α -Synuclein Activates Innate Immunity but Suppresses Interferon- γ Expression in Murine Astrocytes

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Abstract

Glial activation and neuroinflammation contribute to pathogenesis of neurodegenerative diseases, linked to neuron loss and dysfunction. α -Synuclein (α -syn), as a metabolite of neuron, can induce microglia activation to trigger innate immune response. However, whether α -syn, as well as its mutants (A53T, A30P and E46K), induces astrocyte activation and inflammatory response is not fully elucidated. In this study, we used A53T mutant and wildtype α -syns to stimulate primary astrocytes in dose- and time-dependent manners (0.5, 2, 8 and 20 μ g/mL for 24 hour or 3, 12, 24 and 48 hour at 2 μ g/mL), and evaluated activation of several canonical inflammatory pathway components. The results showed that A53T mutant or wildtype α -syn significantly upregulated mRNA expression of toll-like receptor (TLR)2, TLR3, nuclear factor- κ B and interleukin (IL)-1 β , displaying a pattern of positive dose-effect correlation or negative time-effect correlation. Such upregulation was confirmed at protein levels of TLR2 (at 20 μ g/mL), TLR3 (at most doses) and

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Data Accessibility Statement:

Information about the animal model, experimental methods, and data described in this article are available to scientific and medical communities for review, verification, and research studies. For data inquiry, please contact Dr. Jintang Wang at jintangwang@hotmail.com or Dr. Sean Leng at sleng1@jhmi.edu.

IL-1 β (at 3 hour) by western blotting. Blockage of TLR2 other than TLR4 inhibited TLR3 and IL-1 β mRNA expressions. By contrast, interferon (IFN)- γ was significantly downregulated at mRNA, protein and protein release levels, especially at high concentrations of α -syn or early time-points. These findings indicate that α -syn was a TLRs-mediated immunogenic agent (A53T mutant stronger than wildtype α -syn). The stimulation patterns suggest that persistent release and accumulation of α -syn is required for maintenance of innate immunity activation, and IFN- γ expression inhibition by α -syn suggests a novel immune molecule interaction mechanism underlying pathogenesis of neurodegenerative diseases.

Graphical Abstract Text

α -Synuclein (α -syn, A53T mutant or wild-type) stimulated TLR2 and TLR3, as well as TLR1 or TLR4, to activate inflammatory pathway by up-regulating expression of NF- κ B, IL-1 β and IFN- β , whereas IFN- γ expression was significantly down-regulated. Blockage of TLR2 other than TLR4 suppressed expression of TLR3 and IL-1 β . These data indicate a potential interaction mechanism of inflammatory factors in pathogenesis of neurodegenerative diseases. IRF-3: IFN regulatory factor 3

Keywords

α -synuclein; interferon- γ ; toll-like receptors; cytokines; innate immunity; astrocytes

Introduction

Alpha-synuclein (α -syn) is a 140-amino acid protein highly enriched in presynaptic neuronal terminals, and exerts multiple physiological functions such as synaptic vesicle turnover, synaptic plasticity and molecular chaperone (Mori et al., 2002; Norris et al., 2004), whereas its various dysfunctions are strongly implicated in pathogenesis of neurodegenerative diseases or synucleinopathies such as Parkinson's disease (PD), Alzheimer's disease (AD) and multiple system atrophy (MSA) (Goedert, 2001; Norris et al.,

2004). Extensive studies have shown that α -syn serves as a major component of cytoplasmic inclusions such as Lewy bodies/neurites, liposomes and exosomes to trigger intracellular apoptotic mechanism underlying neuronal damage (Lanzillotta et al., 2015; Majd et al., 2015). However, fewer studies show that α -syn functions as antigenic stimulator of glial activation and innate immunity to initiate extracellular inflammatory mechanism by its release from neurons and contribute to neurotoxic pathogenesis (Lee et al., 2010; Ma et al., 2013; Rivest, 2009). A hallmark of this neurotoxicity is derived from conversion of α -syn from constituent soluble monomers to aggregated amyloid-like insoluble forms, as well as formation of other pathologic forms such as missense mutations (A53T, A30P and E46K) and multiple copies of wildtype (WT) α -syn (Eschbach & Danzer, 2014; Goedert, 2001). These molecular changes and high expression of α -syn are persistently observed in substantia nigra of familial or sporadic PD, showing a positive association of α -syn with PD: genetic mutation of α -syn causes familial PD with early-onset and more severe pathogenesis, and multiplication of normal WT form increases risk of sporadic PD (Croisier et al., 2005; Nishioka et al., 2006).

