulated with *E. coli* LPS (1 μg/ml) and Brefeldin A (10 μg/ml) (A and B). After 4 h cells were harvested and intracellular TNF-α expression was measured by flow cytometry. (A) Represents the expression level of TNF-α in control (untreated), LPS-stimulated, Salmeterol-treated and Sal + LPS-treated experimental group. (B) Plot shows the percentage of TNF expressing cells. (C) BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min then stimulated with *E. coli* LPS (1 μg/ml) and after 6 h supernatants were collected. Box-plot represents the TNF-α production in culture supernatant which was measured by ELISA. Data represents mean ± SD of 3–5 independent experiments. ***$p < 0.001$ indicates significant difference from control group and ###p < 0.001 indicates significant difference from LPS-treated group.

production of a number of pro-inflammatory mediators (Qian et al., 2011; Sharma and Flood, 2018a). Here, we wished to determine the effect of Salmeterol on the expression of microglial activation markers. BV2 cells were treated with long-acting β2-AR agonist Salmeterol and then activated with inflammatory stimulus LPS. The intracellular production and release of TNF-α was measured by flow cytometry, qPCR and ELISA. The intracellular staining of TNF-α was enhanced with LPS stimulation and suppressed by Salmeterol treatment (Fig. 1A). The quantitative analysis shown increased number of TNF-α^+ cells in LPS-stimulated group while the number of TNF-α^+ cells was significantly suppressed (by ~ 3-fold; $p < 0.001$) in LPS + Salmeterol group (Fig. 1B). Similarly, the production of TNF-α in culture supernatant was also significantly suppressed (by ~4-fold; $p < 0.001$) in the LPS + Salmeterol group when compared to LPS-alone group (Fig. 1C). Furthermore, upon stimulation with endotoxin, microglia secrete a variety of cytokines and chemokines other than TNF-α that are unique to the M1 inflammatory phenotype. We examined the effect of Salmeterol on mRNA expression of TNF-α and these other M1 inflammatory phenotype makers on LPS-activated BV2 microglial cells. In Fig. 2, we show that LPS exposure upregulates the expression of M1-specific cytokines TNF-α, IL-18, IL-6 which were significantly downregulated in LPS + Salmeterol group ($p < 0.001$ *and* $p < 0.01$; Fig. 2A-C). In addition, stimulation of BV2 cells with LPS also upregulated the mRNA expression of pro-inflammatory M1-specific chemokines CCL2 (MCP-1), CCL3, CCL4 and CXCL10 (IP-10), and pre-treatment with Salmeterol significantly suppresses the expression of these chemokines ($p < 0.001$; Fig. 2D-G). As reported previously, Salmeterol also suppresses the LPS-activated nitric oxide (NO) secretion from microglia cells (Sharma and Flood, 2018a) and here we observe that Salmeterol also significantly downregulates the mRNA expression of LPS-activated iNOS (by ~6-fold; $p < 0.001$, Fig. 2H). In addition, Salmeterol significantly suppresses the LPS-induced generation reactive oxygen species in BV2 cells ($p < 0.001$; Fig. 2I). Collectively, this data suggests that Salmeterol exerts anti-inflammatory effects by inhibiting the expression of M1-specific pro-inflammatory cytokine and chemokine mRNAs and the production reactive oxygen species in endotoxin-activated BV2 cells.

