Master's Thesis

석사 학위논문

TSPO mediates neuroinflammation via Parkin and p62 regulation in microglia

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by

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Science. The study was conducted in accordance with Code of Research Ethics[1)].

12. 04. 2015
Approved by
Professor Seong-Woon Yu (Signature)
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Professor Ji-Woong Choi (Signature)
(Co-Advisor)

1) Declaration of Ethical Conduct in Research**:** I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

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12. 04. 2015

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Abstract

Neuroinflammation has relevance to many neurodegenerative diseases. Although certain aspects of inflammatory responses have beneficial effects, uncontrolled inflammation may impair the maintenance of homeostasis and exacerbate disease states.

Microglia, the major resident brain immune cells, are mainly associated with neuroinflammatory responses in the central nervous system. Under normal condition, deactivated microglia produce anti-inflammatory and neurotrophic factors. However, under exposure of invaded pathogen or tissue damage, microglia are activated and promote a pro-inflammatory response.

The translocator protein 18 kDa (TSPO) is a five transmembrane protein localized in the outer mitochondrial membrane. The expression level of TSPO is increased by neuroinflammation in activated microglia and astrocytes. Although there is a close correlation between a high expression level of TSPO and neuroinflammation, it is not known how TSPO is involved in the regulation of neuroinflammation and microglia activation.

To reveal the role of TSPO in the regulation of inflammation and the related mechanisms through which TSPO regulates neuroinflammation, we examined the effects of two TSPO ligands, PK11195 and Ro5-4864, and TSPO overexpression on the signaling molecules important in mediation of microglia activation. Lipopolysaccharide (LPS) treatment induced a robust increase in the production of inflammatory factors, such as nitric oxide ($NO^{)}$, tumor necrosis factors (TNF) -α and interlukin (IL) -6. TSPO overexpression significantly attenuated the production of pro-inflammatory cytokines. TSPO ligands inhibited mitogen-activated protein kinases (MAPK) pathway, especially Jun N-terminal kinase (JNK) and p38, but not Erk. In addition, TSPO overexpression or pretreatment with TSPO ligands reduced LPS-induced NF-κB transcriptional activity. These

results demonstrate that TSPO negatively regulates neruroinflammation and microglia activation through the suppression of NF-κB and MAPK signaling pathways.

Recently, the role and mechanisms of parkin in the regulation of inflammation has been reported. Because Parkin was recruited to mitochondria following LPS treatment, we confirmed the relationship between TSPO and Parkin in inflammation in microglia. Although Parkin has anti-inflammatory effects, TSPO knockdown blocked the inhibitory effects of Parkin against the production of pro-inflammatory cytokines. Futhermore, TSPO and Parkin suppressed inflammation via autophagy. However, TSPO knock-down increased p62 aggregation that reduced by Parkin.

This study was aimed to investigate whether TSPO negatively regulates neuroinflammation and microglia activation via the regulation of parkin and p62 in microglia. The results obtained from this study will unveil the regulatory mechanisms of TSPO in neuroinflammation, thereby contribute to the anti-inflammatory therapeutic design for the treatment of neurodegernative diseases.

Keywords: Neuroinflammation, Microglia, TSPO, Parkin, p62

Contents

List of figures

1. Introduction

1.1 Microglia

1.1.1Origin and distribution

In 1919, Rio Hortega initially identified microglia of the mesodermal extracerebral origin [1]. Microglia are the resident macrophages specialized in the central nervous system (CNS). Microglia are mainly populated in gray matter, especially, in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra [2]. Microglia comprise 5-20 % population of gila cells depending on the resident regions in CNS [3].

Microglia are distinguished from other glial cells, such as astrocytes and oligodendrocytes by their origin. Previously, microglia was known as the cells derived from the bone marrow pregenitors, similar to macrophages and motocytes. The recent study demonstrated that microglia originate from primitive myeloid progenitors that move to CNS before embryonic day 8 and primitive myeloid progenitors maintain postnatal microglial homeostasis in the healthy brain, independently of circulating monocytes throughout life [4]. Another studies showed that microglia locally increase through self-renewing and they are unusually substituted by bone marrow-derived cells after stimulation, but these cells can not contribute to microglia pool [5].

1.1.2 Microglia morphology and activation

Microglia have the plasticity accompanied bymorphological changes for different cellular functions in responses to changes in their environments. In the healthy condition, microglia of the resting state present ramified forms with the feature of small soma and long branching cellular processes. The ramified microglia survey the surrounding area using their branches. Unlike ameboid microglia of activated state, ramified microglia do not have ability of phagocytosis. Upon changes in surrounding physiological and environmental conditions, microglia transform from ramified to amoeboid forms. The amoeboid microglia present large soma and shortened cellular branches. The amoeboid microglia move freely throughout the neural tissue, and function as scavenger cells trough their ability of phagocytosis [6]. Activated microglia undergo two steps that involve non-phagocytotic and phagocytotic microglia states. Once activated, microglia carry out morphological changes to non-phagocytotic microglia with thickened and rod-like cellular morphology after retraction of their branches. These types of microglia are able to exert common immunomodulatory activity. Activated phagocytic

microglia migrate to the site of the injury, phagocytose foreign materials, and secrete pro-inflammatory factors to promote proliferation of microglia pool. Activated phagocytic microglia also interact with astrocytes and neural cells to prevent the healthy brain cells [7-8]. Interestingly, microglia in vitro showed the heterogeneous shapes that feature rod or amoeboid shape with short and thick branches. In spite of this morphological heterogeneity, they perform normal function when stimulated [9].

Normally, the activation of microglia have two different states, classical activation (M1 type activation states) and alternative activation (M2 type activation states). These two types of activation occur by different stimulators. The classical activation state is typically triggered by toll-like receptor 4 (TLR4) agonist, especially lipopolysaccaride (LPS), tumor necrosis factors (TNF), and interferon-γ (IFN-γ). In contrast, the alternative activation state is induced by interlukin (IL) 4 and 13. The abnormal chronic classical activation by microglia is associated with the progression of diseases, whereas the alternative activation is protective processes [4].

The resting microglia transform to activated form after detection of any signs of tissue injury or presence of foreign materials. Activated microglia turn on inflammatory responses including the expression of major histocompatibility complex (MHC) class II and the production of pro-inflammatory cytokines. These inflammatory responses stimulate adaptive immune responses by activation of T cells. In addition, the pro-inflammatory factors from microglia also activate microglia and astrocytes. Activated astrocytes release the cytokines, such as colony-stimulating factor 1 (CSF1) and TNF-α, and theses cytokines trigger the activation and proliferation of microglia. Theses crosstalk between microglia and astrocytes help to amplify inflammatory responses in the CNS, but chronic inflammation causes the pathology of neurodegenerative diseases [4].

1.2 Neuroinflammation

1.2.1 Microglia in neuroinflammation

The immune system is important to maintain the homeostasis of our body from infection and injury. Microglia are the key players of the immune system in the CNS [10]. Microglia of the resting state survey their surrounding environment and are activated by responses of any changes, and they initiate immune responses, such as inflammation and tissue repair for maintenance of the healthy condition. In addition, the factors produced from microglia induce activation of astrocytes and microglia themselves amplifyng immune responses rapidly. In spite of the beneficial effects of inflammation by mciroglia in the CNS, sustained inflammation

results in the abnormal production of inflammatory factors, and these factors stimulate excessive immune responses as danger signs. These uncontrolled inflammation brings about the production of neurotoxic factors and exacerbates the neurodegeneration.

1.2.2 Neuroinflammation in neurodegenerative disease

- Alzheimer's Diseases (AD)

Several studies suggested the involvement of neuroinflammation by microglia in AD. Microglia were activated by aggregates of amyloid-β (Aβ) and produced the neurotoxic factors including pro-inflammatory cytokines that induce neuronal death [11]. In addition, the inflammatory mediators triggered more production of Aβ in neurons, and amplified inflammation by Aβ-activated microglia. These crosstalk between neurons and microglia deteriorates the disease states.

The recent work reported that mice with conditional deletion of microglia did not increase amyloid plaque formation and neuronal damage compared to control mice [12]. In contrast, another study showed that administration of the growth factor, M-CSF, in APP/PS1 transgenic mice increased micoglia population and decreased Aβ deposits, and cognitive loss [13].

- Parkinson's Diseases (PD)

The loss of dopaminergic neurons is a major factor to promote the pathology of PD. In recent years, the evidences that microglia activation triggers neuronal cell death have been reported. Activated microglia were found in the substantia nigra of PD patients [14], and the expression levels of TSPO that is highly expressed in glia cells also increased in PD patients [15]. In addition, several studies reported that the inflammatory responses by microglia caused loss of dopaminergic neurons in LPS injected model [16-17]. Another study showed that α-synuclein also activated microglia. Microglia activated by α-synuclein produced NADPH oxidase-mediated reactive oxygen species (ROS) [18]. Microglia activation by nitrated α-synuclein was associated with nuclear factor-kappa B (NF-κB) related signaling pathway and promoted expression of neurotrophins [19].

The recent study suggested that nitrated α-synuclein activated not only microglia in the CNS but also macrophages and T cells in peripheral regions. The adaptive immune system also induces the loss of dopaminergic neurons in PD models. The nitrated α-synuclein stimulates macrophages and T cells in

lymphatics, and activated T cells promotes neurodegeneration [20].

1.3 TSPO

1.3.1 Historical perspective and recent controversial topics

Translocator protein 18 kDa (TSPO) was previously known as Peripheral Benzodiazepine Receptors (PBR). In 1977, it was initially described as a specific benzodiazepine receptor that binds diazepam with high affinity in the perpheral nervous system (PNS). It was distinct from the central nervous system benzodiaepine receptor (CBR : the gamma-amionobutyric acid type A ($GABA_A$) receptor) [21]. In 2006, Papadopoulos and other ssuggested that PBR did not reflect its all properties and function. Thus, they renamed PBR as TSPO depending on its structure and molecular function. They proposed new nomenclature by the following reasons with the name 'PBR' : (i) the term 'benzodiazepine' is too limited because TSPO has several endogenous and synthetic ligands. (ii) the term 'peripheral' is not correct because the localization of TSPO in the body is not limited in peripheral regions. TSPO is also highly expressed in the CNS. (iii) 'a receptor' is not enough to explain the function of TSPO [22]

Although there were early attempts to develop TSPO null mice in the 1990s , the absence of functional TSPO reportedly caused embryonic lethality [23]. In recent years, however, the generation of TSPO -/- mice triggered heated controversy about the function of TSPO, especially in the regulation of steroidogenesis and mitochondrial permeability transition pore (MPTP) [24-25]. Recent works demonstrated that TSPO does not regulate steroidogenesis. They generated the conditional knockout mice with lack of TSPO genes in Leydig cells. In contrast to the previous studies, the deletion of TSPO gene did not have any effects about testosteron production, gametogenesis and reproduction in these mice [26]. Subsequently, a follow-up study showed TSPO global knockout mice also had no effects on adrenal and gonadal steroidogenesis. In addition, TSPO global null mice had normal viability without phenotypic defects and abnormalities of ferility [27]. Another study also reported that global C57BL/6-Tspotm1GuWu (GuwiyangWurra)-knockout mice showed normal growth, lifespan, and cholesterol transport [28]. Interestingly, PK11195 (PK), a TSPO ligands, up-regulated the steroidogenesis in TSPO knockout MA-10 cells [29]. Taken together, these recent findings are strikingly opposite to the previous assumed pivotal role of TSPO in mediation of steroidogenesis.

Another recent study further fueled controversy. The conditional TSPO knockout mice with elimination of

Tspo gene in liver and heart showed that TSPO was not associated with the structure and regulation of MPTP. In addition, TSPO ligands were able to regulate MPTP without functional TSPO [30].

1.3.2 Structure and distribution

TSPO is a five transmembrane domain protein localized in the outer mitochondrial membrane (Fig. 1A) [31]. TSPO consists of the functional complex with voltage-dependent anion channel (VDAC), adeninneucleotide transporter (ANT) [32], peripheral benzodiazepine receptor-associated protein 1 (PRAX-1), and PBR-associated protein (PAP7) [33] in steroid synthesizing cells (Fig. 1B).

The molecular structure of TSPO was initially reported from the homologue gene of *Rhodobacter sphaeroides*. TSPO structures from *R.sphaeroides* and human are conserved with 33.5% identity inthe aligned amino acid sequences (Fig. 1C). The structure of TSPO comprises five transmembrane α helices that can form a homodimer [34]. Recently, a human single nucleotide polymorphism (A147T) in TSPO amino acid sequences was reported. This mutations resulted in diminished cholesterol metabolism, reduction of ligand binding affinity and increased vulnerability of neurological diseases [35].

TSPO is widely distributed throughout our body, especially abundant in the tissues related to steroide synthesis. In the CNS, TSPO is highly expressed in glial cells, such as microglia and reactive astrocytes, and some of neuronal cells [36]. The subcellular localization of TSPO is mainly in the outer mitochondrial membrane, in particular inner mitochondrial membrane and outer mitochondrial membrane contact sites. However, TSPO can have various subcellular distribution depending on cell types. TSPO was observed in the nuclear, perinuclear area, the plasma membrane, and other organelle membranes [37-41].

1.3.3 Function

TSPO is important for tissue development, function and survival and is highly conserved through evolution. TSPO has many function for homeostasis in our body. The most studied function of TSPO is steroidogenesis. Especially, TSPO regulates the translocation of cholesterol from the outer mitochondrial membrane to inner mitochondrial membrane [42-43]. In earlier days, because the notion that the lack of functional TSPO in mice caused embryonic lethality was well known, the pharmacological study has been widely used to reveal the role of TSPO. However, the generation of new TSPO -/- mice demonstrated that TSPO -/- mice were generated without developmental defects, and therefore, TSPO is not involved in the regulation of

steroidogenesis. In TSPO-/- mice, steroid hormone was normally produced.

Because TSPO is localized in the outer mitochondrial membrane, it mediates various mitochondrial function, such as protein import, heme biosynthesis, ion transport, cellular respiration, apoptosis, proliferation, differentiation, and immunomodulation [44-46]. In addition, the recent works reported the novel function of TSPO in the mitochondira quality control through the regulation of mitophagy. TSPO suppressed mitophagy by PTEN induced putative kinase 1 (PINK1)-Parkin pathway depending on VDAC1 [47].

1.3.4 Ligands

Over the past two decades, the various endogenous and synthetic TSPO ligands were reported. Cholelstrol (Fig. 2A) and porphyrins are well-known endogenous TSPO ligands. The endozepines which is a neuropeptide family synthesized by glial cells in the CNS are also important endogenous ligands of TSPO. Choleterol mainly binds to conserved CRAC domain of the cytosolic C-terminus in TSPO. However, most of ligands of TSPO bind to N-terminus of TSPO. In addition, the components of TSPO complex, VDAC, helps to strong binding of TSPO ligands.