Traditionally, α -syn is an exclusively intracellular acidic protein and largely located within neurons in substantia nigra, neocortex and hippocampus (Kim et al., 2004). Also, α -syn can be expressed in astrocytes (Tanji et al., 2001), oligodendroglia (Mori et al., 2002) and peripheral immune cells such as T-cells, B cells, natural killer cells and monocytes (Shin et al., 2000), and be secreted or released into biological fluids such as cerebrospinal fluid and blood plasma (Lee et al., 2006; Sui et al., 2014). Additionally, α -syn can be transferred to neighboring astrocytes or neurons to promote formation of inclusion bodies, leading to inflammatory responses and neuron death (Desplats et al., 2009; Lee et al., 2010). This widespread cell expression and release of α -syn provides multiple potential stimulatory sources responsible for glial activation and innate immunity (Rivest, 2009). While microglia are known as a resident immune cell in the brain to contribute to synucleinopathies, astrocytes are also found to express toll-like receptors (TLRs) and release proinflammatory cytokines and participate in α -syn-induced neuroinflammation (Farina et al., 2007). Therefore, α -syn plays a critical role in the etiology of synucleinologies: its closer relevance to innate and adaptive immune systems and its multi-systematic involvement in pathogenesis of synucleinologies.

Substantial evidence supports the central role of immune system in pathogenesis of PD. In human postmortem tissues and animal models of PD, abnormal α -syn expression is always accompanied by inflammation and immune responses including activated astrocytes and microglia, increased pro-inflammatory cytokines, infiltration of CD4 lymphocytes, MHCII expression and IgG deposition (Brochard et al., 2009; Harms et al., 2013; Hirsch & Hunot, 2009). This inflammatory process involves at least four components of canonical inflammatory pathway (González-Reyes et al., 2017): 1) the endogenous or exogenous stimulators, including microbial factors such as pathogen-associated molecular patterns (PAMPs), non-microbial factors such as toxic compounds, self-debris or damage-associated molecular patterns (DAMPs) (Béraud et al., 2011), and senescence-associated secretory phenotype (SASP) derived from tissue injury/malfunction and cell apoptosis/necroptosis (Pasparakis & Vandenabeele, 2015); 2) the receptors such as TLRs and cytokine receptors to recognize molecule alteration in extracellular microenvironment (Roodveldt et al., 2013); 3)

the downstream mediators such as nuclear factor (NF)- κ B, etc., to crosstalk with other stimulators or signal pathways and impact inflammatory outcomes; and 4) the effectors such as cytokines, vasoactive substances, complement components and proteolytic enzymes released from immune cells (e.g., macrophages) and tissue cells, determining neurotoxicity and cell fate. In this study, A53T mutant or WT α -syn was used as an antigenic stimulator to induce activation of canonical inflammatory pathway involving TLRs 1, 2, 3 and 4, NF- κ B and cytokines such as interleukin (IL)-1 β , interferon (IFN)- γ , IFN- β and tumor necrosis factor (TNF)- α .

Taken together, α -syn, as an abnormal metabolite of neurons, is released into extracellular space and induces activation of glial cells and inflammatory response, reflecting its immunogenic attribution (Lee et al., 2014; Roodveldt et al., 2013; Sanchez-Guajardo et al., 2013). However, correlation between α -syn stimulation and neurotoxic outcomes in astrocytes remains to be fully elucidated, especially in its dose and temporal stimulation patterns. In this study, we utilized A53T mutant or WT α -syn to stimulate astrocytes in dose- and time-dependent manners and systematically evaluated activation pattern of innate immunity and inflammatory outcomes by expression profiles of inflammatory pathway components (TLRs 1–4, NF- κ B, TNF- α , IL-1 β , IFN- γ and IFN- β) and use of TLR antagonists. Our findings provide a novel insight into the neurotoxic feature of α -syn and inflammatory mechanism underlying pathogenesis of synucleinopathies.