3.2. Salmeterol stimulates expression of M2 phenotypic markers in LPS-activated BV2 cells

In response to inflammatory stimuli such as LPS, microglia change

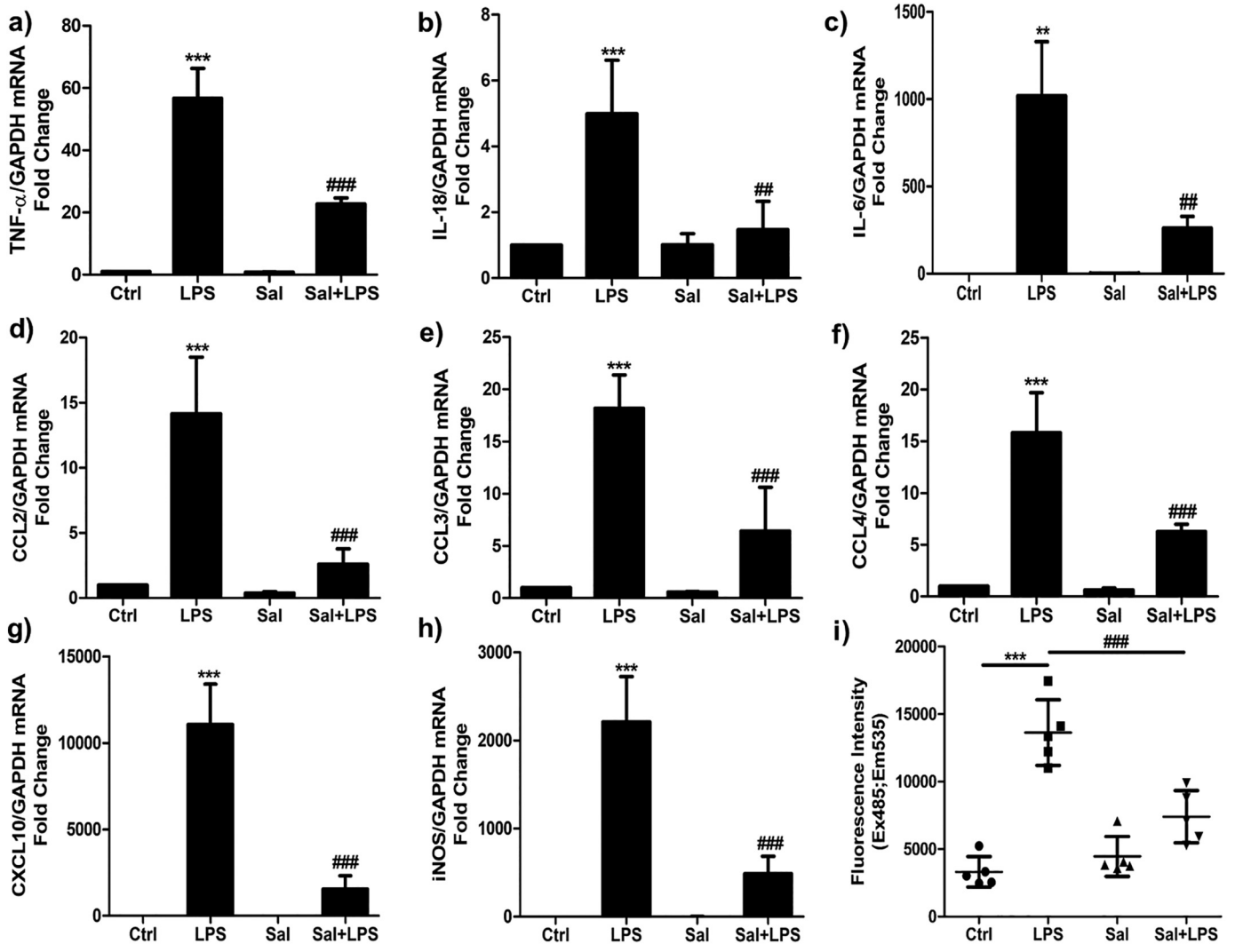

Fig. 2. Engagement of beta 2 adrenergic receptor with agonist Salmeterol downregulates LPS-stimulated expression of M1 phenotypic markers of microglia. BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with *E. coli* LPS (1 μg/ml). After 4 h cells were harvested, RNA was isolated from each experimental group and qRT-PCR analysis was performed. Bar graphs represent the expression level of (A) *TNFA*, (B) *IL-18*, (C) *IL-6*, (D) *CCL2*, (E) *CCL3*, (F) *CCL4*, (G) *CXCL10*, and (H) iNOS. The level of intracellular reactive oxygen species (ROS) was measured in culture supernatant 1 h post-LPS stimulation (I). Data represents mean ± SD of 3 independent experiments. ***p < 0.001 and **p < 0.01 represents significant difference from control group. ###p < 0.001 and ##p < 0.01 represents significant difference from LPS-treated group.

their phenotype from resting to the activated pro-inflammatory type (M1) (Tang and Le, 2016). Conversely, activation of TLRs with LPS can also weakly enhance the expression of the M2 phenotypic marker IL-10 in immune cells including microglia (Samarasinghe et al., 2006). Recent studies have shown that adrenergic receptor activation can robustly increase the expression of IL-10 in dendritic cells (Nijhuis et al., 2014) and activated macrophages (Grailer et al., 2014). Consequently, we tested if pre-treatment with Salmeterol can convert the pro-inflammatory M1-like phenotype to the M2-like or anti-inflammatory phenotype by increasing the expression of not only IL-10 but of other M2 markers as well. The intracellular production and release of IL-10 was measured by flow cytometry, qPCR and ELISA. While the intracellular staining of IL-10 was slightly enhanced in LPS-activated BV2 cells compared to unstimulated cells, IL-10 staining was robustly increased after Salmeterol treatment in these cells. Interestingly, no increased expression of IL-10 was observed in Salmeterol alone treated BV2 cells (Fig. 3A). The quantitative analysis shows a small increase in the number of IL-10^{+} cells in LPS-stimulated group, which was significantly enhanced by Salmeterol treatment (approximately by 3-fold; p < *0.001*) in LPS + Salmeterol group when compared to LPS alone group (Fig. 3B). Also, pre-treatment with Salmeterol enhances the mRNA expression of IL-10 in LPS-activated BV2 cells (p < *0.01*; Fig. 3C). Similarly, the production of IL-10 in culture supernatant was also significantly enhanced (by ~4-fold; p < *0.001*) in LPS + Salmeterol group when compared to LPS-alone group (Fig. 3D). When we further examined the effect of Salmeterol on the expression of other M2-specific phenotype makers of activated microglia. We found that Salmeterol significantly enhances the expression of arginase-1 and CXCL14 in LPS-activated BV2 microglia (p < *0.001*; Fig. 3E-F). Consistent with IL-10 expression, there was no enhanced expression of arginase-1 and CXCL14 observed in Salmeterol alone treated group.

3.3. Salmeterol-mediated anti-inflammatory effect and phenotypic conversion is specific to activation of β2-AR

Given the evident that Salmeterol is a specific agonist of β2-AR, we sought to examine if other β2-AR agonist shows similar effects to Salmeterol. For these experiments, we used the ultra-long-acting β2-AR agonists Vilanterol and Indacaterol. BV2 cells were pre-treated with Vilanterol and Indacaterol and then stimulated with LPS, and the