The several synthetic ligands of TSPO have been developed. PK (Fig. 2B) and benzodiazepine 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864; Ro, Fig. 2C) are the most famous synthetic TSPO liands. PK binds solely to TSPO with high affinity. On the other hands, Ro binds to TSPO with other TSPO complex [44].

Because TSPO expression levels are up-regulated in neuroinflammation, TSPO lignads have been used as a diagnostic tool, such as positron emission tomography (PET) imaging, to measure neuroinflammation in many neurodegenerative diseases [48]. Futhermore, the therapeutic effects by TSPO ligands in neurodegenerative diseases also have been suggested. Barron, et al. showed that the TSPO ligand, Ro decreased the progression of neurophatology, gliosis, and behavioral impairment in 3xTgAD mice models. In addition, coadministration of PK and Ro reduced the production of soluble amyloid-β in non-Tg mice [49]. The previous study from our lab also showed that TSPO ligands, PK and Ro, signifficantly suppressed neuroinflammation in microglia and attenuated neurotoxicity by activated microglia and increased the viability of MN9D cells [50].

1.3.5 TSPO in neuroinflammation and neurodegenerative disease

Chronic neuroinflammation is a risk factor for progression of neurodegenerative diseases. Both microglia

and astrocytes trigger neuroinflammation in neurodegenerative diseases. In most neurodegenerative diseases, a remarkable increase of TSPO expression in microglia was shown at the sites of degenerative changes, and the level of TSPO expression returned to the normal level after recovering from tissue damages [51-56]. Thus, TSPO is considered to be associated with neuroinflammation and regenerative processes.

In the periperal region, the specific TSPO ligands, PK and Ro, prevented microglia activation and production of proinflammatory factors [57]. Another TSPO ligand, etifoxine also regulated macrophage activation and the production of pro-inflammatory cytokines [58].

In the CNS, a TSPO ligand, PK effectively suppressed microglia activation and the production of pro-inflammatory cytokines and the expression of inducible nitric oxide sythase (iNOS) in the rat striatum following the administration of quinolinic acid (QUIN) [59]. In addition, PK reduced reactive gliosis in the brain in the absence of neuronal death following LPS treatment in rats [60]. Ro also attenuated reactive gliosis and protected hippocampal hilar neurons in hippocampus injected with kianic acid [61]. The previous study in our lab reported the role of TSPO in regulation of neuroinflammation in microglia. The over-expression of TSPO reduced the production of pro-inflammatory cytokine through blocking NF-κB signaling pathway [50].

1.4 Parkin

1.4.1 Parkin Structure and Function

Parkin is a 52 kDa protein encoded by *PARK2* gene in human. Parkin is highly expressed in many human tissues and especially abundant in the brain, such as the substantia nigra. In 1998, Kitada, et al. reported the mutations in the newly identifed gene from the patients of autosomal recessive juvenile Parkinson's disease (AR-PD) and named the protein product of this gene as 'Parkin' [62].

Parkin is a member of RBR (Ring between Ring) E3 ubiquitin ligases. Loss of ubiquitin-protein ligase activity and dysfunction of protein degradation have been reported in AR-PD patients that have mutations inparkin gene [63]. Parkin ubiquitinates a wide variety of cytosolic and outer mitochondrial member proteins upon mitochondrial depolarization. It forms multiple types of ubiquitin chains, most frequently K63, K48, K11, and K6 linkage. Parkin consists of a ubiquitin like domain at its N terminus and four zinc-coordinating RING like domains: RING0, RING1, IBR, RING2. Parkin RING2 domain has catalytic cysteine with conserved chemistry across the RBR family of E3 ligase for ubiquitin transfer to the target protein [64-65].

Parkin is important for motochondria homeostasis and immune responses. Parkin has many function for homeostasis in our body. Especially, Parkin controls mitochondria quality through mitochondria biogenesis and transport, mitophagy, and fission and fussion. In parkin deficient mice, mitochondria dysfunction and oxidative stress were increased [66]. In addition, Parkin can function as a tumor suppressor and the deletion or inactivation of Parkin has been reported in a variety of human cancers. Parkin is related to immunomodulation through regulation of inflammation [67] and pathogen defense system [68]. Parkin has been reported to promote cell survival through preventing cell death [64].

1.4.2 Parkin and neuroinflammation in neurodegenerative diseases

Recent works suggested that chronic inflammation is involved in progression of neurodegenerative disease and abnormal microglia activation is a risk factor to neuronal loss. Therefore, the role of Parkin in the regulation of neuroinflammation also has been reported. Casarejos, et al. reported the relationship between microglia and Parkin. Their works showed that the proportion of microglia was increased following rotenone in Parkin null mice and microglia from parkin null mice enhanced sensitivity of dopaminergic neurons to toxicity of rotenone [69]. Frank-Cannon, et al. reported a novel role for Parkin in the regulation of neuroinflammation. Their results showed that Parkin deficiency triggers inflammation-induced nigral degeneration and Parkin has a protective effects in neuroinflammation [67].

In addition, the mechanisms that Parkin regulates neuroinflammatin also have been reported (Fig. 3). Parkin protein and mRNA levels were down-regulated depending on NF-κB signaling pathway by LPS and TNF stimulation in microglia [70]. The anti-inflammatory mechanisms by Parkin also were reported. Parkin attenuated neuroinflammation and cytokine-induced cell death thorugh the proteasomal degradation of TNF-α receptor-associated factor 2/6 (TRAF 2/6). Parkin directly interacted with TRAF 6 and eliminated it. Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD and human PD tissues showed increased expression of TRAF 6. However, Parkin mutnats, R42P and T204R showed decreased anti-inflammatory effects and degradation of TRAF 6 [71].

2. Methods and Materials

2.1 Chemicals and reagents

LPS was purchased from Calbiochem (La Jolla, CA, USA, 437627). TSPO ligands, PK and Ro were purchased from Sigma-Aldrich (St. Louis, MO, USA, PK;C0424 , Ro;C5174). TSPO, PINK1 antibody was purchased from Abcam (Cambridge, MA, USA, TSPO; ab109497, PINK1; ab23707). LC3B antibody was purchased from Novus Biologicals (Littleton, CO, USA, NB100-2220). p62 antibody was purchased from Sigma-Aldrich (p0067). Parkin antibody was purchased from Biolegend (#808501). Antibodies against Erk, phospho-Erk, p38, phospho-p38, JNK, phospho-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA, Erk; #4695, phospho-Erk; #4370, p38; #9212, phospho-p38; 4511, JNK; #9258, phospho-JNK; #4671). Antibodies against beta-actin, SBP were purchased Santa Cruz (Dallas, Taxas, USA, beta-actin; sc47778, SBP; sc101595).

2.2 Cell cultures

The murine BV2 microglial cells were cultured in DMEM/F12 (Thermo Fisher scientific, #12400-024) supplemented with 5% heat-inactivated FBS (Hyclone, SH30919.03) and 1% penicillin-streptomycin (Hyclone, SV30010). HEK 293T cells were grown in DMEM (Corning, 10-013-CVR) supplemented with 5% FBS and 1% penicillin-streptomycin.

2.3 Nitrite assay

The production of nitric oxide (NO) was measured 24 h after 100 ng/ml LPS treatment. NO level was determined by measuring the accumulated level of the NO metabolite nitrite (NO_2^-) in the supernatant using colorimetric reaction with Grisess reagent (0.1% naphthylethylenediamine, 1% sulfanylamide and 2.5% H_2PO_4; Promega, G2930) following the manufacturer's instructions. Absorbance was measured at 550 nm with a microplate reader (BioTek, Synerge HT microplate reader).

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-6 and TNF-α in the supernatant were measured 24 h after 100 ng/ml LPS treatment using an ELISA kit (R&D system, IL-6; DY406-05, TNF-α; DY410-05) following the manufacturer's instructions. Absorbance was measured at 450 nm with a microplate reader (BioTek, Synerge HT microplate reader).

2.5 NF-κB luciferase reporter assay

The pGL4.32 NF-κB luciferase reporter vector and the pGL4.74 Renilla luciferase vector were purchased from Promega (pGL4.32; E8491, pGL4.74; E6921) and co-transfected to BV2 cells using Amaxa^{TM} SF Cell Line 4D-Nucleofactor^{TM} X Kit (Lonza, Basel, Switzerland, V4XC-2024) with 4D-Nucleofactor® Core Unit (Lonza) according to the manufacturer's instructions. Renilla luciferase was used as an internal control reporter vector to normalize differences in transfection efficiency. BV2 cells were transfected for 24 h and treated with 100 ng/ml LPS for 3 h. The cells were lysed in dual luciferase lysis buffer, and 10 μL aliquots of the cell lysate were assayed using a dual luciferase assay system (Promega, E2920) with a luminescence microplate reader (Molecular Devices, SpectraMax L). The transcriptional activity of NF-κB was calculated by normalizing the firefly luciferase activity with the corresponding Renilla luciferase activity and was reported as fold of induction.

2.6 Western blot Analysis

The cells were washed with cold PBS and harvested at the indicated time points and lysed in cell lysis buffer (1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 1 mM EDTA (pH 8.0), 1 mM dithiotheritol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in 20 mM Tris-HCl (pH 7.2)) containing 1× protease cocktail inhibitors (Thermo Scientific, #87786) and 1× phosphatase cocktail inhibitors (Thermo Scientific, #78420) for 30 min on ice. After lysis, the samples were centrifuged at 14,000 rpm for 10 min and the supernatant was collected. The protein concentration of cell lysates was measured using a BCA protein assay kit (Thermo Scientific, #23224). Typically, 10-20 μg of proteins were loaded for 12% sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis. The proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes

(Millipore, Bedford, MA, USA, IPFL00010) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h at room temperature in a blocking solution of 5% nonfat skim milk in Tris buffered saline-0.1% Tween 20 (TBST). The membranes were incubated with each primary antibody at 4 °C overnight. After washing with TBST, the membranes were incubated for 1 h at room temperature in blocking solution containing horseradish peroxidase conjugated secondary antibodies. After washing with TBST, the protein bands were detected using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, #34080).

2.7 Immunoprecipitation

The samples were precleared by incubation with protein A Sepharose beads (Thermo Scientific, #20333) for 30 min at 4 °C. Precleared samples were incubated overnight at 4°C with rabbit anti-Parkin (Abcam, ab15954), rabbit anti-HA (Santa Cruz, sc-805), rabbit anti-TSPO (Abcam, ab109497), rabbit anti-p62 (Sigma-Aldrich, p0067), or control IgG (purified rabbit IgG; Santa Cruz, sc-2027). The antigen–antibody complexes were captured by addition of protein A Sepharose beads for 1 h at room temperature and washed three times with washing buffer (1% Triton X-100, 100-200 mM NaCl, 250 mM sucrose, 1 mM EDTA (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF) in 20 mM Tris-HCl (pH 7.2)). Proteins were released from the beads by heating at 100 °C for 5 min in 2× Laemli's buffer (Bio-Rad, #1610737) and proteins were loaded for 12% SDS-gel electrophoresis with each input of protein samples. The protein levels were determined by Western blot Analysis.

2.8 DNA transfection and knockdown

The pIRES2-EGFP vector containing sequence for streptavidin binding protein (SBP) tag was kindly provided by Cheolju Lee (Korea Institute of Science ans Technology, Korea) (Fig. 4A). TSPO cDNA was purchased from Origene (Rockville, MD, USA, sc111597) and cloned into the pIRES2-EGFP vector and performed point mutation (T147A) (Fig. 4B-C). The pRK5 Myc tagged parkin vector was kindly supplied by Yunil Lee and performed point mutation (R275W) (Fig. 4D-E). Parkin cDNA from pRK5 Myc tagged Parkin vector was cloned into the EGFP C1 vector (Fig. 4F-G). The HA tagged p62 vector was obtained by Addgene

(Plasmid #28027) (Fig. 4H-I). DNA transfection in BV2 cells was performed using Amaxa™ SF Cell Line 4D-Nucleofactor™ X Kit (Lonza, V4XC-2024) with 4D-Nucleofactor® Core Unit (Lonza) according to the manufacturer's instructions. DNA transfection in HEK cells was performed using Lipofectamine® 2000 (Thrmo Fisher Scientific, #11668-027). Over-expression levels of all proteins were determined by Western blot Analysis with each antibody against tag or proteins at 24-72 h after DNA transfection. The ON-TARGET plus SMARTpool TSPO small interfering RNA (siRNA) and non-targeting (NT) siRNA were purchased from Dharmacon (Lafayette, CO, USA, TSPO siRNA; L-040291-02-0005, NT siRNA; D-001810-01-20). BV2 cells were transfected with TSPO or NT siRNA using DharmaFECT transfection reagent (Dharmacon, T-2001-02). Knock-down levels of TSPO protein were determined by Western blot Analysis with TSPO antibody (Abcam, ab109497) at 48-96 h after siRNA transfection.

2.9 Statistical Analyses

All data were presented as the mean ± standard deviation (SD). Statistical comparisons between different groups were determined by either a Student’s t-test or one-way Analysis of Variance (ANOVA) with Tukey’s multiple comparison tests. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Translocator Protein 18 kDa (TSPO) is a negative regulator in neuroinflammation in microglia.

TSPO is a sensitive biomarker of inflammation and reactive gliosis in neurodegenerative diseases, so TSPO ligands are used as a potent diagnosic tool [72-73]. A large body of evidence shows the importance of TSPO in inflammation. The strong upregulation of TSPO expression in microglia and reactive astrocytes is observed in most neurodegenerative diseases, such as AD, PD, multiple sclerosis (MS), Huntington'sdisease (HD), amyotrophic lateral sclerosis (ALS) [74-80]. Futhermore, the strong upregulation of TSPO expression is observed during not only inflammaory responses but also regeneration processes [81]. The selective TSPO ligands reduced the inflammatory responses in neurodegenerative diseases [82-83]. However, The functional roles and underlying regulatory mechanisms of TSPO in neuroinflammation remain unclear.

To determine whether TSPO regulates inflammation in BV2 cells, we confirmed the change of TSPO protein expression levels following LPS treatment in BV2 cells. As expected, TSPO protein expression levels were increased at 24 h after LPS treatment in BV2 cells (Fig. 5A). To investigate the role of TSPO in the regulation of neuroinflammation in microglia, we performed TSPO over-expression. BV2 cells were transfected with 2 μg of SBP tagged TSPO plasmid and treated with LPS (100 ng/ml) 24 h after transfection for 24 h. TSPO over-expression was confirmed by SBP tagged TSPO expression in BV2 cells (Fig. 5B). TSPO over-expression suppressed the levels of pro-inflammatory factors, such as NO, IL-6, TNF-α following LPS treatment (Fig. 5C-E). These data showed that TSPO is a negative regulator in neuroinflammation in microglia.