Materials and Methods

Experimental design

Our aim is to determine α -syn-induced activation pattern of canonical inflammatory pathway and neurotoxic outcomes. In experimental design, we gave the following considerations. 1) Choice of stimulator α -syns. A53T mutant α -syn is neurotoxic in familial PD, but no conclusive evidence shows that WT α -syn has a neurotoxic role in astrocyte-mediated neuroinflammation, although high amount of α -syn can self-assemble and trigger protein aggregation, a PD-inducing factor (Eschbach & Danzer, 2014). Thus, we mainly evaluated the neurotoxicity of A53T mutant α -syn, with WT α -syn as a control to compare their possible difference in stimulation effect; 2) Doses and time-points of stimulation. The varied physiological concentrations of α -syn in blood and CFS are detected at nanogram/milliliter level (Koehler et al., 2015; Lin et al., 2017), which are up to tenfold in serum and plasma and up to 10,000-fold higher in whole blood due to its abundant expression in hematopoietic system (Mollenhauer et al, 2011, 2012). Previous studies *in vitro* show a range of concentrations of α -syn available from 0.1 to 10 μ g/mL at one time-point (24 hour) (Roodveldt et al., 2010, 2013). In this study, we used four concentrations of 0.5, 2, 8, 20 μ g/mL with a fixed time-point 24 hour and four time-points of 3, 12, 24, 48 or 72 hour with a fixed concentration of 2 μ g/mL, to sufficiently show dose and temporal stimulation effects; 3) Choice of 90% cell confluency. α -Syn stimulation was not observed to effectively inhibit cell growth, and overgrowth (100%) of the cells gave rise to plenty of floating or dead cells, which may potentially interfere with the sensitive detection by qRT-PCR; 4) Determination of inflammatory pathway components: TLRs are the well-established receptors of innate immunity, and TLR2 or TLR3 is a representative of cell surface receptors and intracellular

receptors, respectively. TLR3 activation by α -syn is likely attributed to endocytosis of α -syn as evidenced previously. TLR1 and TLR4 were also detected for supporting purpose. NF- κ B is a core regulator of canonical inflammatory pathway that is known to crosstalk with many other bioactive factors. IL-1 β , TNF- α and IFN- γ are common effectors of inflammatory outcomes and in this study we focused on expression profile of IL-1 β as a representative of cytokines, although a similar induction pattern of TNF- α was also observed; and 5) Data normalization. Ct values from qRT-PCR can be normalized at Ct level, Ct level and Ct level according to the manufacturer's instruction (ThermoFisher Scientific, Waltham, MA, USA). In this study, we placed normalization at Ct values, which are closer to the final calculation by 2^{-Ct} and exclude calculation error and experimental error by control value calibration, and so the control values were all assigned at 1 in the mRNA-related data. Differently, control values from ELISA were directly calculated into concentration by index selection on Microplate Reader and applied to group comparison.

Chemicals and Reagents

Cell culture reagents were obtained from Gibco Fisher Scientific (Waltham, MA, USA): Advanced Dulbecco's modified Eagle medium (DMEM)-nutrient mixture F12 Ham (DMEM-F12), Hank's Balanced Salt Solution (HBSS), Fetal bovine serum (FBS), Penicillin/Streptomycin, Trypsin-EDTA, and TRIzol[®] Reagent. Cell treatment reagents were: WT and A53T mutant α -syns from rPeptide (Bogart, GA, USA), TLR4-IN-C34 (C34, TLR4 antagonist) and CU-CPT22 (CPT22, TLR2 antagonist) from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies were all rabbit anti-mouse IgG against: glial fibrillary acidic protein (GFAP), TLR2 and TLR3 from Abcam (Cambridge, MA, USA); IL-1 β , IFN- γ and β -Actin from ThermoFisher Scientific (Waltham, MA, USA). Other reagents included: Alexa Fluor[®] 514 goat anti-rabbit secondary antibody, reagent kits for reverse transcription and quantitative real-time PCR (qRT-PCR) and 4',6-Diamidino-2-Phenylindole (DAPI) purchased from ThermoFisher Scientific (Waltham, MA, USA); Enzyme-linked Immunoabsorbent Assay (ELISA) kits from R&D System (Minneapolis, MN, USA); lysis buffer, complete protease inhibitor cocktail and HRP-conjugated secondary antibody from Cell Signaling Technology (Beverly, MA, USA); IgG from rabbit serum (negative control) and Fluoromount[™] Aqueous Mounting Medium from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male and female C57BL/6 mice were purchased from Jackson Laboratories (Sacramento, CA) and were bred in animal facilities of Johns Hopkins University School of Medicine. All experimental procedures were approved by Institution's Animal Care Committee and performed in accordance with the guidelines by Institution of Laboratory Resources, National Research Council (Department of Health and Human Service, National Institutes of Health, Bethesda, MD).