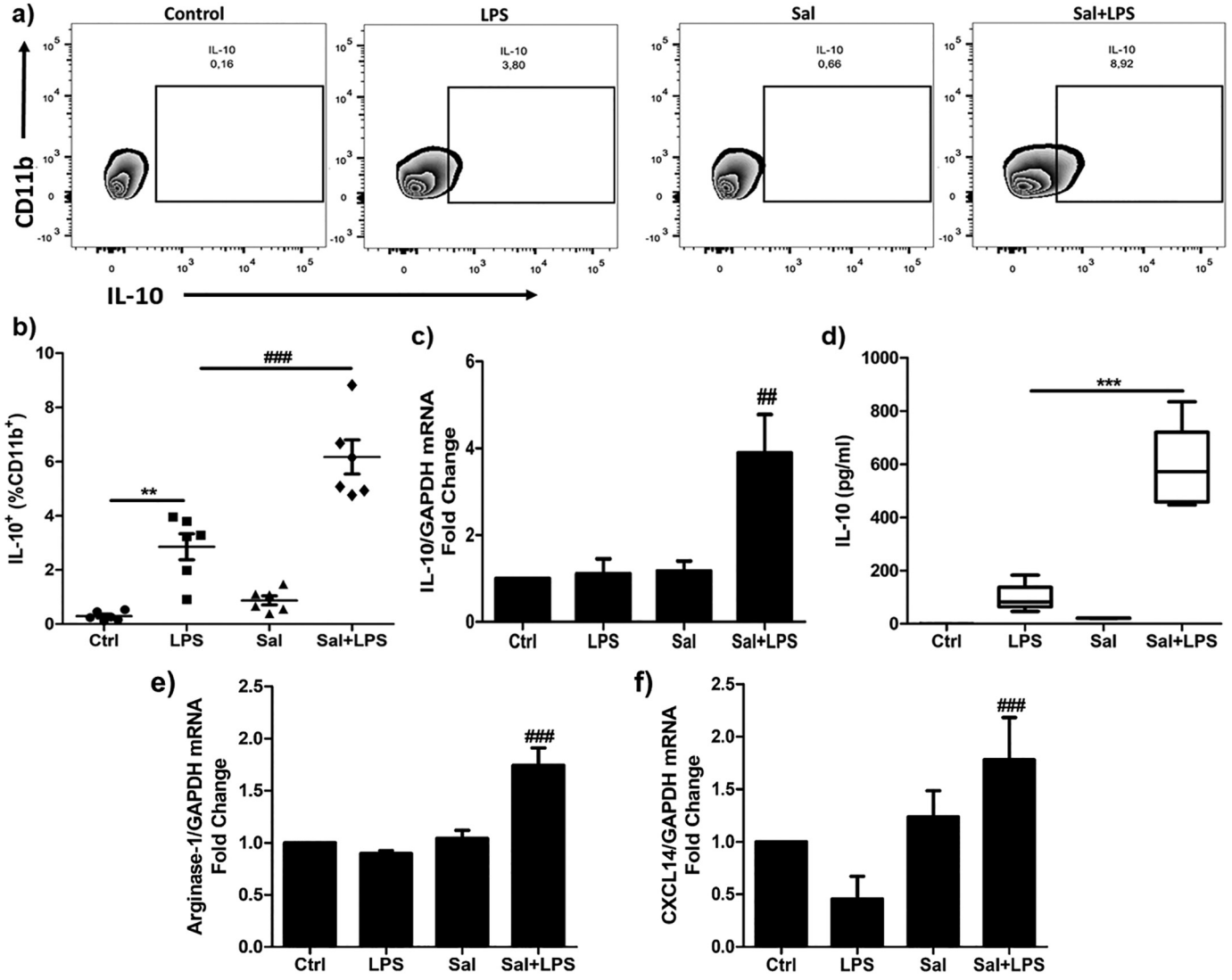

Fig. 3. Treatment with Salmeterol converts immunological phenotype of LPS-stimulated microglia. BV2 cells were treated with Salmeterol (10^{-9} M) for 45 min and then stimulated with *E. coli* LPS (1 μg/ml) and Brefeldin A (10 μg/ml). After 4 h cells were harvested, and intracellular IL-10 expression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

production of inflammatory mediators TNF-α and IL-10 was measured in culture supernatant. Results shows both Vilanterol and Indacaterol significantly suppressed the LPS-induced production of TNF-α and enhanced the LPS-induced production of IL-10 in BV2 microglia ($p < 0.001$; Fig. 4A-B). Furthermore, the blockade of β2-AR with the specific antagonist ICI 118,551HCl reverses the immunoregulatory effects of Salmeterol in LPS-activated BV2 microglial cells (Fig. 4E-F).

3.4. Salmeterol-mediated enhancement of IL-10 is β-arrestin2-independent

After binding with agonist, β2-AR activation leads to two different signaling pathways: the classical or G-protein-dependent pathway and the alternate β-arrestin-dependent or G-protein-independent pathway. Previously, we have found that anti-inflammatory effects of Salmeterol is β-arrestin2-dependent by demonstrating that following the silencing β-arrestin2 by siRNA Salmeterol had no effect on the reduced production of TNF-α in LPS-activated BV2 cells (Sharma and Flood, 2018a). Consequently, we sought to examine whether the increased production of IL-10 is also regulated by β-arrestin2-mediated signaling by transfecting BV2 cells with siRNA against β-arrestin2 and then treating these cells with Salmeterol, followed by stimulated with LPS. Our results indicate that cells exposed to the scrambled siRNA (Si control) showed significant increased production of the IL-10 in LPS + Salmeterol-treated group compared LPS alone ($p < 0.001$), and the silencing of β-arrestin2 with siRNA to *Arrb2* had no effect on the enhancement of IL-10 production in LPS + Salmeterol activated BV2 cells ($p < 0.01$; Fig. 5A). The effect of Si-RNA on the inhibition of β-arrestin2 protein expression was verified by western blot (Fig. 5C).

3.5. Salmeterol-mediated IL-10 enhancement in activated microglia requires CREB activation

It has been shown that the classical G-protein-dependent or cAMP/PKA/CREB signaling pathway is responsible for converting M2