3.2 TSPO suppresses MAPK activation and NF-κB activity in microglia.

Neuroinflammation by the activation of mitogen-activated protein kinases (MAPK) signaling pathways contributes to promoting disease progression in neurodegenerative diseases. Aggregates of Aβ42 produce ROS and pro-inflammatory cytokines through activation of MAPK signaling pathway in activated microglia in AD. In addition, inhibitors of p38 blocked production of pro-inflammatory cytokin and reduced pathological symtoms in AD mouse model. α-Synuclein also increased neuroinflammatory responses by activation of p38,

exreacellular signal-regulated kinases (ERK), and c-Jun N-terminal kinases (JNK) pathways in human microglial cells [84].

To examine whether TSPO regulates the MAPK activation in microglia, we tested whether TSPO ligands decrease MAPK signaling pathway. First, we checked peak time points of p38, JNK and ERK phosphorylation following LPS treatment. The levels of P-p38 and P-JNK peaked at 30 min after LPS treatment and the level of P-ERK peaked 60 min after LPS treatment (Fig. 6A-C). Next, to investigate the effects of TSPO ligands on MAPK signaling pathway, we pretreated TSPO ligands for 1 h before LPS treatment. Interestingly, TSPO ligands attenuated phosphorylation of p38 and JNK, but not ERK at each peak time point (Fig. 6D-F).

NF-κB regulates the production of pro-inflammatory cytokines and the expression of enzymes that make inflammatory products, such as iNOS through activating transcriptional activity of specific cellular genes. Cytokines defendent on NF-κB stimulation can also activate the NF-κB pathway and amplify the inflammatory responses through the autoregulatory loop [85].

We wondered whether TSPO mediates inflammation via down-regulation of NF-κB activity. To reveal the relationship between TSPO and NF-κB, we measured NF-κB transcriptional activity using luciferase reporter assay. LPS treatment induced NF-κB activity by 3-5 fold. As expected, TSPO over–expression significantly reduced NF-κB activity (Fig. 7A). In addition, TSPO ligands also repressed NF-κB activity (Fig. 7B). Taken together, we suggested that TSPO suppresses neuroinflammation via attenuation of MAPK activation and NF-κB transcriptional activity in microglia.

3.3 Parkin suppresses neuroinflammation in microglia

Recent reports showed the importance of Parkin in inflammation. Parkin is negatively associated with the production of pro-inflammatory cytokines following TNF-α or LPS treatment in cells or mice. In particular, macrophages and microglia from parkin null mice or cells increased the production of cytokines following LPS treatment [68,70]. In addition, Parkin suppressed inflammation by reducing the expression level of TRAF2/6, an essential regulator of NF-kB pathway activity [71]. But the exact mechanisms by which Parkin regulates neuroinflammation remains to be clarified.

To determine whether parkin also regulates inflammation in BV2 cells, we confirmed the change of Parkin protein expression levels in LPS treatment in BV2 cells. Parkin expression levels were decreased 24 h

after LPS treatment in BV2 cells (Fig. 8A). We performed Parkin over-expression to verify the function of parkin in inflammation in BV2 cells. BV2 cells were transfected with pRK5 vector or pRK5 myc tagged Parkin vector for 24 h before LPS treatment. Parkin over-expression levels were detected by Western blot (Fig. 8B). Interestingly, Parkin over-expression significantly supressed the production levels of NO and IL-6, but not TNF-α following LPS treatment for 24 h (Fig. 8C-E). These data showed that Parkin also a negative regulator of neuroinflammation in microglia.

3.4 TSPO modulates neuroinflammation via Parkin in microglia.

To investigate the relationship between TSPO and Parkin in the regulation of neuroinflammation, we performed combined knockdown and over-expression experiments with TSPO and Parkin. BV2 cells were transfected with TSPO-targeting siRNA or NT siRNA (20 nM each) for 24 h for TSPO knockdown and were transfected with pRK5 or pRK5 myc tagged Parkin vector (2 μg each) for another 24 h prior to LPS treatment. The levels of TSPO knockdown and Parkin over-expression was detected by Western blot (Fig. 9A). Interestingly, TSPO knockdown blocked the anti-inflammatory effects conferred by Parkin over-expression. The released NO and IL-6 levels, but not that of TNF-α from TSPO knock-downed and Parkin over-expressed BV2 cells were increased as compared with only Parkin over-expressed BV2 cells (Fig. 9B-D).

Next, we confirmed whether TSPO regulates parkin protein levels in neuroinflammation. BV2 cells were transfected with pIRES2-EGFP vector and pIRES2-EGFP TSPO vector 24 h prior to LPS treatment. TSPO over-expression increased parkin expression levels after LPS treatment (Fig. 9E). In addition, we investigated whether TSPO ligands also up-regulate parkin protein level following LPS treatment. As expected, PK increased parkin protein levels following LPS treatment, whereas Ro did not change parkin protein levels (Fig. 9F).

Taken together, these data showed that TSPO is an up-regulator of Parkin in the regulation of neuroinflammation in microglia and TSPO may control neuroinflammation via Parkin regulation in BV2 cells.

Because previous study reported that Parkin moves to mitochondria following LPS treatment, we investigated whether TSPO directly interacts Parkin for the regulation. HEK cells were transfected with pIRES2-EGFP TSPO and GFP-Parkin vector for 24 h and performed co-immunoprecipitation. TSPO did not interact with parkin, although VDAC1 interacted with TSPO (Fig. 10). These results suggested that TSPO may

does not regulate Parkin through direct interaction in inflammation.

3.5 TSPO and Parkin regulate inflammation via autophagy

Because TSPO and Parkin are associated with autophagy and mitophagy, we confirmed whether TSPO or Parkin increases autophagy to reduce inflammation in BV2 cells. To determine autophagy regulation, we checked autophagic markers, p62 and LC3, in TSPO or Parkin over-expression in inflammation in BV2 cells. Both of TSPO and Parkin over-expression reversed increase of p62 caused by LPS treatment. In addition, a decrease in LC3-Ⅱ expression level following LPS treatment was prevented by TSPO over-expression, although parkin failed to increase LC3-II level (Fig. 11A). Next, we investigated the effects of TSPO ligands against autophagy. BV2 cells were pretreated with TSPO ligands 1 h before LPS treatment. The results showed that TSPO ligands also attenuated LPS-triggered up-regulation of p62 level (Fig. 11B).

To determine whether TSPO regulates autophagy through parkin in inflammation, we again performed combined knockdown and over-expression experiments with TSPO and Parkin. p62 level was increased in TSPO knock-downed and Parkin over-expressed BV2 cells compared with only Parkin over-expressed BV2 cells (Fig. 11C).

Next, to investigate how Parkin or TSPO regulate p62 aggregation in inflammation in microglia, we performed co-immunoprecipitation to check interaction between Parkin or TSPO and p62. HEK cells were transfected with GFP-Parkin and HA tagged p62 vectors for 24 h and BV2 cells were treated with LPS for 24 h for endogenouse Parkin or TSPO and p62 co-immunoprecipitation. As a result, Parkin and p62 were co-immunoprecipitated in HEK cells and BV2 cells, but not TSPO (Fig. 11D-E).

Taken toether, these data demonstrate that TSPO negatively regulates neuroinflammation via up-regulation of autophagy by Parkin in microglia. Parkin regulates autophagy for the regulation of inflammation through the interaction with p62.

3.6 Parkin R275W mutant is associated with the regulation of inflammation in microglia.

To determine which domains are associated with the regulation of inflammation in Parkin in microglia, we performed over-expression of Parkin mutant. We used the Parkin R275W mutant. Parkin R275W mutant is a

well-known RING1 domain pathogenic mutant with defect in the regulation of mitophagy. When mitochondria were depolarized, Parkin R275W mutant was recruited to mitochondria and induced mitochondrial clustering, similarly to wild-type Parkin. However, this mutant fails to clear damaged mitochondria and triggers dysfunction of mitophagy [86]. BV2 cells were transfected with Parkin wild-type or R275W mutants 24 h prior to LPS treatment. Parkin over-expression levels were detected by Western blot (Fig. 12A). We expected that Parkin R275W mutant may increase the production of pro-inflammatory cytokines following LPS treatment, because Parkin suppressed inflammation through autophagy. However, the results showed that Parkin R275W mutant has increased inhibitory effects about the production of pro-inflammatory cytokines, especially NO and IL-6 (Fig. 12B-D). These results suggested that Parkin R275W (the RING1 domain mutant) may be important to regulate inflammation in microglia.

4. Discussion

In this report, we demonstrated the role and mechanisms of TSPO in the regulation of neuroinflammation in microglia. Over several decaeds, the synthetic TSPO ligands were generally used for pharmachological approaches. The previous study in our lab reported the anti-inflammatory effects of TSPO ligands, PK and Ro. TSPO ligands, PK and Ro also significantly decreased pro-inflammatory factors in vitro and in vivo [50]. The protein expression levels of TSPO significantly increased in murine microglial BV2 cell lines. Although TSPO is highly expressed in the tissues undergoing inflammation and TSPO have protective effects on inflammation, the exact role in the regulation of inflammation is not clear.

We adopted genetic approaches to reveal the function of TSPO in regulation of inflammation. TSPO over-expression suppressed the generation of pro-inflammatory factors, such as NO, IL-6, and TNF-α in BV2 cells. These results described that TSPO negatively regulates neuroinflammation in microglia and the induction of TSPO protein is an adaptive response to resolve the harmful neuroinflammation.

MAPK and NF-κB signaling pathway are involved in the inflammatory responses by activated microglia in the CNS. The regulatory mechanisms of TSPO in inflammation also included MAPK and NF-κB signaling pathway. The pharmacological approaches using TSPO ligands, PK and Ro suggested that TSPO ligands attenuated the phosphorylation of MAPK proteins. In addition, both of TSPO over-expression and TSPO ligands decrasead NF-κB transcriptional activity in BV2 cells. Taken together, our results established that TSPO negatively modulates neuroinflammation thorugh inhibition of MAPK and NF-κB signaling pathways in microglia (Fig. 13).

In recent years, neuroinflammation was proposed as a key risk factor of PD. In familiar PD patients, the mutation or deletion of Parkin was frequently reported. Thus, relationship between parkin and inflammation has emerged as a potential pathogenic mechanism of PD. The previous study reported that Parkin may inhibit inflammation depending on NF-κB related signaling pathway. Parkin is implicated in the regulation of inflammatory cytokine production following TNF-α, or LPS treatment cells or mice. But the exact mechanisms

that Parkin regulates neuroinflammation remains to be clarified. In the present study, we confirmed the effects of Parkin over-expression following LPS treatment. Parkin over-expression effectively suppressed the production of pro-inflammatory factors such as NO and IL-6, but not TNF-α.

Because another study reported that Parkin is recruited to mitochondria following LPS treatment, we performed the genetic approaches combining TSPO knockdown and Parkin over-expression to determine the relationship between TSPO and Parkin following LPS treatmet. Interestingly, TSPO knock-down blocked the inhibitory effects of inflammation by Parkin over-expression. Furthermore, TSPO over-expression and TSPO ligands increased the protein expression levels of Parkin following LPS treatment. These results suggest that Parkin is downstream of TSPO in the regulation of inflammatory signaling pathway.

Mitophagy is important to maintain the homeostasis of mitochondria. Recently, the mechanisms that parkin mediated mitophagy through VDAC1 and p62/SQSTM1 were reported [87]. Gatliff, et al. reported that TSPO modultates parkin mediated mitophagy through VDAC1. This study suggested that the relative ratio between TSPO and VDAC1 is the key factor to regulate parkin mediated mitophagy. When TSPO is highly expressed compared to that of VDAC, ROS is increased and mitophagy is suppressed [47]. Although TSPO negatively regulates parkin, both proteins are involved in the mechanisms of mitophagy. Thus, we expected that TSPO and Parkin regulates inflammation through autophagy. As expected, TSPO or Parkin over-expression and pre-treatment of TSPO ligands reduced the aggregation of p62, one of autophagic markers, following LPS treatment. In addition, Parkin interacted with p62 in HEK cells and BV2 cells. Based on our findings, we concluded that TSPO negatively regulates neuroinflammation via parkin-mediated mitophagy in microglia (Fig. 14). Although our study suggested some of evidence that Parkin and TSPO inhibit the neuroinflammation through mitophagy in microglia, the link between inflammation and mitophagy depending on TSPO and Parkin is not clear. So, additional experiments will be required to reveal the unsolved problems in this study.

We expected that Parkin mutant R275W inducing dysfuction of mitophagy may decrease the anti-inflammatory effects by Parkin wild-type over-expression. However, Parkin mutant R275W showed conflicting results. These results provided an evidence that the inflammation may be regulated by different mechanisms by the TSPO-Parkin-p62 linked regulation, not mitophagy. In previous studies, p62 also have the role in the regulation of inflammation. LPS stimulated the aggregation of p62 and p62 interacted with TRAF 6. Interaction

between p62 and TRAF 6 leaded to the K63-linked polyubiquitination of TRAF 6 and subsequent activation of the NF-κB pathway [88]. Thus, we suggested the hypothesis that Parkin by regulated TSPO may degradate p62 through proteosomal activity following LPS treatment and suppress MAPK and NF-κB related inflammatory signaling pathway (Fig. 14). In future study, the additional experiments will be required to reveal this hypothesis.

Our study will provide the clues to understand the role and underlying regulatory mechanisms of TSPO in neuroinflammation and contribute to the therapeutic design for anti-inflammatory effects.

5. Figure Legends

Fig. 1 TSPO structure in mitocondrial outer membrane

(A) Monomeric TSPO structure. TSPO is localized in mitochondrial outer membrane, and it consists of five alpha-helices similar to a channel like structure. (B) TSPO related complex structure. The functional complex of TSPO is associated with VDAC1, PRAX-1, and PAP7. VDAC1 directly interacts with TSPO in outer mitochondrial membrane (OMM) – inner mitochondrial mambrane (IMM) contact sites. (C) TSPO is highly conseved in the evolution. These figure showed that the conserved region in the species of six kinds.

Fig. 2 TSPO ligands.

(A) Steroid cholestrol is a well known endogenous TSPO ligand. The binding of cholesterol with TSPO regulates the production of steroid hormone. (B-C) PK-11195 and Ro5-4864 are the prototypical TSPO ligands. These ligands selectively bind to TSPO with nanomolar affinity. The binding of these ligands changes the channel like structure of TSPO and has any effects on the function of TSPO.

Fig. 3 Parkin and Neuroinflammation in neurodegenerative diseases

(A) Parkin protein and mRNA expression are regulated by NF-κB signaling pathway by LPS and TNF-α stimulation in microglia. The activated NF-κB signaling pathway by LPS blocks the transcription of Parkin [70]. The down-regulation of Parkin increases the production of pro-inflammatory cytokines. (B) Parkin directly interacts with TRAF 6 and performs the proteosomal degradation of protein levels of TRAF 6. It leads to decrease of inflammation depending on the activaiton of MAPK and NF-κB signaling pathway [71].