Astrocyte Isolation and Cell Culture

According to an established protocol (Gorina et al., 2011, Han et al., 2000), mixed glial cells were prepared from 80 cerebral cortices of new-born (3–4 days) C57BL/6 mouse pups with hypothermia (on ice 20 minutes) anesthesia, and astrocytes were separated by differential

adhesion of cell types according to previously described method. Briefly, cerebral cortex was removed from brain and homogenized into single-cell suspension by mincing, trypsinizing and passaging through nylon mesh. Primary glial cells were washed and cultured for 16 hours in poly-D-lysine-coated 75 cm² flasks (4 pups/flask) with complete DMEM/F12 containing 10% FBS (heat-inactivated), 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified 5% CO₂, 95% air atmosphere at 37°C. After 16 hour incubation, horizontal shakes by hands and washes with HBSS were performed to remove oligodendrocytes and microglia, and astrocytes were adhered for further culture. Purity of astrocytes was determined by immunostaining for GFAP (a specific marker for astrocytes) and immunofluorescence microscopy. All astrocytes were cultured for a total of 7–10 days, and then shaken and washed again before they were detached from plates by trypsinization (0.05% trypsin EDTA), resuspended and plated in complete DMEM-F12 medium for planned experiments.

Immunofluorescence microscopy

Astrocytes were seeded and cultured on coverslips until confluent, and fixed in ice-cold solution of methanol and acetone (1:1) for 5 min. After they were rinsed twice in phosphate-buffered saline (PBS, pH7.4), the main steps below were performed with PBS washes of 10–15 minutes in between: 1) Glycine (2.25%, 20 minutes) was put to remove endogenous fluorescent; 2) Blocker buffer containing 1% bovine serum albumin (BSA), 0.3% Triton X-100 and 10% goat serum in PBS was made for blocking nonspecific staining at room temperature for 1 hour; 3) Primary antibody for GFAP (rabbit anti-mouse, 1:1000) and IgG from rabbit serum (negative control) were used at 4°C overnight; 4) Alexa Fluor® 514 fluorescein-conjugated goat-anti-rabbit antibody (1:1000) was added at room temperature for 1 hour; 5) DAPI was used for nuclear and chromosome counterstaining at 1 µg/mL for 10 minutes. After final wash in PBS, coverslips were mounted with Fluoromount™ Aqueous Mounting Medium and examined under fluorescence microscope (Nikon Model: ECLIPSE Ni-U, Tokyo, Japan).

Astrocyte treatment

Astrocytes were divided into multiple treatment groups in 6-well plates and cultured to about 90% confluency. Culture medium was replaced with fresh complete DMEM/F12 containing appropriate final concentration of individual drugs or their combinations (Mollenhauer et al, 2011, 2012). For dose dependency, WT α -syn or A53T mutant was set at 0.5, 2, 8, 20 µg/mL for a fixed time-point (24 hour); For time dependency, time-points were set at 3, 12, 24, 48 hour at a fixed concentration (2 µg/mL) of α -syn; For combination treatment, α -syn (2 µg/mL) and CPT22 (5 µg/mL) or C34 (5 µg/mL) co-incubated for a fixed time 24 hours. These treatment groups and control groups were cultured in a humidified 5% CO₂, 95% air atmosphere at 37°C for 24 hours unless otherwise indicated. Supernatants from each group were collected and stored at –80°C, and astrocytes were harvested with TRIzol Reagent (0.5 mL/well) or lysis buffer (0.2 mL/well) and properly stored according to instructions on qRT-PCR or western blotting analysis.