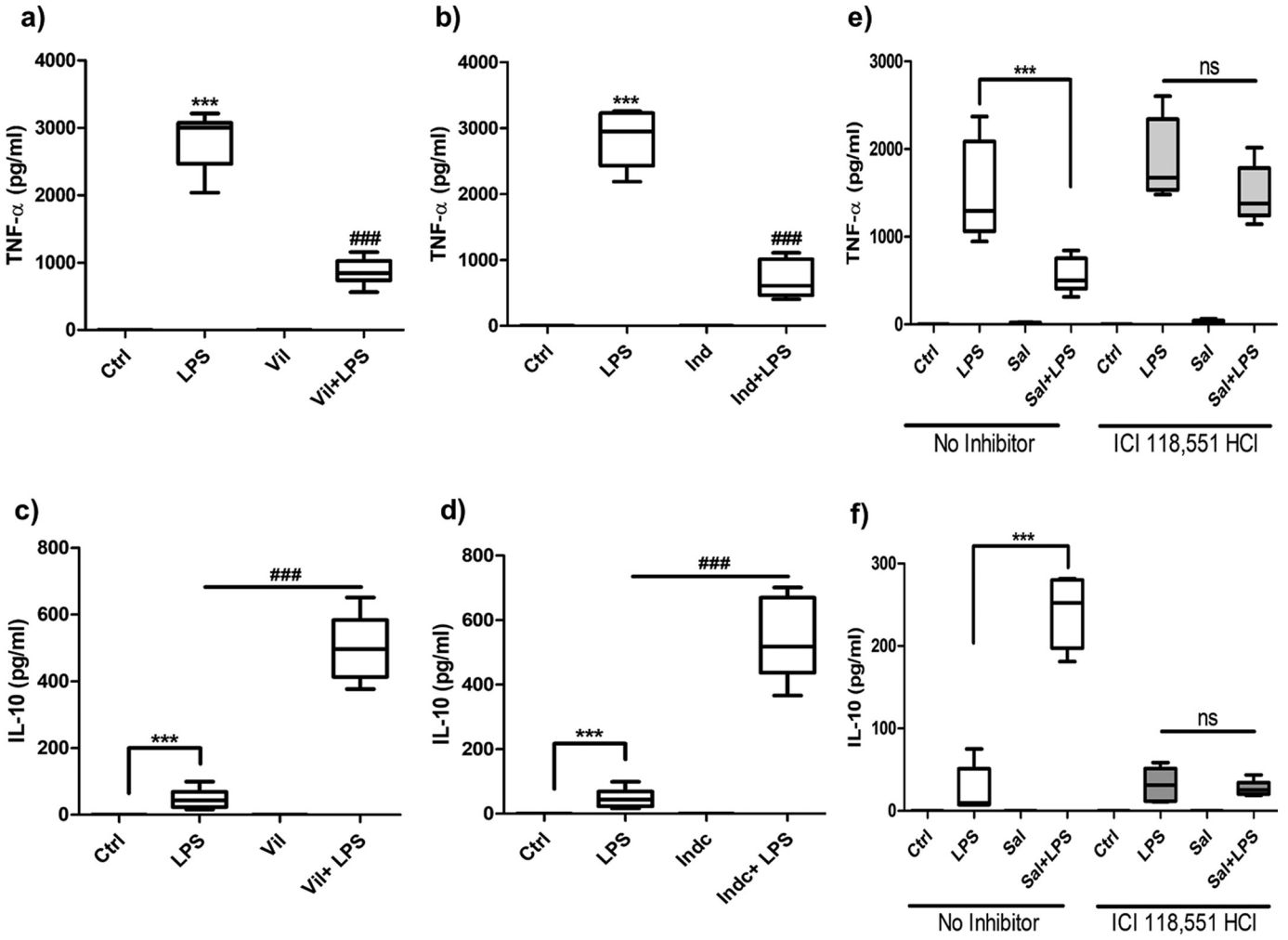

Fig. 4. Inflammatory conversion of microglia by is mediated by β2-AR activation and blocked by β2-AR antagonists. BV2 cells were treated with the ultra-long acting β2-AR specific agonists Vilanterol and Indacaterol (10^{-9} M) for 45 min and further stimulated with *E. coli* LPS (1 μg/ml). Production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

polarization in microglia activated by GPCR such as cannabinoid CB2 receptors (Navarro et al., 2016). (Wen et al., 2010). We therefore first examined if the LPS-dependent Salmeterol-activated enhancement of the production of IL-10 is CREB dependent. To test this, BV2 cells were transfected with siRNA against *CREB* (Si-Creb) and then treated with Salmeterol, followed by stimulated with LPS. The release of IL-10 was measured by ELISA and results indicate that cells exposed to the scrambled siRNA (Si-Control) showed significantly increased production of the IL-10 in LPS + Salmeterol-treated group compared LPS alone ($p < 0.001$). In contrast, BV2 cells transfected with Si-Creb showed a much smaller but still slightly significant production of IL-10 in LPS + Salmeterol treated group ($p < 0.05$, Fig. 5B) when compared with the cells transfected with Si-Control. This shows IL-10 production was significantly reduced by Salmeterol in LPS-activated BV2 cells after the silencing of *CREB* ($p < 0.001$; Fig. 5B) when compared with Si-Control group. Effect of Si-RNA on protein expression of Creb was measured by western blot (Fig. 5D).

Activation of CREB has been shown to involve activation of the PKA signaling pathway, but can also be mediated by activation of MAPK (Delghandi et al., 2005; Naqvi et al., 2014) and PI3K (phosphoinositide 3-kinase) (Vergadi et al., 2017). Therefore, we examined whether LPS + Salmeterol-mediated enhancement of IL-10 production via CREB is mediated by these and/or other signaling molecules. BV2 cells were treated with the indicated pharmacological inhibitors targeting intracellular signaling molecules for 1 h prior to treatment with Salmeterol and LPS. Next, production of IL-10 in culture supernatant was measured. Results shows inhibition of ERK1/2 (extracellular signal-regulated kinase) (by 10 μM of U0126) and JNK (c-Jun N-terminal kinase) (by 10 μM of SP600125) was not capable to affecting the Salmeterol-mediated production of IL-10 (Fig. 6A-B), while p38 MAPK inhibition (via 10 μM of SB203580) significantly inhibited Salmeterol-mediated increased production of IL-10 ($p < 0.001$; Fig. 6C). Likewise, it has been previously shown that PI3K activation is required for M2 activation of macrophages (Vergadi et al., 2017), and we find that treatment with PI3K inhibitor (Wortmannin; 1uM and) also abrogates the Salmeterol-mediated IL-10 production ($p < 0.001$; Fig. 6D). As expected, protein kinase A inhibition (by KT5720; 1 μM) also significantly inhibited IL-10 enhancement in LPS + Salmeterol-activated BV2 cells ($p < 0.001$; Fig. 6E), which further indicates the finding that Salmeterol-mediated IL-10 production is dependent on the classical cAMP/PKA/CREB signaling pathway (Fig. 7).