Fig. 4 Vector maps

(A) The pIRES2-EGFP SBP vector is the backbone vector of TSPO vectors. (B) The pIRES2-EGFP SBP tagged

TSPO vector expresses SBP conjugated TSPO protein (28 kDa). (C) The pIRES2-EGFP TSPO vector has not tags. This vector was carried out point mutation (T147A). (D) The pRK5 vector is the backbone vector of myc tagged Parkin and R275W Parkin vectors. (E) The pRK5 myc tagged Parkin vector expresses myc tagged Parkin protein. (F) The EGFP C1 vector is the backbone vector of GFP-Parkin. (G) The GFP-Parkin vector expressed GFP and myc conjugated parkin protein (80 kDa). (H) The pcDNA4/TO vector is the backbone vector of HA tagged p62 vector. (I) The HA-p62 vector expresses HA tagged p62 protein.

Fig. 5 TSPO negatively mediates neuroinflammation in BV2 cells.

(A) BV2 cells were treated with LPS (100 ng/ml) for 24 h. Protein expression levels were normalized to β-actin. Results are the mean ± S.D. (n=3). *Asterisks* indicate significant difference between control and LPS treated groups, *p<0.05. (B) BV2 cells were transfected with 2 μg of SBP or SBP-tagged TSPO vectors for 24 h using Amaxa^{TM} SF Cell Line 4D-Nucleofactor^{TM} X with 4D-Nucleofactor® Core Unit. LPS (100 ng/ml) was treated for additional before analysis. TSPO over-expression level were measured by Western blot compared with SBP control vector. (C-E) The released NO, TNF-α and IL-6 were measured from supernatants using Nitrite (NO_2^-) and ELISA (TNF-α and IL-6) assays. Results are the mean ± S.D. (n=5). *Asterisks* indicate significant difference between LPS treated groups, **p<0.01.

Fig. 6 TSPO ligands decrease MAPK activation

(A-C) BV2 cells were treated with LPS (100 ng/ml) and harvested at each time point. LPS treatment triggers phosphorylation of MAPK signaling pathways. The level of P-p38 peaked at 30 min following LPS treatment, and the levels of P-JNK and P-Erk peaked at 60 min following LPS treatment. (D-E) The levels of MAPK activation were measured 30 min after LPS treatment by Western blot. TSPO ligands (10 μM) were pretreated 1 h prior to LPS treatment in BV2 cells. The levels of P-p38 and P-JNK decreased by pretreatment of TSPO ligands, but not (F) the level of P-Erk. Protein expression levels were normalized to β-actin. The data are presented the mean ± S.D. (n=3). *Asterisks* indicate significant difference between LPS only treated groups and TSPO ligands + LPS treated groups, *p<0.5, **p<0.01, ***p<0.001.

Fig. 7 TSPO and TSPO ligands modulate NF-κB activity

(A) BV2 cells were transfected with 2 μg of SBP or SBP-tagged TSPO vectors for 24 h using Amaxa^{TM} SF Cell Line 4D-Nucleofactor^{TM} X Kit with 4D-Nucleofactor® Core Unit. LPS (100 ng/ml) was treated for additional 24 h before analysis. The data are presented the mean ± S.D. (n=3). *Asterisks* indicate significant difference between SBP + LPS groups and TSPO + LPS groups, $^{**}p<0.01$ (B) BV2 cells were pretreated with TSPO ligands (10 μM) 1 h prior to LPS (100 ng/ml) treatment. Luciferase activity was measured 3 h after LPS treatment (100 ng/ml). The data are presented the mean ± S.D. (n=3). *Asterisks* indicate significant difference between LPS only treated groups and TSPO ligands + LPS treated groups, $^{**}p<0.01$, $^{***}p<0.001$.

Fig. 8 Parkin suppresses neuroinflammation in microglia

(A) Parkin protein levels were reduced at 24 h after LPS (100 ng/ml) treatment. Results are the mean ± S.D. (n=3). *Asterisks* indicate significant difference between control and LPS treated groups, $^{***}p<0.001$.

(B) BV2 cells were transfected with 2 μg of pRK5 control vector or Parkin vectors and treated with LPS (100 ng/ml) for 24 h. Parkin over-expression is confirmed by Western blot Analysis with anti-Parkin antibody. (C) Nitrite (NO_2^-), IL-6 and TNF-α levels were measured from the supernatants 24 h after LPS (100 ng/ml) treatment using Nitrite (NO_2^-) and ELISA (TNF- α and IL-6) assays. The production of NO and IL-6 were significantly suppressed by Parkin over-expression. The data are presented the mean ± S.D. (n=3). *Asterisks* indicate significant difference between LPS only treated group and TSPO ligands + LPS treated group, $^{**}p<0.01$.

Fig.9 TSPO modulates neuroinflammation via Parkin in microglia.

BV2 cells were transfected with TSPO and NT siRNA for 48 h using DharmaFECT reagent and transfected with 2 μg of pRK5 control vector or Parkin vectors using using Amaxa^{TM} SF Cell Line 4D-Nucleofactor^{TM} X Kit with 4D-Nucleofactor® Core Unit for additional 24 h. Subsequently, LPS (100 ng/ml) was treated for 24 h. (A) TSPO knock-down and Parkin over-expression is detected by Western blot with anti-TSPO and anti-Parkin antibody. (B-D) The released NO_2^-, IL-6 and TNF-α levels were measured from the supernatants 24 h following

LPS (100 ng/ml) treatment using NO_2^- and ELISA (TNF- α and IL-6) assays. TSPO knock-down increased NO and IL-6 levels despite Parkin over-expression. (E) BV2 cells were transfected with 2 μg pIRES2-EGFP (pIRES) control vector and TSPO vector using Amaxa™ SF Cell Line 4D-Nucleofactor™ X Kit with 4D-Nucleofactor® Core Unit for 24 h and LPS (100 ng/ml) was treated for additional 24 h. TSPO over-expression increased Parkin protein levels following LPS treatment. (F) TSPO ligands, PK (20 μM) and Ro (20 μM) were pretreated 1 h prior to LPS treatment and harvested at 24 h after LPS treatment. All protein expression levels were normalized by β-actin.

Fig. 10 TSPO does not interact with Parkin.

HEK 293T cells were transfected with TSPO and GFP tagged Parkin for 24 h using Lipofectamine 2000. The cells were harvested and subjected to immunoprecipitation with anti-TSPO (A) and anti-Parkin (B) followed by Western blot with anti-Parkin and anti-TSPO antibody.

Fig. 11 TSPO and Parkin regulate inflammation via autophagy

(A) p62 level was regulated by TSPO and Parkin over-expression following LPS treatment in BV2 cells. BV2 cells were transfected with 2 μg of pIRES2, TSPO or Parkin vectors for 48 h followed by LPS treatment for additional 24 h. TSPO and Parkin over-expression was confirmed by Western blot with anti-GFP and anti-Parkin antibodies. (B) TSPO ligands also decreased p62 level in LPS-treated BV2 cells. TSPO ligands, PK (20 μM) and Ro (20 μM) were pretreated 1 h prior to LPS treatment and harvested at 6 h after LPS treatment. (C) TSPO knock-down inhibited the down-regulation of p62 aggregation by Parkin over-expression. BV2 cells were transfected with TSPO and NT siRNA for 48 h using DharmaFECT reagent and transfected with 2 μg of pRK5 control vector or Parkin vectors using Amaxa™ SF Cell Line 4D-Nucleofactor™ X Kit with 4D-Nucleofactor® Core Unit for additional 24 h. Subsequently, LPS (100 ng/ml) was treated and harvested at 24 h. (D) HEK 293T cells were transfected with HA tagged p62 and GFP tagged Parkin for 24 h using Lipofectamine 2000. The cells were harvested and subjected to immunoprecipitation with anti-HA and followed by Western blot with anti-Parkin antibody. (E) BV2 cells were treated LPS (100 ng/ml) for 24 h. The cells were harvested and subjected to immunoprecipitation with anti-p62 and followed by Western blot with anti-Parkin.

Fig. 12 Parkin R275W mutant is associated with the regulation of inflammation in microglia.

(A) BV2 cells were transfected with 2 μg of pRK5 control vector, Parkin wild-type or Parkin R275W vectors and treated with LPS (100 ng/ml) for 24 h. Parkin and Parkin R275W over-expression is confirmed by Western blot with anti-Parkin antibody. (B-D) Parkin R275W showed increased inhibitory effects in inflammation compared with Parkin wild-type. NO_2^-, IL-6 and TNF-α levels were measured from the supernatants 24 h after LPS (100 ng/ml) treatment using NO_2^- and ELISA (TNF- α and IL-6) assays.

Fig. 13 TSPO negatively regulates neuroinflammation through MAPK and NF-κB signaling pathway

TSPO ligands decreased the phosphorylation of JNK and p38 following LPS stimulation and it might lead to the blockade of the transcriptional activity of AP-1. TSPO ligands and over-expression also reduced the tanscriptional activity of NF-κB following LPS stimulation. These down-regulation of MAPK and NF-κB activation might induce attenuation of the production of pro-inflammatory cytokines.

Fig. 14 The link between TSPO, Parkin and TSPO in the regulation of inflammation

(1) TSPO may regulates inflammation through up-regulation of mitophagy by parkin. (2) Parkin by regulated TSPO may degradate p62 through proteosomal activity following LPS treatment and suppress MAPK and NF-κB related inflammatory signaling pathway.

6. Figures

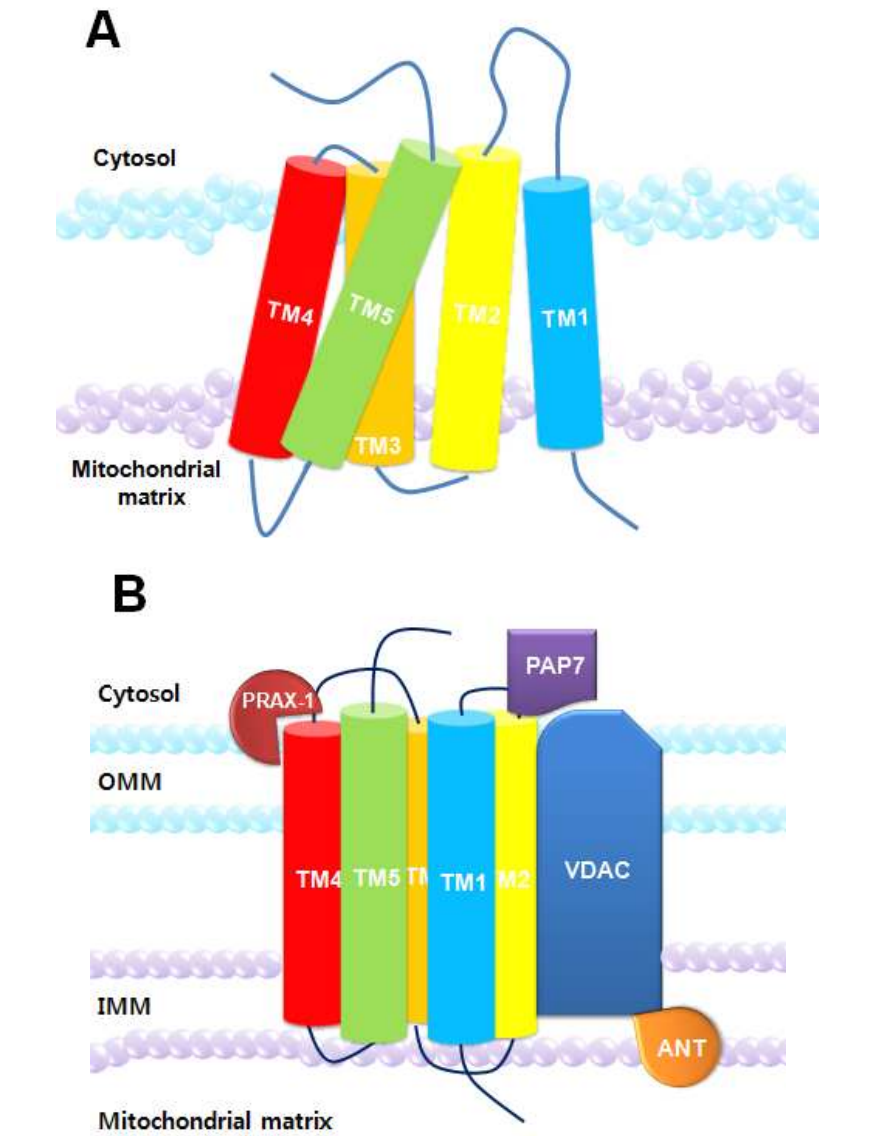
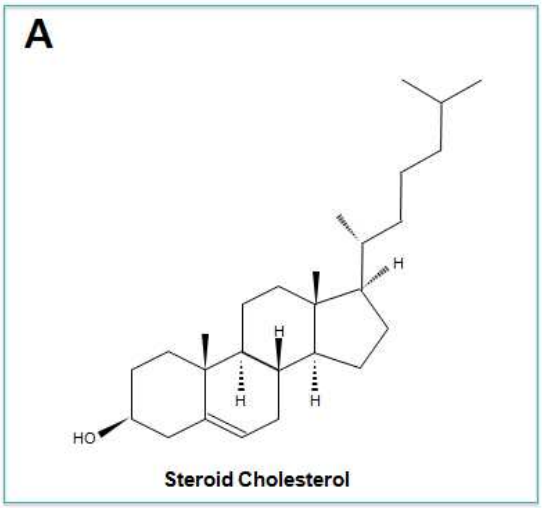
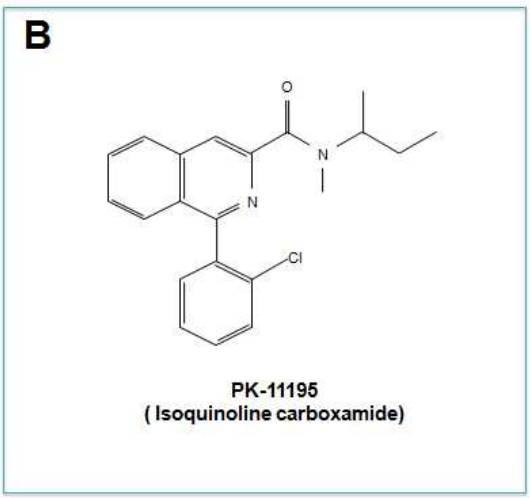