Determination of TLRs and cytokine gene expression

Expression of genes for TLRs 1–4, NF- κ B, IL-1 β , IFN- γ , IFN- β , and glyceraldehyde phosphate dehydrogenase (GAPDH, an internal control) was determined by using two-step qRT-PCR method. Total RNA was extracted from astrocytes of each group with TRIzol Reagent; RNA concentration was measured with NanoDropTM 2000 Spectrophotometer from Biotek (Winooski, VT, USA); RNA (2 μ g) was reverse-transcribed with High Capacity cDNA Reverse Transcription Kit according to manufacturer's protocol; and qRT-PCR was performed with *TaqMan*® Fast Advanced Master Mix and *TaqMan* primer and probe sets by using Stratagene Mx3000P Real Time PCR Systems from Bio-Rad (Hercules, CA, USA) according to manufacturer's protocol. Multiple transcripts prepared on a 96-well PCR plate were analyzed simultaneously under standardized thermal cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target transcripts were measured with *TaqMan* probes for TLR1 (Mm01208874_m1), TLR2 (Mm00442346_m1), TLR3 (Mm00628112_m1), TLR4 (Mm00445273_m1), NF- κ B (Mm00476361_m1), IL-1 β (Mm01336189_m1), IFN- γ (Mm01168134_m1), and IFN- β (Mm00439552_s1), and their relative abundance was normalized with respect to the expression of GAPDH (Mm03302249_g1). Changes in gene expression levels were determined by using C_t (cycle threshold) value taking off GAPDH, and fold differences in expression level of individual gene under each treatment condition over untreated control was calculated by the C_t method: C_t values were obtained by subtracting C_t values of untreated sample from that of treated sample according to manufacturer's instruction, with fold difference calculated by formula of 2^{-C_t} . All qRT-PCR reactions were run in duplicates and their average values were used in data analysis.

Western blotting

Protein expression of TLRs 2 and 3, IL-1 β and IFN- γ was determined by western blotting with β -actin as internal control. Briefly, astrocytes (1 $\times$ 10⁶ cells/well) treated in a 6-well plate were washed once with ice-cold PBS and lysed in lysis buffer supplemented with complete protease inhibitor cocktail and 1mM phenylmethanesulfonyl fluoride (PMSF, before use). Cell debris was removed by centrifugation at 13,000 g, 4°C for 30 minutes, and protein concentration was determined by Bradford reagent (Bio-Rad, Hercules, CA). A total of 30 μ g lysate protein for one lane was added to appropriate denaturing sample buffer and heated at 95°C for 5 minutes, and then was resolved by Mini-PROTEIN TGX Precast Gels from Bio-Rad and transferred to 0.45 μ m polyvinylidenedifluoride (PVDF) membrane (Millipore, USA) by using Bio-Rad Trans-Blot Semi-Dry Transfer Cell and System. After blocked in 5% milk at room temperature for 1 hour, the membrane was probed with appropriately diluted primary antibodies (TLR2 and TLR 3 at 1:300; IL-1 β and IFN- γ at 1:800, β -actin at 1:2000) in 5% milk at 4°C overnight and then hybridized with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) at room temperature for 1 hour. Results were visualized by using Enhanced Chemiluminescence (ECL) Substrate Kit (Bio-Rad, Hercules, CA, USA) and films were exposed and developed in dark room according to manufacturer's instructions. To confirm equal loading of proteins in each lane, membrane was re-probed for β -actin following removal of background bands by incubation at 37°C for 30 minutes with Gentle ReviewTM Stripping Buffer (Amresco-inc, Solon, USA).

Cytokine release measurements

Supernatants collected from each group were centrifuged at 300 g at 4°C for 10 minutes, and then stored at -80°C or used for measurement of cytokine release. IFN- γ was chosen as a typical representative of proinflammatory cytokines to assess neurotoxicity in astrocytes. IFN- γ protein levels in each supernatant were measured by using specific sandwich ELISA kits according to manufacturer's instruction, and the normalized concentrations were automatically generated through 460 nm filter plus 570 nm subtraction on Microplate Reader (Bio-Rad, Hercules, CA, USA).

Data analysis

Data are expressed as means $\pm$ standard deviation and scatter charts, displaying sample distribution, sample number (n), means ($-$) and p values in group comparison, and statistically analyzed on Ct values. Fold difference in means was determined by $2^{-\Delta\Delta Ct}$ calculation and $2^{-\Delta\Delta Ct}$. Dose- and time-dependent effects of single drugs were evaluated statistically by one-way randomized blocks ANOVA, in combination with Fisher's least significant differences (LSD), for comparison of different treatment levels, whereas paired comparison between one and combined treatments at each level were performed by two-way ANOVA methods combined with Margin Means. Normality and homogeneity of variance were examined by Shapiro-Wilk test and Levene test at a 0.05 significance level, respectively. When either assumption was significant, statistical analysis was performed on suitably transformed data. In addition, release levels (IFN- γ) by ELISA are statistically presented as concentration values, and protein levels (TLR2, TLR3, IL-1 β and IFN- γ) are statistically presented as grayscale values from densitometry (ImageJ software). All statistical analyses were conducted using SAS software (SAS 9.1.3, SAS Institute Inc., Cary, NC), and statistically significant differences were defined as $p < 0.05$, with p values labeled in figures.