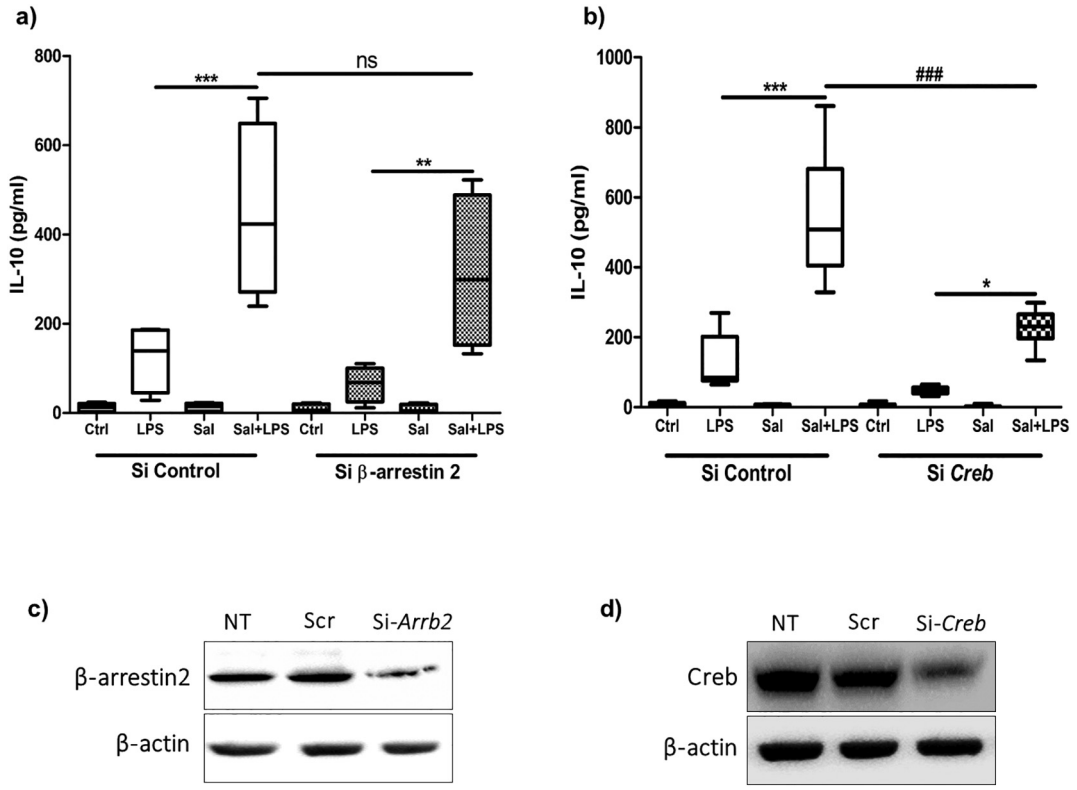

Fig. 5. Sal/β2-AR-mediated enhancement of IL-10 production is dependent on classical GPCR signaling pathway. BV2 microglia were transfected with β-arrestin2 siRNA (Siβ-arrestin2) and scrambled siRNA (SiControl or Scr). In separate experiments BV2 cells were transfected with Creb siRNA (Si-Creb) and scrambled siRNA. After 24 h of transfection cell were treated with Salmeterol and followed by stimulated with LPS. After 6 h cell culture supernatants were collected and production of IL-10 was measured by ELISA. Box plots depict the effect of silencing of β-arrestin2 (A) and Creb (B) on IL-10 production in all experimental groups. Representative western blots (C) and (D) show reduced expression of β-arrestin2 and total Creb protein after transfection. Data represents mean ± SD of independent experiments (n = 4). ***p < 0.001, **p < 0.01 and *p < 0.05 indicates significant difference from LPS-treated group and ###p < 0.001 indicates significant difference between SiControl and Siβ-arrestin2 and Si-Creb experimental groups. ns- not significant.

4. Discussion

Inflammatory processes in the brain are mainly regulated by microglia and these microglial cells acquire different activation states in order to perform their molecular and cellular functions. Once activated, the M1 microglia responds to the toxic stimuli and promote inflammation. On the other hand, the M2 microglia secrete anti-inflammatory mediators and promote repair and tissue homeostasis (Ransohoff, 2016b; Tang and Le, 2016). Our results indicate treatment of LPS-activated microglia with Salmeterol suppresses M1-type activation and promotes M2-type activation of microglia. Previously, we have studied the neuroprotective and anti-inflammatory properties of β2-AR in MPTP and LPS model of PD but the underlying mechanism is still not completely understood (Qian et al., 2011; Sharma and Flood, 2018a). Here, we find that the β2-AR agonist Salmeterol significantly inhibits the cytokine/chemokine expression such as TNF-α, IL-1β, IL-6, IL-18, IP-10 and MCP-1 (Figs. 1 and 2), as well as other M1- phenotypic markers such as iNOS, reactive oxygen species, and even COX2 (cyclooxygenase-2, data not shown). These pro-inflammatory mediators have been shown to play crucial role in neuroinflammation and progression of PD and other neurodegenerative diseases (Chhor et al., 2013; Lehnardt, 2010; Nagatsu et al., 2000; Nolan et al., 2013). On the other hand, we found that Salmeterol significantly enhances the production of the anti-inflammatory cytokine IL-10 suggesting that its mode of action is not only to inhibit the pro-inflammatory phenotype, but to induce the production of anti-inflammatory cytokines that may help to control the continued destruction of dopaminergic neurons by other neighboring microglial cells.

The importance of inflammation in the destruction of dopaminergic neurons in PD has long been suggested (Sharma et al., 2016; Flood et al., 2011; Sharma and Flood, 2018b). Elevated levels of pro-inflammatory mediators have been found in blood and cerebrospinal fluid of PD patients (Blum-Degen et al., 1995; Starhof et al., 2018). The increased production of pro-inflammatory mediators have been associated with several neurodegenerative conditions including PD (Alam et al., 2016; Sharma et al., 2016; Tansey and Goldberg, 2010). A number of studies have shown an association between the use of NSAIDs and risk of Parkinson's disease (Chen et al., 2005; Etminan et al., 2008; Etminan and Suissa, 2006). However, it has been suggested that non-specific shut-down of inflammation may not have sufficient beneficial effects on the disease pathogenesis (Peña-Altamira et al., 2016). Therefore, suppressing neuroinflammation and toxicity is an important therapeutic approach but strengthening the neuroprotective and restorative properties of microglia may also be required to halt the disease progression. Results from our current study also address this hypothesis.