Figure. 1 TSPO structure in mitocondrial outer membrane





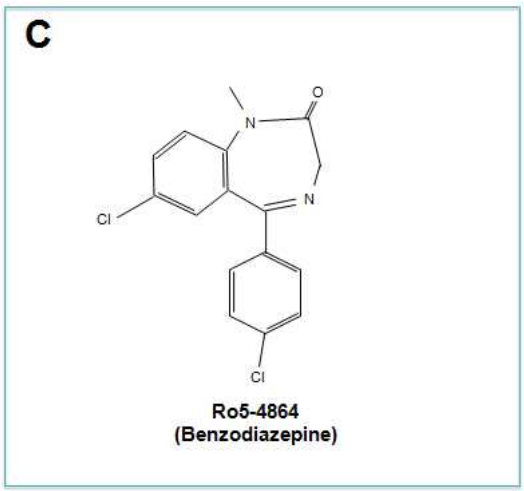


Figure. 2 TSPO ligands

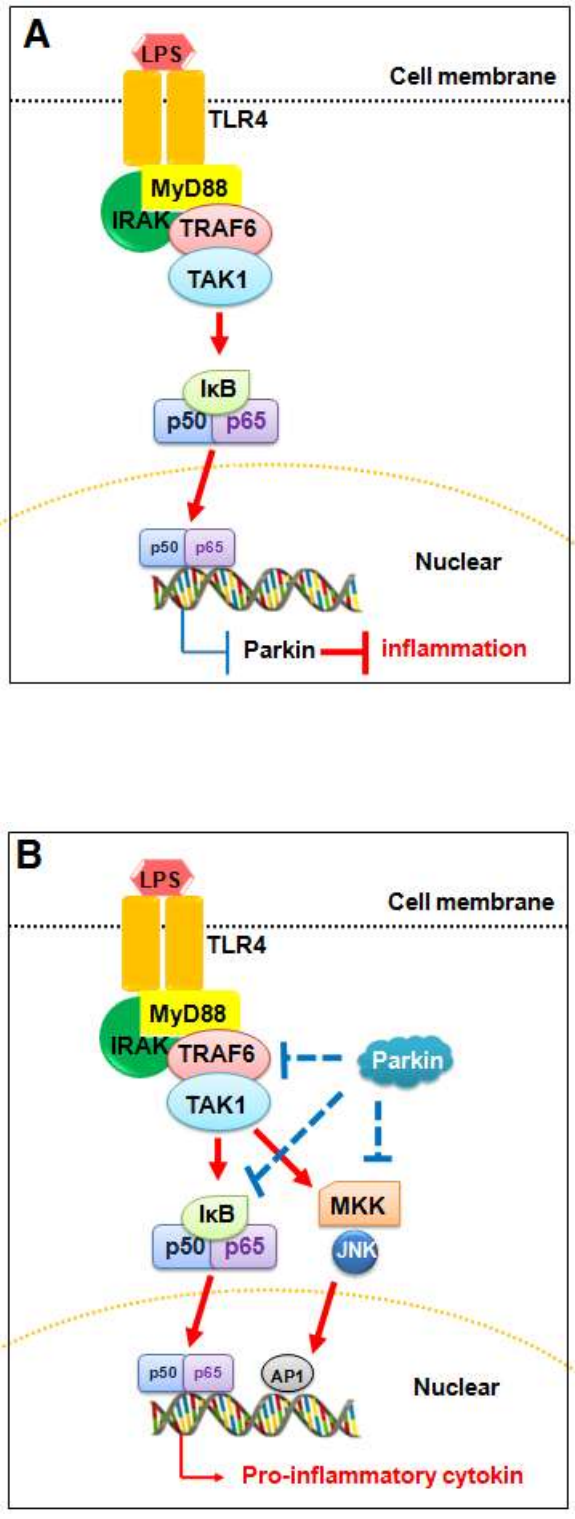


Figure. 3 Parkin and Neuroinflammation in neurodegenerative diseases

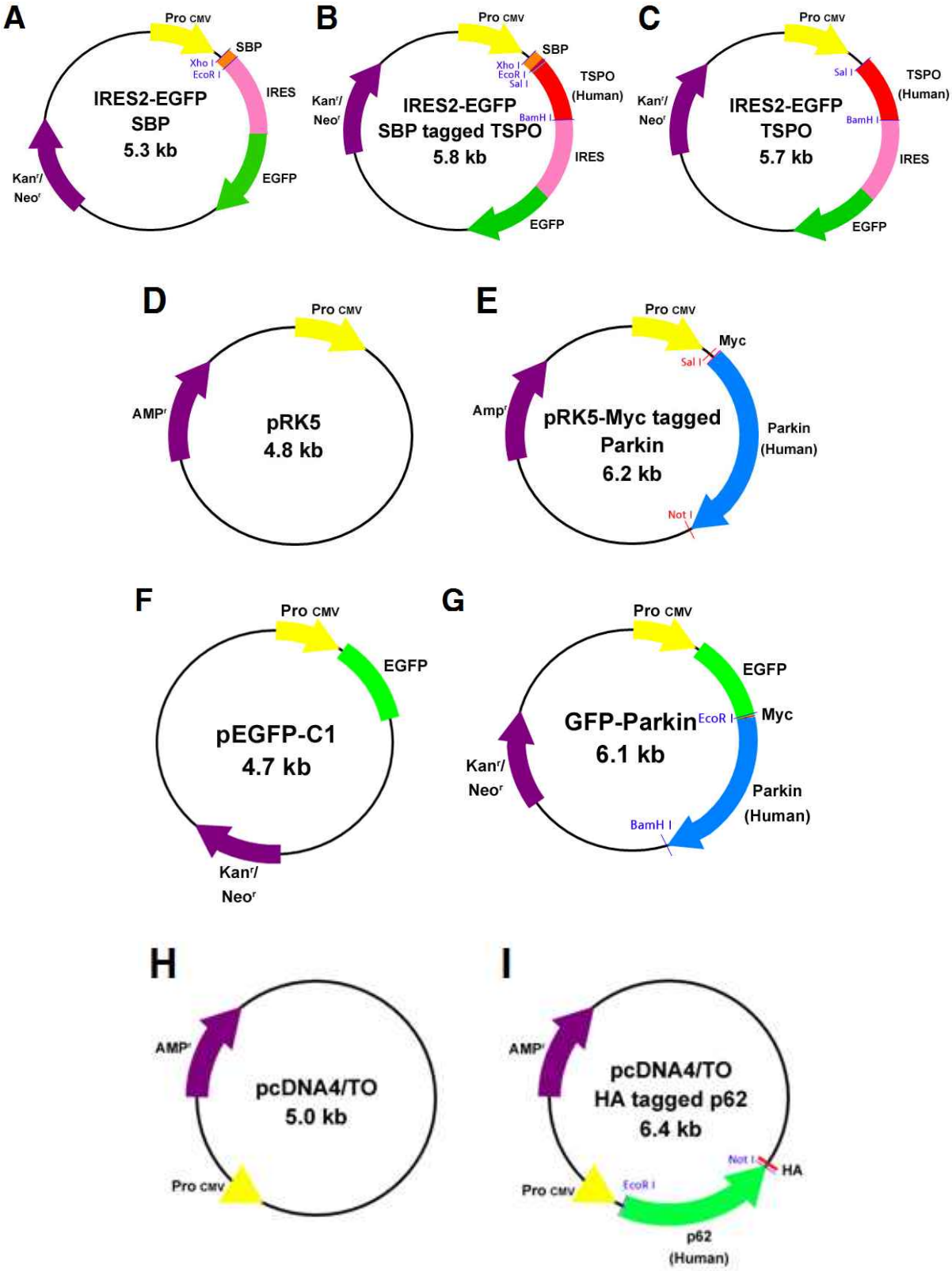


Figure. 4 Vector maps

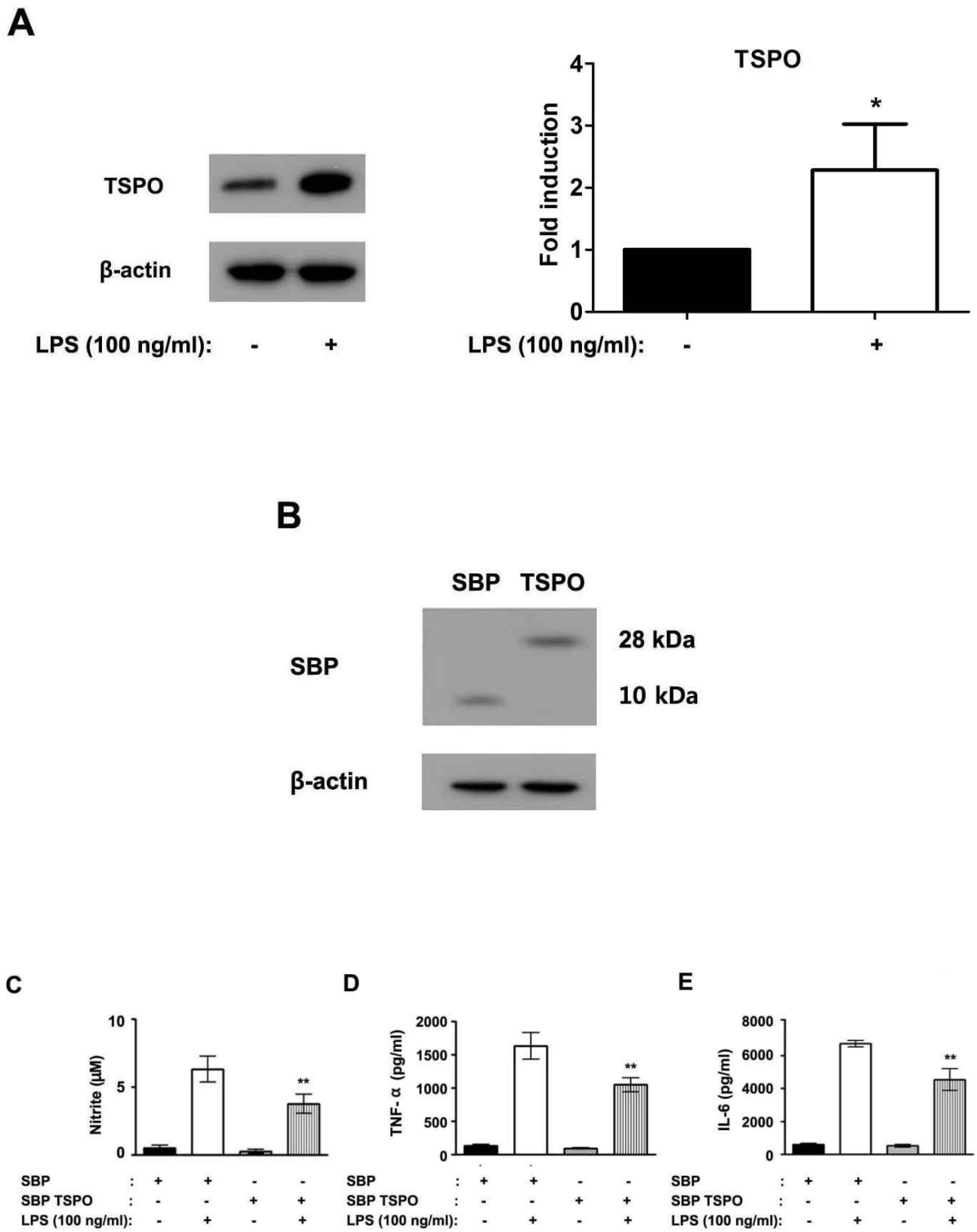
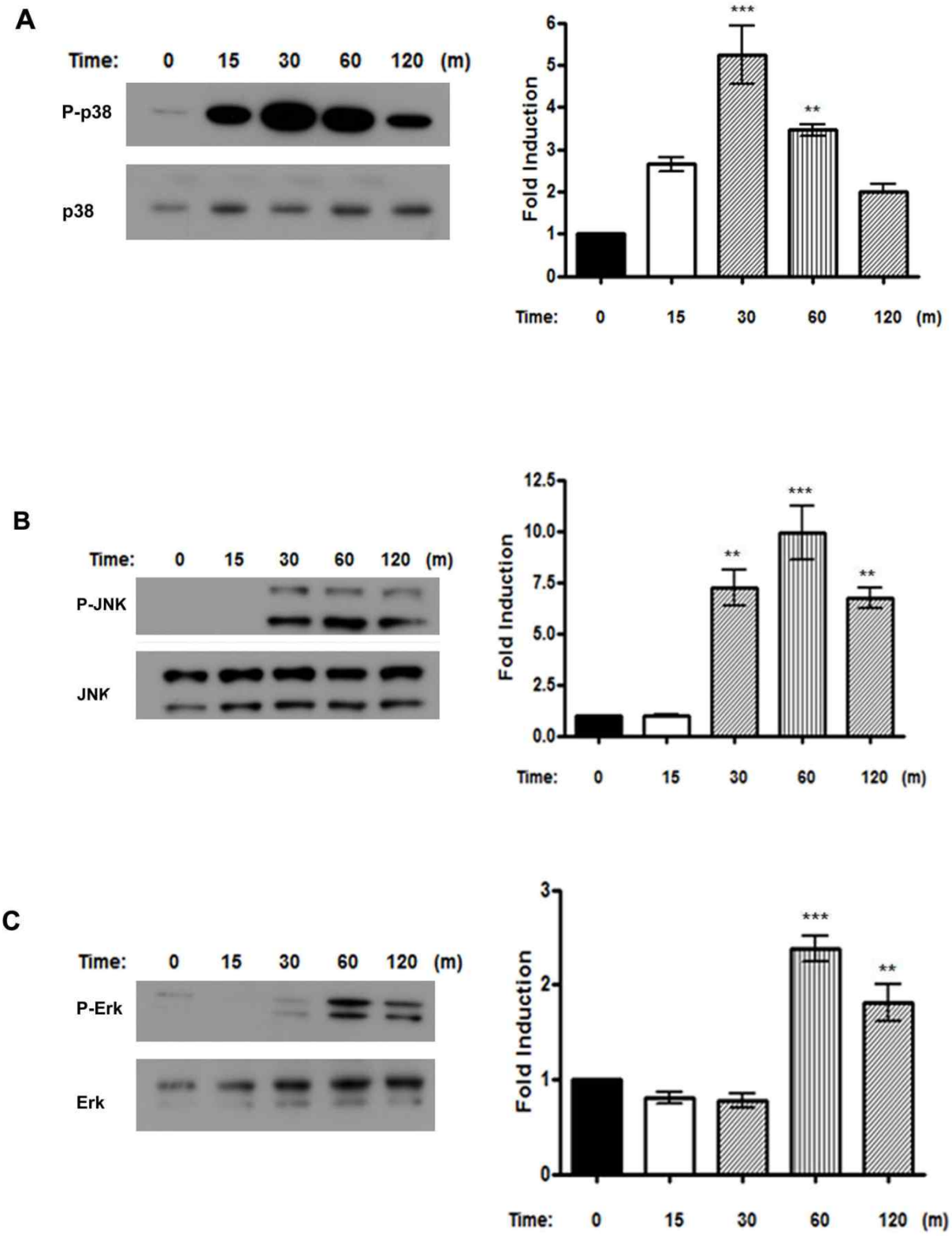