Results

Identification of primary astrocytes

GFAP is an intermediate filament specifically expressed in astrocytes, and immunofluorescence microscopy showed that the GFAP-positive astrocytes comprised a heterogeneous population: small-, medium- and large-sized cells with multiple primary and secondary branches that generally extend to outer space in a long distance (Fig. 1a). This morphological characteristic may reflect multiple cell division phases of astrocytes, which is different from appearance of microglia (1–2 very short branches). Purity of astrocytes was determined by calculating the proportion of GFAP-positive cells in total numerated cells (DAPI-positive nuclei staining) within five randomly-selected areas under microscope, showing that approximately 94% ($93.96 \pm 4.03\%$) of DAPI-positive cells were GFAP-positive. Meanwhile, IgG from rabbit serum, the equivalent to GFAP antibody, was used as negative control for the immunostaining procedure and microscope indices, and no GFAP-positive cells were observed (Fig. 1b).

α -Syn induced activation of TLRs 1, 2, 3 and 4 and NF- κ B in astrocytes

TLRs are a group of type I transmembrane glycoprotein, responsible for activation of innate immunity by recognizing foreign pathogens such as PAMPs or endogenous antigens such as cell debris, DAMPs and SASP. In the thirteen TLR functional components detected so far, TLRs 1, 2, 4, 5 and 6 are expressed on cell surface for extracellular recognition and TLRs 3, 7, 8 and 9 are localized on endosomes or lysosomes for detecting intracellular molecules such as viral or bacterial nucleic acids. In this study, TLR2, TLR3 and NF- κ B, three components of canonical inflammatory pathway, were determined following stimulation of A53T mutant or WT α -syn, with TLR1 and TLR4 detection only for the supporting purpose.

In dose-dependent stimulation, A53T mutant and WT α -syns increasingly upregulated mRNA expression of TLR2 and TLR3 with dose increase, displaying a induction pattern of positive dosage-effect correlation (PDEC) (Fig. 1c,d). The significant difference at Ct ($p < 0.05$) between dose treatment groups was statistically shown as: **TLR2** (Fig. 1c): A53T 2 μ g/mL (A53T-2) (-1.406 ± 0.272 , $n = 9$) versus A53T-8 (-1.948 ± 0.231 , $n = 5$) at $p = 0.0442$; WT 0.5 μ g/mL (WT-0.5) (-0.530 ± 0.243 , $n = 4$) versus WT-20 (-1.020 ± 0.948 , $n = 4$) at $p = 0.0445$; **TLR3** (Fig. 1d): A53T-0.5 (-0.226 ± 0.107 , $n = 5$) versus A53T-2 (-0.679 ± 0.223 , $n = 11$) at $p = 0.0019$; A53T-2 (-0.679 ± 0.223 , $n = 11$) versus A53T-8 (-10.84 ± 0.085 , $n = 5$) at $p = 0.0049$; WT-0.5 (0.260 ± 0.131 , $n = 4$) versus WT-2 (-0.270 ± 0.183 , $n = 4$) at $p = 0.0134$. In time-dependent stimulation, maximal mRNA level of TLR2 was observed at 3 hour (early time-point), whereas TLR3 maximal level appeared at 12 hour, and then both levels declined at later time-points (24 or 48 hour) (Fig. 1c,d), showing a tendency of negative time-effect correlation (NTEC). This delayed upregulation in TLR3 expression may be related to its intracellular localization compared to TLR2 expression. The significant difference at Ct ($p < 0.05$) between time treatment groups was statistically shown as: **TLR2** (Fig. 1c): A53T 3 hour (-3.112 ± 0.266 , $n = 5$) versus A53T 12 hour (-2.590 ± 0.225 , $n = 4$) at $p = 0.0463$; A53T 12 hour (-2.590 ± 0.225 , $n = 4$) versus A53T 24 hour (-1.406 ± 0.272 , $n = 9$) at $p = < 0.0001$; WT 12 hour (-2.027 ± 0.705 , $n = 4$) versus WT 24 hour (-0.803 ± 0.440 , $n = 4$) at $p = 0.0006$; **TLR3** (Fig. 1d): A53T 3 hour (-0.145 ± 0.288 , $n = 6$) versus A53T 12 hour (-2.077 ± 0.504 , $n = 6$) at $p = < 0.0001$; A53T 12 hour (-2.077 ± 0.504 , $n = 6$) versus A53T 24 hour (-0.679 ± 0.223 , $n = 11$) at $p = < 0.0001$. Stimulation at 72 hour tended to reverse this decline in TLR2 or TLR3 expression. In almost all treatment groups, A53T mutant α -syn had higher induction amplitude than WT α -syn in TLR2 or TLR3 mRNA expression (Fig. 1c,d), as statistically shown at Ct between treatment groups: **TLR2** (Fig. 1c): WT-2 (-0.802 ± 0.440 , $n = 4$) versus A53T-2 (-1.406 ± 0.272 , $n = 9$) at $p = 0.0428$; **TLR3** (Fig. 1d): WT-2 (-0.270 ± 0.183 , $n = 4$) versus A53T-2 (-0.679 ± 0.223 , $n = 11$) at $p = 0.0164$; WT-8 (-0.583 ± 0.163 , $n = 4$) versus A53T-8 (-1.084 ± 0.085 , $n = 5$) at $p = 0.0094$. As a supporting research, observation on TLR1 and TLR4 expression also showed an upregulation with dose increase (Fig. 2b), as statistically shown as: **TLR1** (Fig. 2b): A53T-0.5 (-1.054 ± 0.215 , $n = 3$) versus A53T-2 (-0.541 ± 0.114 , $n = 4$) at $p = 0.0482$; A53T-2 (-0.541 ± 0.114 , $n = 4$) versus A53T-8 (-0.343 ± 0.072 , $n = 3$) at $p = 0.0357$; **TLR4** (Fig. 2c): A53T-0.5 (-1.441 ± 0.197 , $n = 3$) versus A53T-20 (-1.027 ± 0.852 , $n = 3$) at $p = 0.0475$. Western blotting analysis (Fig. 1e) showed that protein level of TLR2 or TLR3 were significantly enhanced by A53T mutant α -syn at 20 μ g/mL or 0.5–20 μ g/mL, respectively (Fig. 1f,g), indicating a difference in their sensitivity to α -syn stimulation. The significant