One of the important features of our findings is that Salmeterol not only suppresses neurotoxic phenotype of microglia (M1) via inhibiting TNF-α, IL-6, IL-1β, IL-18, CCL2, CCL3, CCL4, CXCL10, nitric oxide and ROS production, but also elicits anti-inflammatory effects by inducing IL-10 production and promoting the microglial conversion from the M1- to the M2-like phenotype (Figs. 1 and 2). It has been found that M1 cytokines and chemokines play a major role in neurodegenerative diseases. For example, in addition to the known neuropathic effects of TNF-α (Ferger et al., 2004; Rousselet et al., 2002; Sriram et al., 2002) and IL-1β (Tanaka et al., 2013), the higher expression of CCL2/MCP-1 exacerbates the chronic inflammation in many neurodegenerative conditions (Sawyer et al., 2014). Increased levels of chemokines

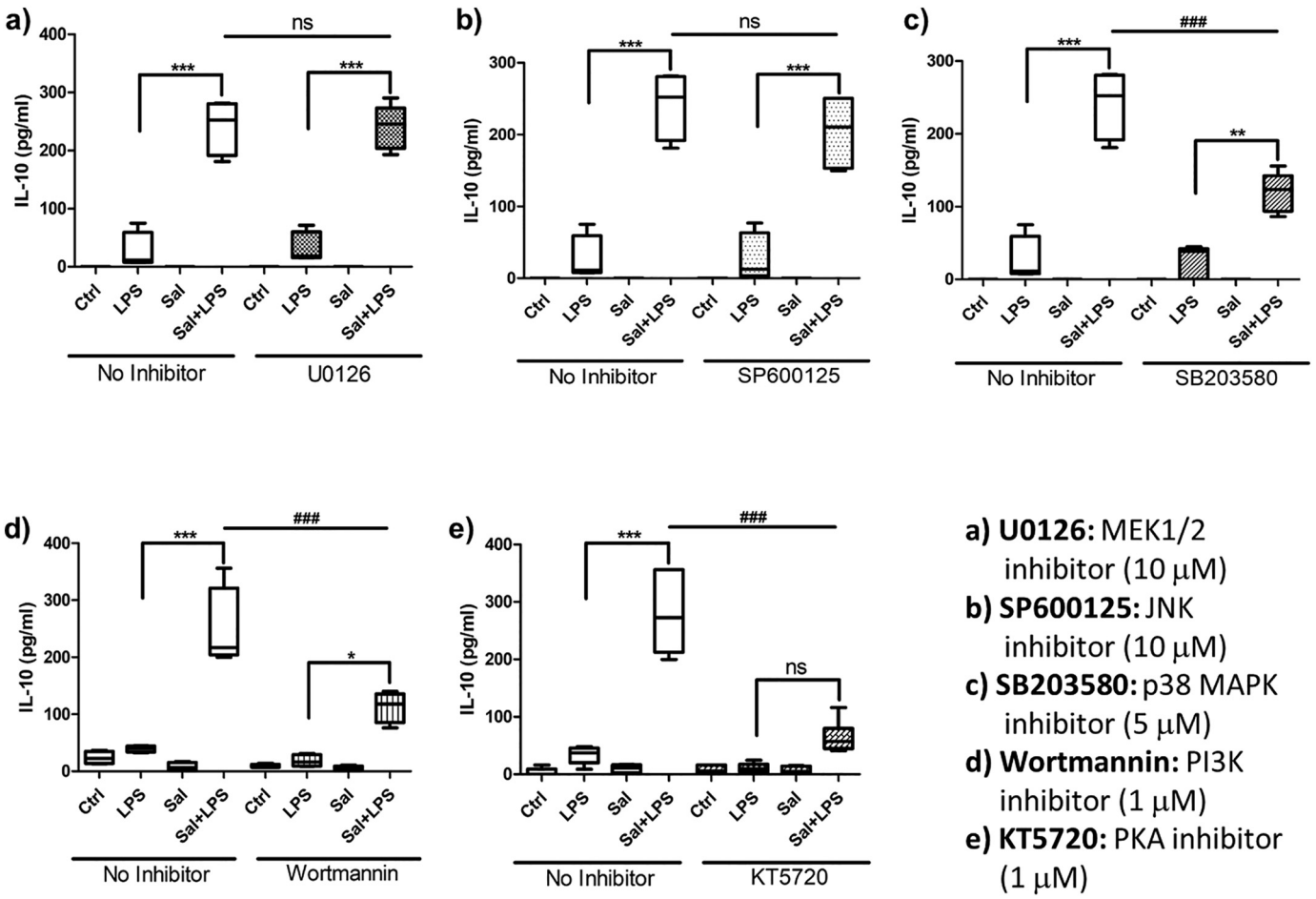

Fig. 6. Effects of blockade of various signaling pathways on Salmeterol-mediated production of IL-10. BV2 cells were incubated with inhibitors indicated for 1 h prior. Then, cells were treated with Salmeterol (10^{-9} M) for 45 min and further stimulated with *E. coli* LPS (1 μg/ml). Production of IL-10 culture supernatant was measured by ELISA. (A-E) Box plots represent the IL-10 production by Salmeterol in presence and absence of pharmacological inhibitors of indicated signaling molecules. Data represents mean ± SD of independent experiments ($n = 5$). ***p < 0.001 and **p < 0.01 indicates significant difference between LPS-treated and LPS + Salmeterol-treated group. ###p < 0.001 indicates significant difference between experimental groups treated with inhibitors and no inhibitors. ns- not significant.

including MCP-1, CCL3, CCL4 and CXCL10 have been associated with depressive symptoms and cognitive impairment in PD patients (Lindqvist et al., 2013; Rocha et al., 2014). Salmeterol also reduces the expression of IL-18 which contributes to dopaminergic neurodegeneration (Sugama et al., 2004) and several other inflammation-related disorders (Pawlik et al., 2006; Yu et al., 2009).

Conversely, we find that β2-AR agonists Salmeterol enhances the expression and production of IL-10 in LPS-activated BV2 microglia and also enhances the expression of M2 markers arginase-1 and CXCL14 (Fig. 3). IL-10 has been found as an anti-inflammatory and neurotrophic factor which helps in tissue repair and homeostasis process (Burmeister and Marriott, 2018; Lobo-Silva et al., 2016). Previously, we have shown neuroprotective effects of anti-inflammatory cytokine (such as IL-10 and TGF-β) therapies in PD models (Qian et al., 2006a; Qian et al., 2006b; Qian et al., 2008). IL-10 is also known to protect LPS-induced dopaminergic neurodegeneration in SN and mesencephalic culture (Zhu et al., 2015). Similarly, IL-10 also suppresses programmed-cell death in ventral mesencephalic neurons via JAK-STAT3 pathway (Zhu et al., 2017). IL-10 has been shown to prevent glutamate-induced excitotoxicity in brain ischemia. Protective and immunoregulatory effects of IL-10 have been explained in gut inflammation and also in cancer (Mannino et al., 2015), infection (Peñaloza et al., 2016), autoimmune diseases (Dambuza et al., 2017), and neurodegenerative diseases (Burmeister and Marriott, 2018; Lobo-Silva et al., 2016). These findings give further insight to the idea that neuroprotective role of β2-AR agonists are not only due to the suppression of pro-inflammatory mediators but also due to the conversion of microglia from pro- to anti-inflammatory-like phenotype.