Figure. 5 TSPO negatively mediates neuroinflammation in BV2 cells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Time: 0 15 30 60 120 (m)
B
Time: 0 15 30 60 120 (m)
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JNK
Fold Induction
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C
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Erk
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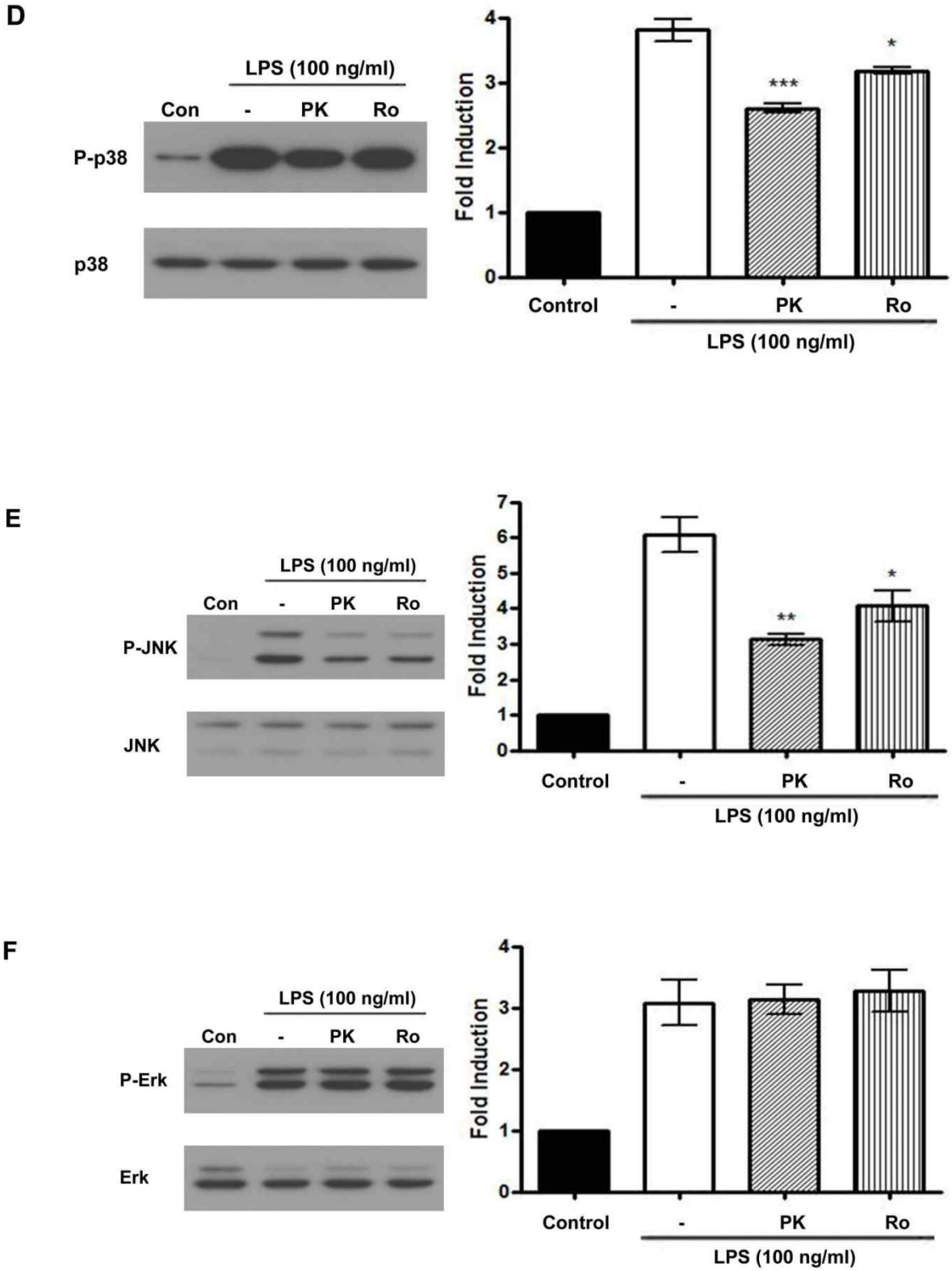


Figure. 6 TSPO ligands decrease MAPK activation

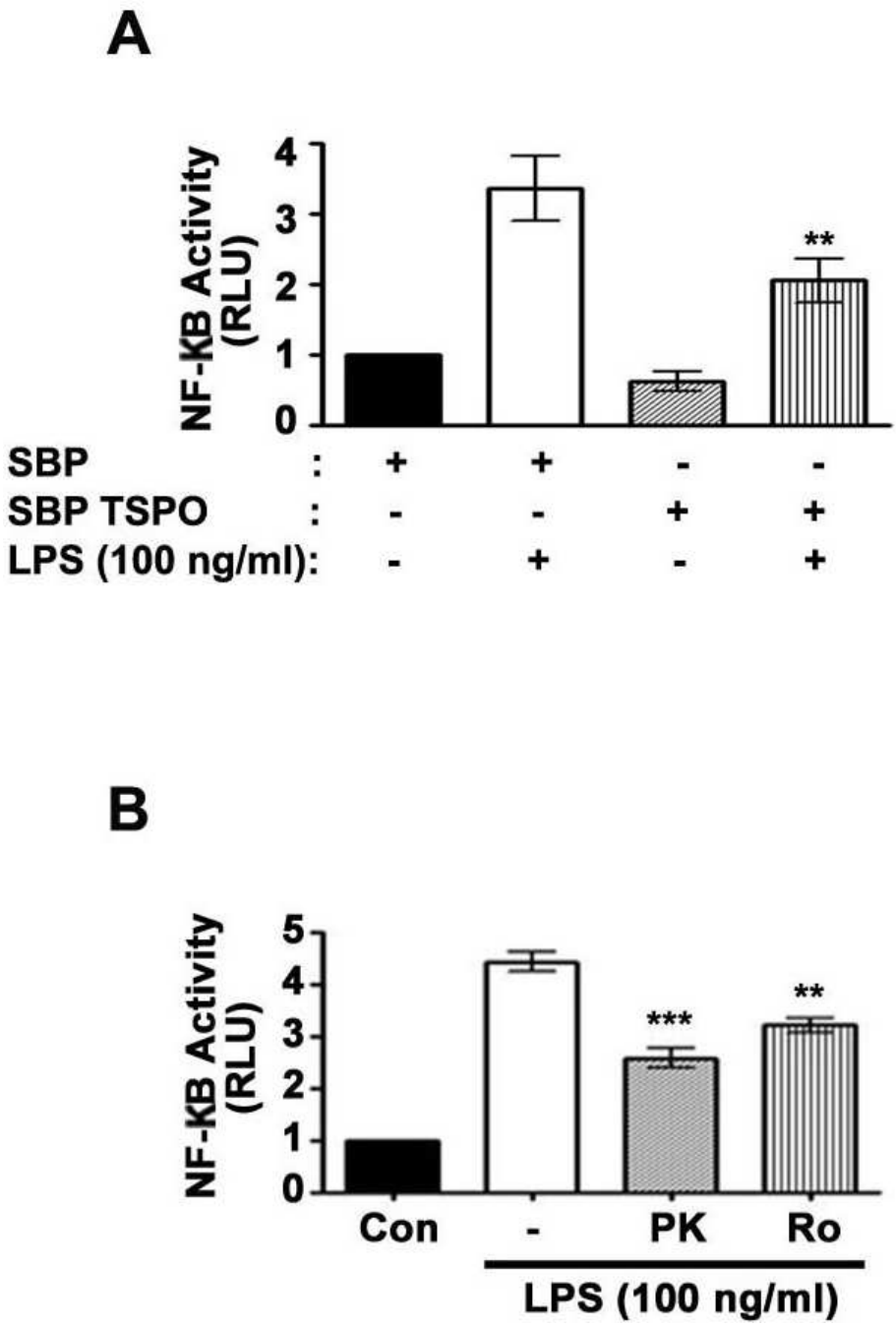


Figure. 7 TSPO and TSPO ligands modulate NF-κB activity

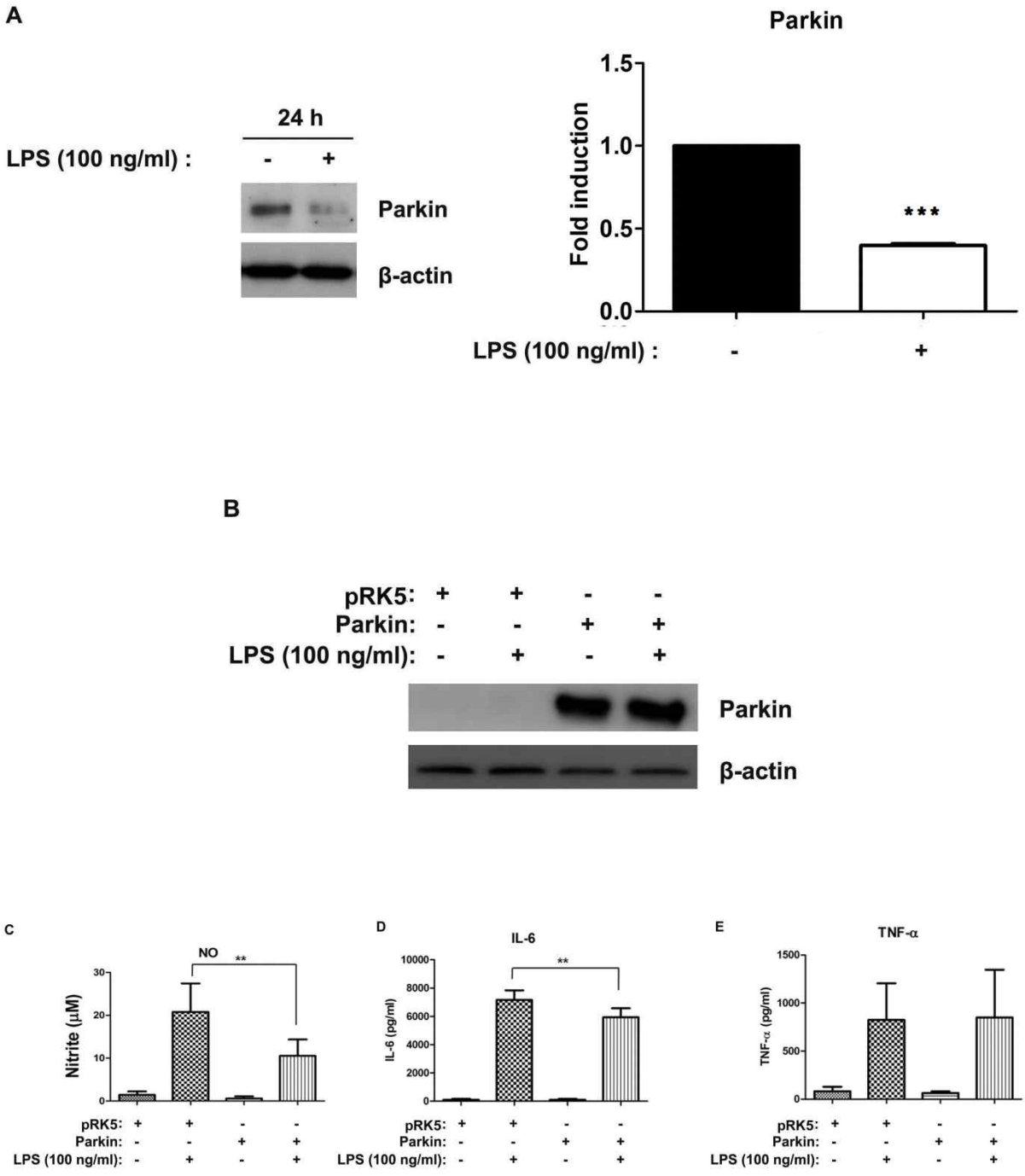
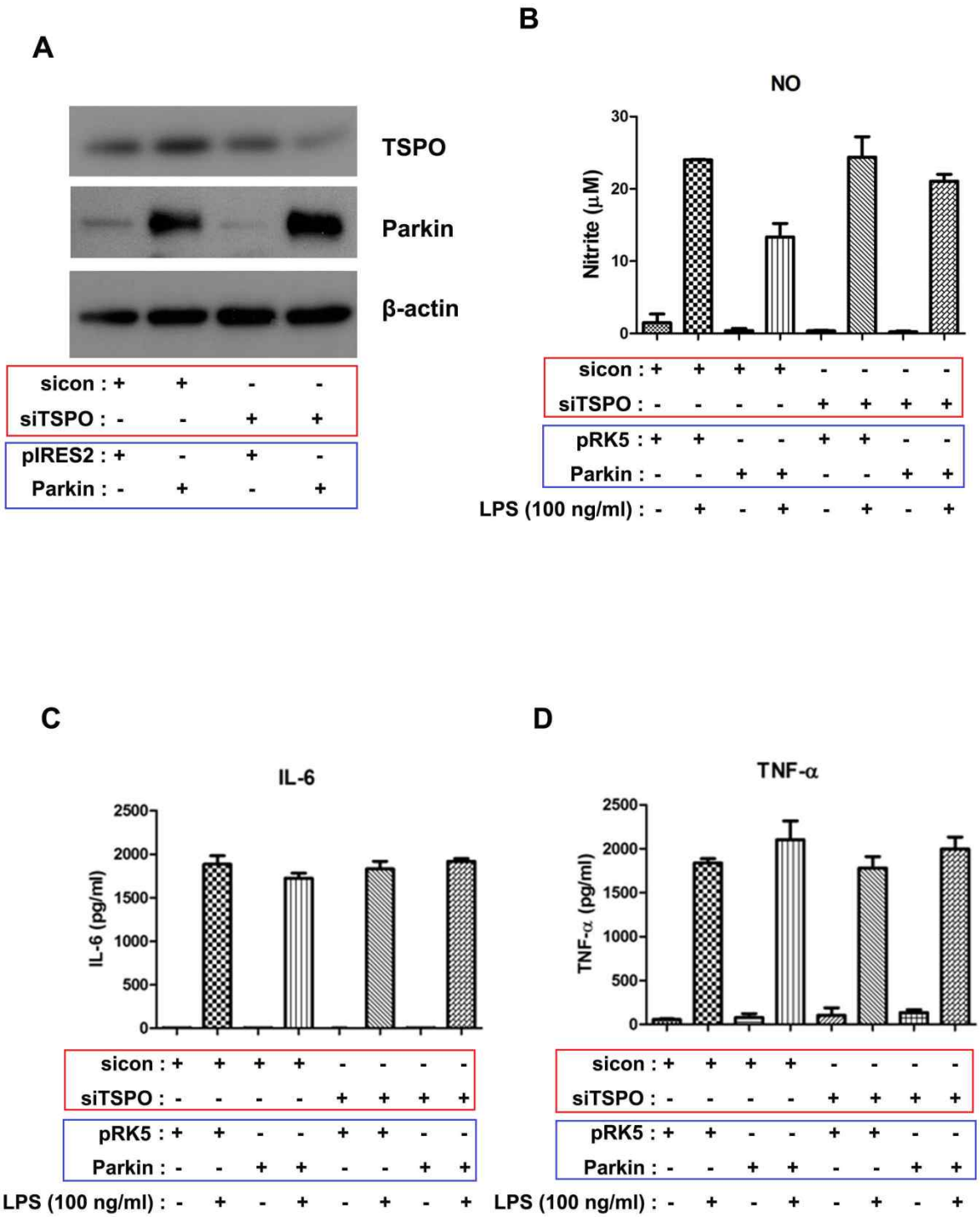


Figure. 8 Parkin suppresses neuroinflammation in microglia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TSPO
Parkin
β-actin
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pIRES2 : + - + -
Parkin : - + - +
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NO
Nitrite (μM)
30
20
10
0
sicon : + + + + - - - -
siTSPO : - - - - + + + +
pRK5 : + + - - + + - -
Parkin : - - + + - - + +
LPS (100 ng/ml) : - + - + - + - +
C
IL-6
IL-6 (pg/ml)
2500
2000
1500
1000
500
0
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pRK5 : + + - - + + - -
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D
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TNF-α (pg/ml)
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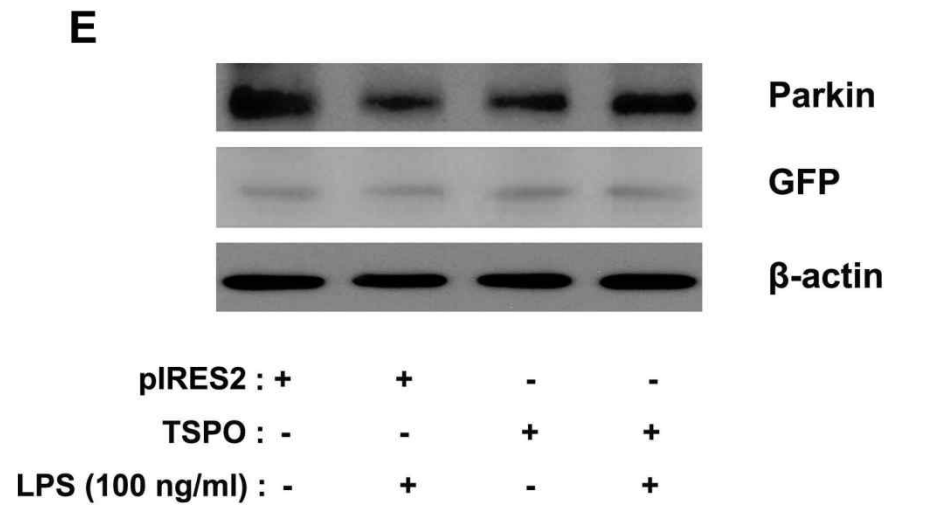
sicon : + + + + - - - -
siTSPO : - - - - + + + +
pRK5 : + + - - + + - -
Parkin : - - + + - - + +
LPS (100 ng/ml) : - + - + - + - +



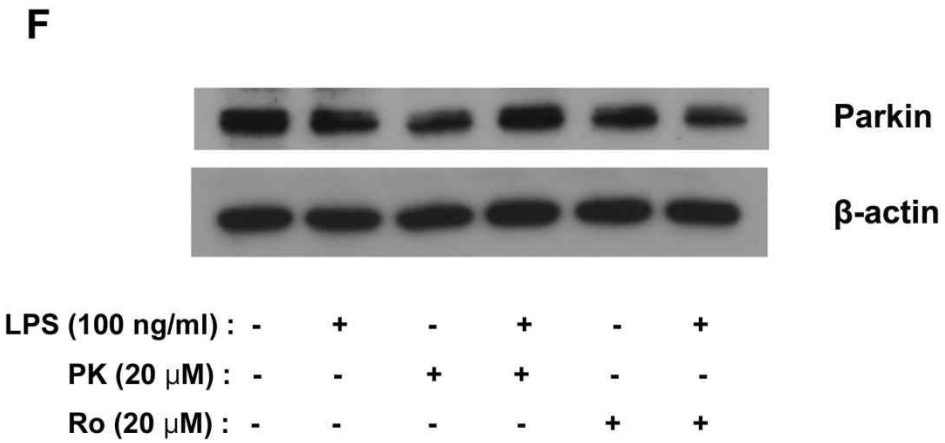


Figure. 9 TSPO modulates neuroinflammation via Parkin in microglia

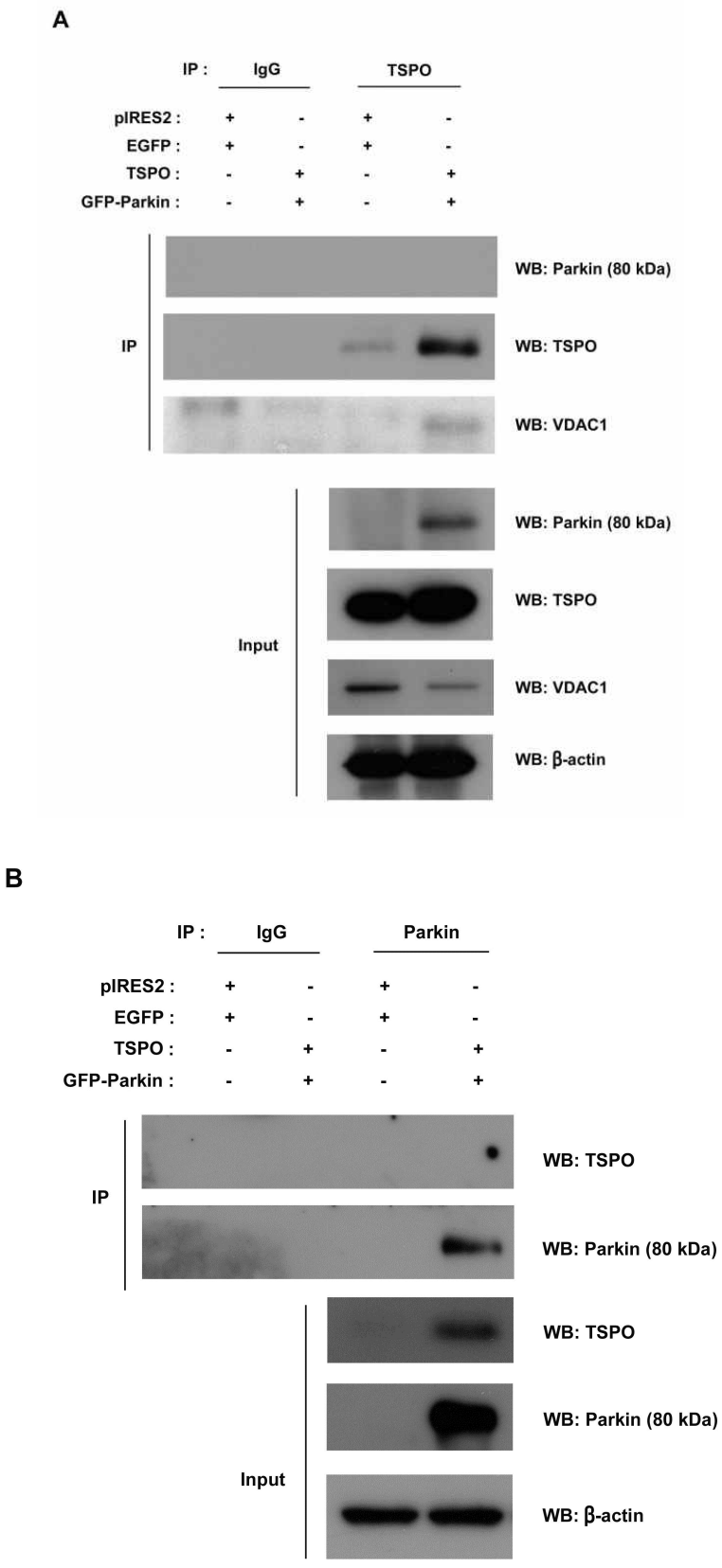
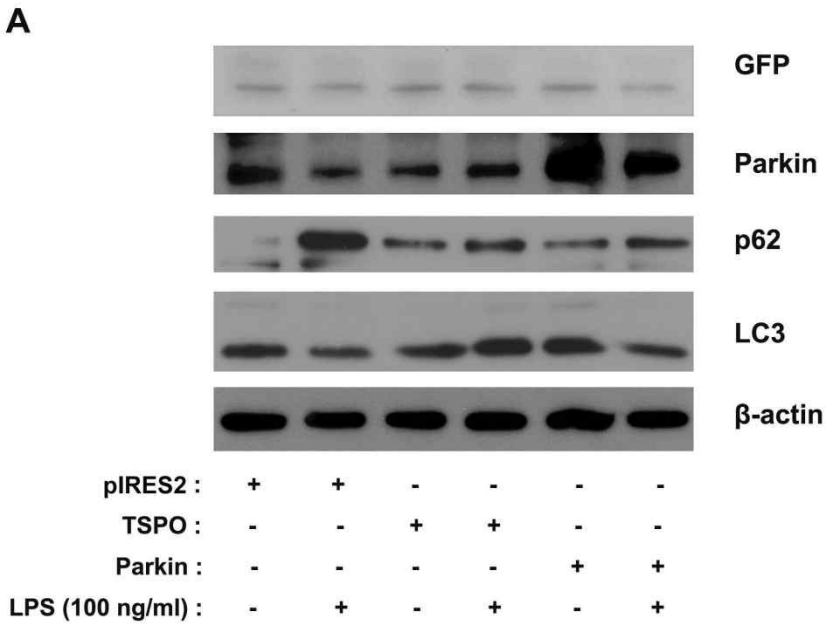
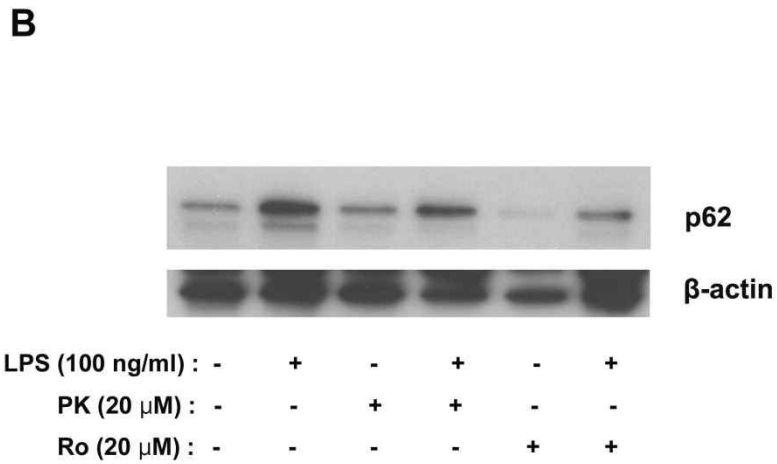
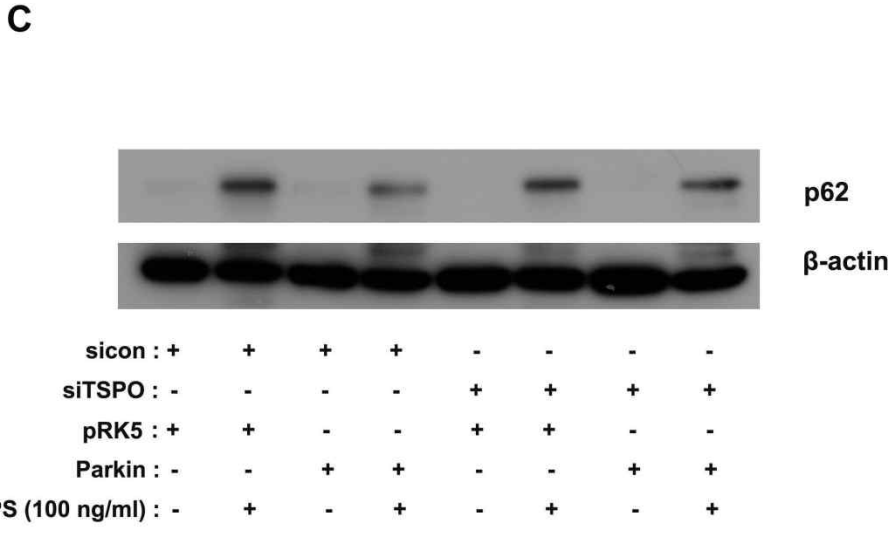


Figure. 10 TSPO does not interact with Parkin


A
GFP
Parkin
p62
LC3
β-actin
pIRES2 : + + - - - -
TSPO : - - + + - -
Parkin : - - - - + +
LPS (100 ng/ml) : - + - + - +


B
p62
β-actin
LPS (100 ng/ml) : - + - + - +
PK (20 μM) : - - + + - -
Ro (20 μM) : - - - - + +


C
p62
β-actin
sicon : + + + + - - - -
siTSPO : - - - - + + + +
pRK5 : + + - - + + - -
Parkin : - - + + - - + +
LPS (100 ng/ml) : - + - + - + - +