Similar to our findings, activation of β2-AR in murine macrophages promotes an M2-like phenotype and shows protection against endotoxemia and acute lung injury (Grailer et al., 2014). Furthermore, β2-AR activation by NE in macrophages robustly enhances the IL-10 production in vitro and in vivo, and consequently, *ADRB2* knockout mice were more susceptible to infection and LPS challenge (Ağaç et al., 2018). Transcriptome analysis of β2-AR-activated macrophages showed transcriptome with up-regulation of M2-spectrum gene expression which is regulated by CREB, C/EBPβ (CCAAT-enhancer-binding protein-beta) and ATF (activating transcription factor) transcription factors (Lamkin et al., 2016). However, we are able to define β2-AR-stimulated microglia only as M2-like microglia because classically defined M2 macrophages are activated by IL-4 and the transcription factors involved in the M2 phenotype are different than β2-AR-related transcription factors (Hayakawa et al., 2016; Lamkin et al., 2016). Further studies need to be done to determine the exact nature of the conversion of activated microglia to the M2-like phenotype we see in BV-2 cells.

Early degeneration of locus coeruleus (LC) adrenergic neurons and their circuitry to substantia nigra have been found in PD patients, suggested a role for the noradrenergic system in the neuropathology of PD (Weinshenker, 2018). Additionally, pharmacological approaches to mimic the effects of noradrenaline exhibit neuroprotective effects