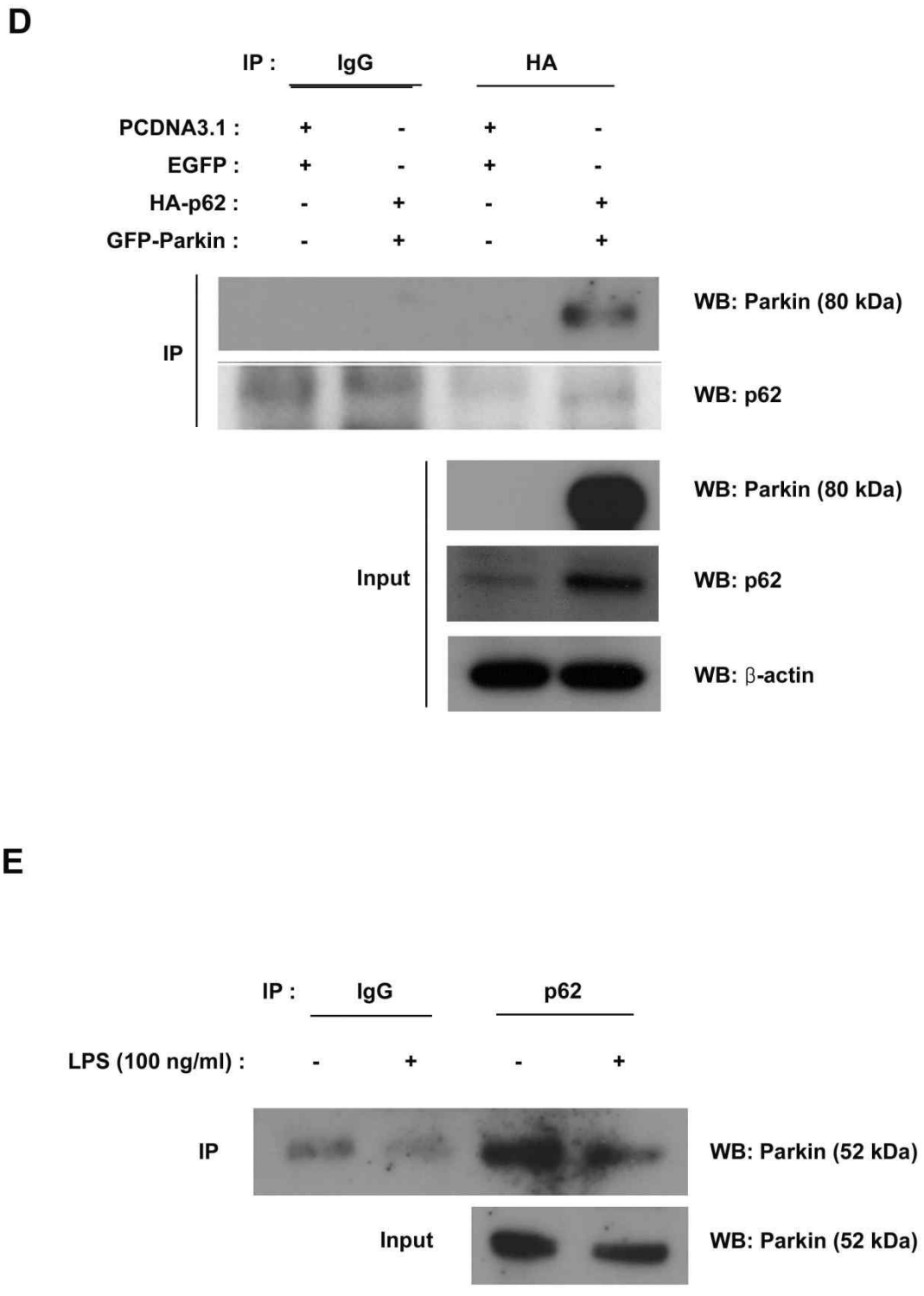


Figure. 11 TSPO and Parkin regulate inflammation via autophagy

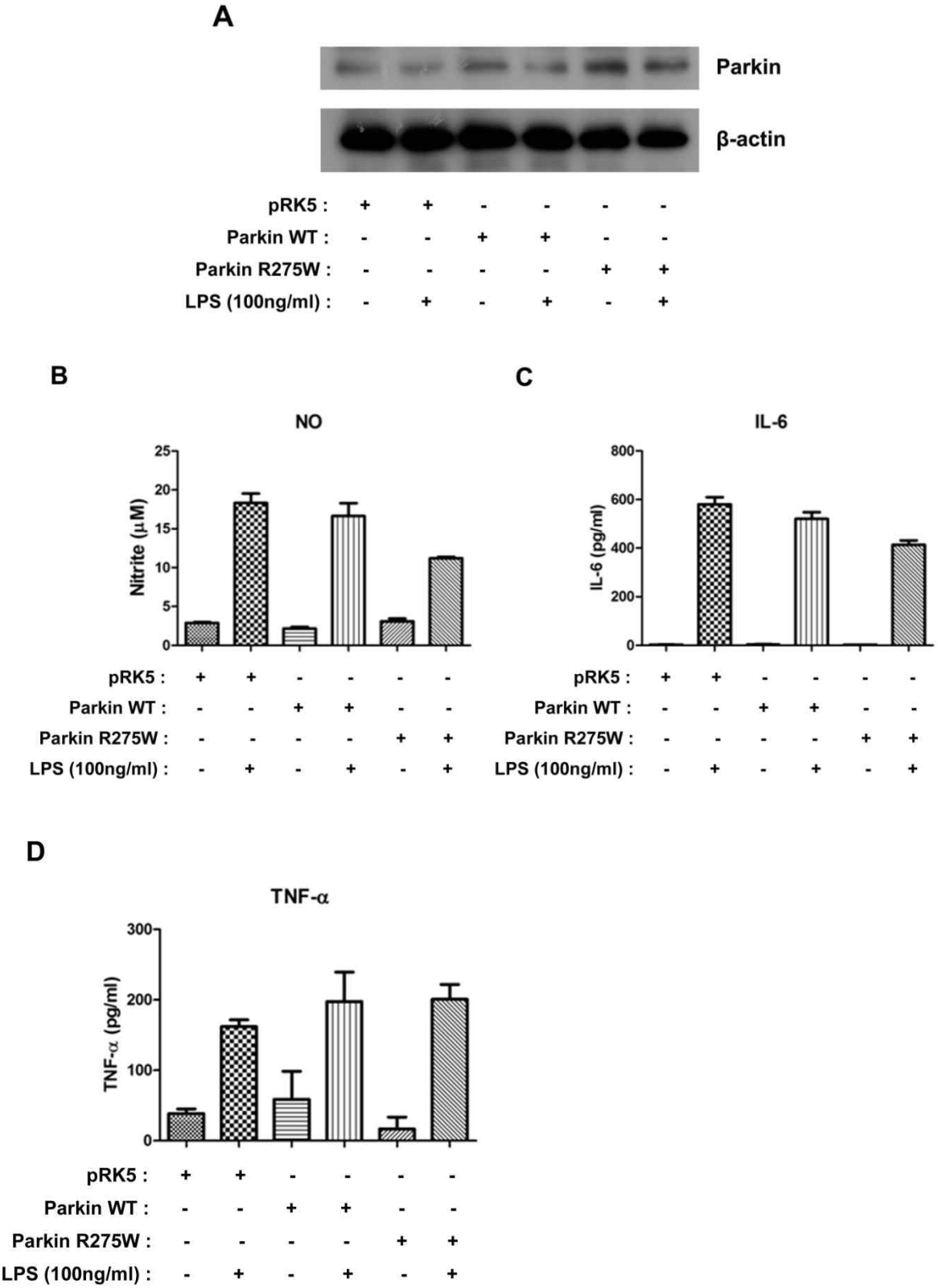


Figure. 12 Parkin R275W mutant is associated with the regulation of inflammation in microglia

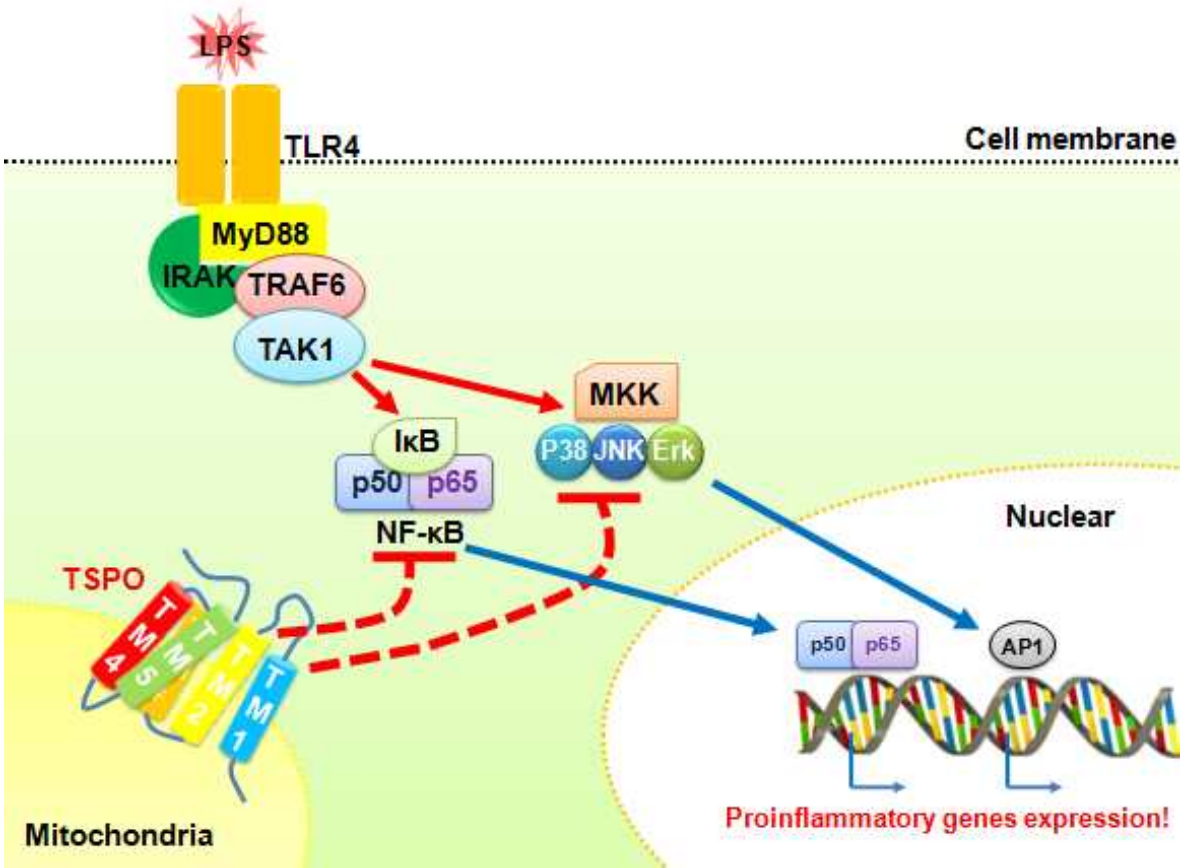


Figure. 13 TSPO negatively regulates neuroinflammation through MAPK and NF-κB signaling pathway

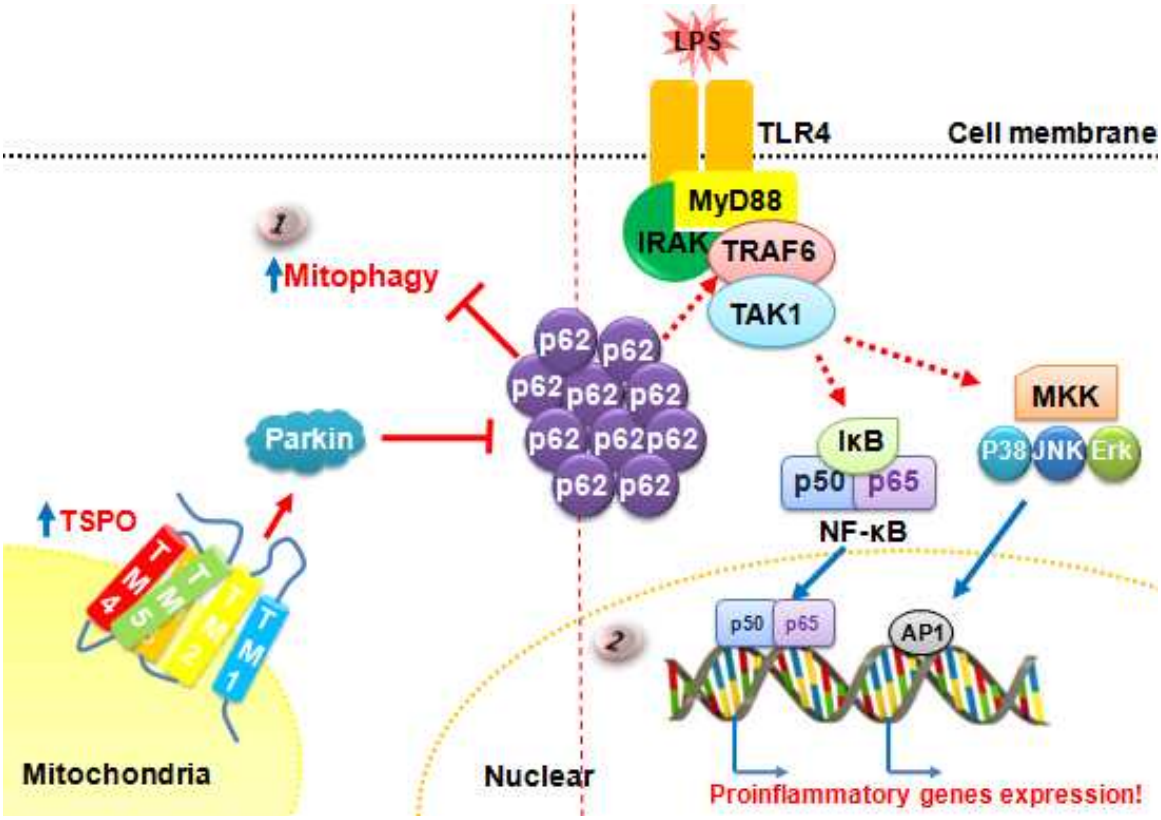


Figure. 14 The link between TSPO, Parkin and TSPO in the regulation of inflammation

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요 약 문

미세아교세포에서의 Parkin 및 p62를 통한 TSPO의 신경 염증 반응 조절

신경염증반응은 많은 신경퇴행성 뇌질환과 관련이 있다. 비록 염증 반응이 유익한 측면도 있지만, 잘못 조절된 염증 반응은 항상성을 파괴하고, 병의 진행을 더욱 빠르게 한다. 주요한 뇌신경계 면역세포인 미세아교세포는 이러한 신경 염증 반응에서 중요한 역할을 한다. 정상적인 상태에서 미세아교세포는 활성화 되지 않은 상태로 존재하며, 신체에 이로운 신경영양인자들을 분비하지만, 병원체의 침입 및 조직의 손상이 유발된 경우, 미세아교세포는 활성화 상태로 변화하여 염증반응을 유발한다. Translocator protein 18 kDa (TSPO)는 미토콘드리아 바깥쪽 막에 있는 five transmembrane protein 이다. TSPO 는 정상 상태에서는 낮은 발현을 보이나, 신경염증 반응에 의해 미세아교세포가 활성화 되면 TSPO 의 발현이 증가된다. 비록 신경 염증에 의한 TSPO 발현의 증가양상은 알려졌으나, TSPO 가 신경염증반응에서 어떤 역할을 하는지는 명확히 알려진 바가 없었다. 본 연구에서는 TSPO 의 리간드 및 과발현 실험을 통해 염증반응에서의 TSPO 의 역할과 조절기작을 밝혔다. TSPO 의 과발현은 신경염증반응에 의한 염증성 사이토카인의 분비를 억제시켰으며, MAKP (JNK 와 p38)와 NF-κB 에 의한 염증반응 유발 경로 또한 효과적으로 줄이는 양상을 보여주었다. 이러한 결과들은 TSPO 가 신경염증반응을 억제하는 조절자라는 것을 입증하였다. 최근 Parkin 에 의한 신경염증반응 조절에 대한 연구가 활발히 보고되고 있다. Parkin 은 LPS 에 의해 세포질에서 미토콘드리아로 이동하여 염증반응에 관여한다고 보고되었기 때문에, 신경염증반응 조절에 있어서 TSPO 와 Parkin 의 연관성에 대해 연구하였다. Parkin 의 과발현은 항염증 효과를 보여주었으나, TSPO 의 발현을 억제할 경우 Parkin 에 의한 항염증 효과도 줄어드는 것을 확인할 수 있었다. 뿐만

아니라, TSPO 및 Parkin 은 autophagy marker 인 p62 의 응집을 완화시키는 것이 관찰 되었다. 하지만, TSPO 의 발현을 억제한 경우 parkin 에 의한 p62 의 응집이 완화되는 현상은 줄어드는 것으로 보아, TSPO 가 Parkin 을 통해 p62 의 증가된 응집을 완화시키고, autophagy 를 통해 염증반응을 줄여주는 것으로 사료된다. 본 연구는 뇌신경염증반응에 있어서 TSPO 의 역할 및 조절기작을 밝혀, 항염증 약물의 개발에 도움을 줄 것이며, 나아가 신경퇴행성 뇌질환의 치료의 발판을 마련할 것으로 기대된다.

핵심어 : 신경 염증 반응, 미세아교세포, TSPO, Parkin, p62