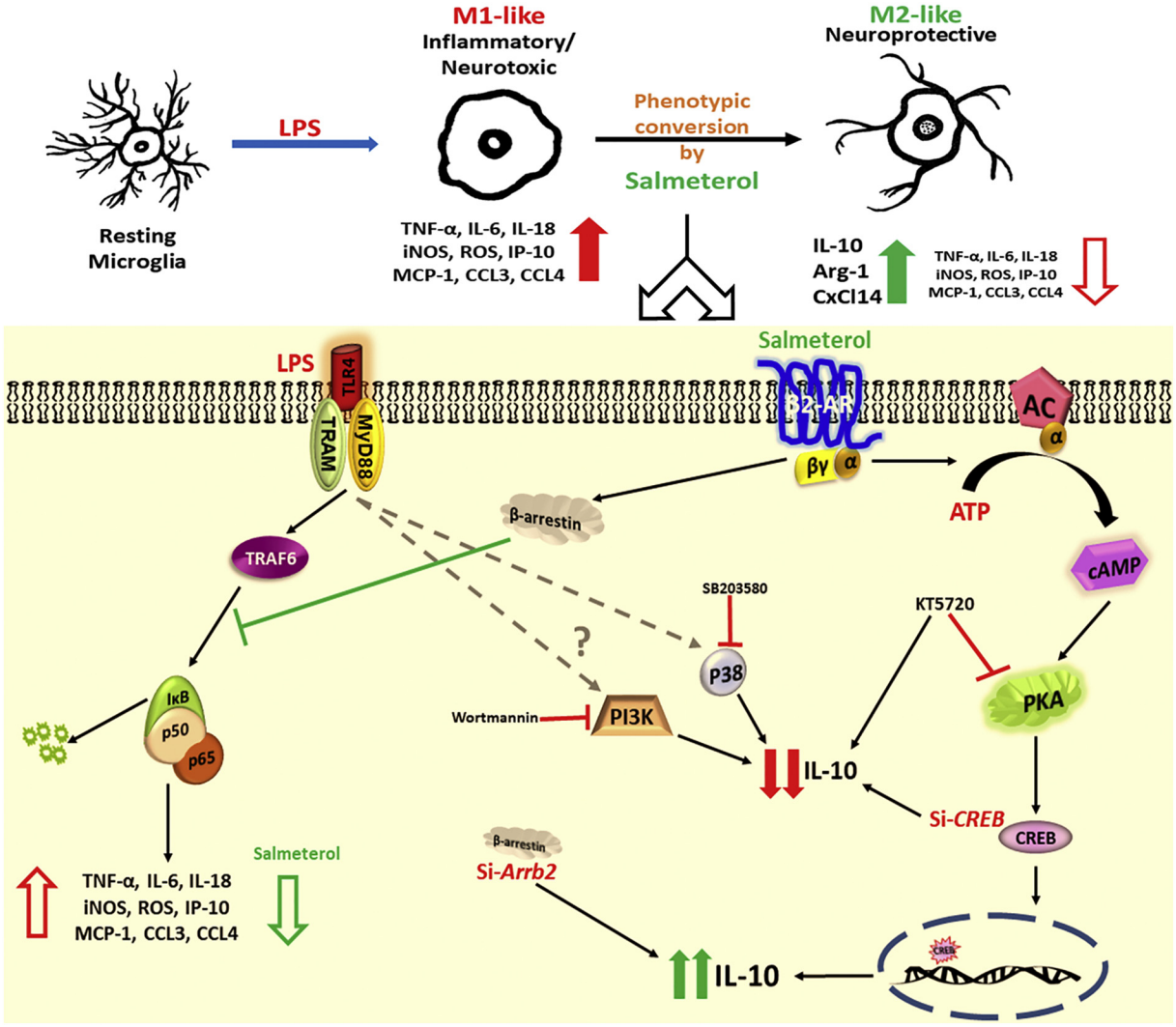

Fig. 7. Schematic diagram showing immunomodulatory effect of Salmeterol on inflammatory conversion of LPS-stimulated BV2 cells. Resting microglia, once activated with endotoxin, expresses an M1-like phenotype which is neurotoxic. These M1-like activated microglia robustly enhance production of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-18, MCP-1, CCL3, CCL4, ROS and CXCL10/IP-10). In contrast, treatment of LPS-activated microglia with Salmeterol changes their phenotype to an M2-like phenotype which is characterized by the inhibition of pro-inflammatory cytokines and the enhancement of anti-inflammatory cytokines and chemokines IL-10, arginase-1, and CXCL14. Stimulation of microglia with LPS triggers TLR4 signaling pathway, leading to the activation of NF-κB microglia, which further enhances the production of pro-inflammatory cytokines and chemokines. In contrast, treatment of LPS-activated microglia with Salmeterol also increases the production of anti-inflammatory cytokine IL-10. Inhibition of classical signaling (cAMP/PKA/CREB) pathway of β2-AR via silencing *CREB* by siRNA and inhibiting protein kinase A (PKA) abrogates Salmeterol-mediated production of IL-10. Similarly, blockade of PI3K and p38 also inhibit the Salmeterol mediated IL-10 enhancement. In contrast, while inhibition of the M1-like phenotype by Salmeterol is accomplished through activation of β-arrestin2 (Sharma and Flood, 2018a), silencing of β-arrestin2 via siRNA does not affect the Salmeterol-mediated production of IL-10.

(Cacabelos, 2017; Delaville et al., 2011). For example, it has been found that activation of β2-AR via NE leads to suppressed microglial activation and to the production of growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor-1 (NGF-1) by astrocytes (Culmsee et al., 1999; Gao et al., 2016), thereby providing neuroprotective and also limit the cytotoxicity of DA-neurons (Gleeson et al., 2010; Zorec et al., 2018). In addition, since β2-ARs belong to the seven transmembrane G-protein-coupled receptor superfamily, the engagement of β2-ARs with their agonists initiates either the classical G-protein-dependent or the alternate β-arrestin-dependent signaling pathway, both of which can result in immunomodulatory actions (Shenoy and Lefkowitz, 2011; Shirshev, 2011).

Consequently, we have further investigated the molecular mechanism of the immunoregulatory effects of Salmeterol. In a recent study we have shown that the anti-inflammatory effects or regulation of production of pro-inflammatory mediators by Salmeterol is dependent on the alternative/β-arrestin-dependent pathway of G-protein coupled signaling. Silencing of β-arrestin2 by using siRNA against *Arrb2* gene reverses the inhibition of TNF-α and IL-1β production by Salmeterol (Sharma and Flood, 2018a). In this study we show that silencing of *Arrb2* gene did not alter the enhanced IL-10 production by Salmeterol (Fig. 5A). Rather, in our results blockade of PKA by pharmacological inhibitor reduces the Salmeterol-mediated production of IL-10, so it appears that cAMP/PKA activation leading to CREB activation through the classical pathway of G-protein coupled activation leads to IL-10 enhancement. Similar mechanisms have been suggested by Ghosh et al. where they have found that treatment with cAMP in combination with Th2 cytokine (IL-4) promoted microglia polarization towards to the M2 phenotype (Ghosh et al., 2016) and this M2 conversion requires the activation of PKA. In addition to that, Salmeterol also suppress LPS-induced systemic inflammation via inhibition of NLRP3 inflammasome and this anti-inflammatory effect of Salmeterol is dependent on both classical GPCR/cAMP pathway as well as β-arrestin2 pathway (Song et al., 2018). Collectively, data from our previous study (Sharma and

Flood, 2018a) and this study suggest that the suppression of pro-inflammatory phenotype (M1) of microglia is dependent on β-arrestin pathway while enhancement of anti-inflammatory-like phenotype (M2) requires the activation of the classical cAMP/PKA/CREB pathway of GPCR signaling.

Another interesting finding of our study is Salmeterol alone did not enhance the IL-10 production in resting microglia and only induce IL-10 production in LPS-activated microglia. Activation of TLR pathway not only activates the NF-κB pathway but also activates MAPK pathway. Blockade of ERK1/2 and JNK MAPK did not affect the Salmeterol-mediated IL-10 production but blockade of p38 and PI3K reverse the enhanced production of IL-10 by Salmeterol. It is known that p38 and PI3K pathway play a role in the regulation of the production of IL-10 in macrophages (Grailer et al., 2014; Li et al., 2014). The PI3K pathway also regulates macrophage activation and their M1/M2 polarization. Therefore, it appears that IL-10 enhancement by Salmeterol involves both the PI3K/p38 pathway as well as the PKA pathway, both of which appear to be needed to get optimal activation of CREB leading to IL-10 production.

We have yet to determine if the immune-conversion effects are the major therapeutic benefits of b2-AR agonists in the treatment of dopaminergic neurodegeneration. Interestingly, β2-AR activation has also been linked with the inhibition of transcription of α-synuclein in animals and in vitro models of PD. In this study, it was shown that β2-AR agonists Clenbuterol and Salbutamol suppresses the expression of *SNCA* gene via histone-3-lysine-27 acetylation of its promoter and enhancer region. Interestingly, it was also found that patients on Salbutamol, had a reduced risk of developing PD (Mittal et al., 2017). In contrast, patients using a β2-AR antagonist Propranolol were found to be at increased risk of developing PD (Mittal et al., 2017). A similar study by Gronich et al. also shows that within a large cohort of the Israeli population, the use of various β2-agonists was associated with reduced risk of PD while patients treated with Propranolol appear to have higher risk of developing PD (Gronich et al., 2018). Interestingly, in a mouse model of Alzheimer's disease, investigators showed that the β2-AR activation enhances hippocampal neurogenesis, ameliorates memory deficits, and increases dendritic branching and spine density (Chai et al., 2016). Taken together, we suggest that β2-AR agonist may have therapeutic benefits against neurodegeneration via: i) suppression of pro-inflammatory mediator production and conversion of microglia activation from neurotoxic/M1- phenotype to neuroprotective/M2-like phenotype, ii) promoting the release of neurotrophic factors from glial cells, iii) regulating the gene and protein expression of SNCA and iv) promoting restoration of neurons.

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Authors contributions

MS performed the experimentation, and MS, NA and PMF analyzed the data, interpreted the results, and wrote the paper.

Ethics approval

Animal or human samples were not used in this study.

Competing interest

Authors declare no conflict of interest in these studies.

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