difference at grayscale ($p < 0.05$) between treatment groups was statistically shown as: **TLR2** (Fig. 1f): control (0.311 ± 0.045 , $n = 3$) versus A53T-20 (0.446 ± 0.052 , $n = 3$) at $p = 0.0419$; **TLR3** (Fig. 1g): control (0.386 ± 0.081 , $n = 3$) versus A53T-2 (1.078 ± 0.149 , $n = 3$) at $p = 0.0353$; control (0.386 ± 0.081 , $n = 3$) versus A53T-8 (1.155 ± 0.181 , $n = 3$) at $p = 0.0284$; control (0.386 ± 0.081 , $n = 3$) versus A53T-20 (1.257 ± 0.153 , $n = 3$) at $p = 0.0197$; A53T 3 hour (0.487 ± 0.099 , $n = 3$) versus A53T 12 hour (2.375 ± 0.259 , $n = 3$) at $p = < 0.0001$; A53T 12 hour (2.375 ± 0.259 , $n = 3$) versus A53T 24 hour (1.078 ± 0.149 , $n = 3$) at $p = 0.0014$. Moreover, at 12 hour, protein level of TLR3 was maximally enhanced, and declined to an appropriate level (Fig. 1g), which is a similar pattern to its mRNA expression. Collectively, A53T mutant and WT α -syns can activate expression of TLR2 and TLR3 at both mRNA and protein levels, with A53T mutant having more activation effect than WT α -syn.

NF- κ B, as a core regulator in inflammatory signal pathway, has a widespread molecular connection with its upstream molecules such as TLRs, its downstream effectors cytokines and other signal pathways. Stimulation of A53T mutant moderately upregulated NF- κ B mRNA expression with a significant difference between high dose (8, 20 μ g/mL) and low dose (0.5, 2 μ g/mL) groups or between early (3, 12 hour) and later (24, 48 hour) time-point groups (Fig. 2a). The significant difference at Ct ($p < 0.05$) between treatment groups was statistically shown as (Fig. 2a): A53T-2 (-0.49 ± 0.394 , $n = 8$) versus A53T-8 (-1.263 ± 0.376 , $n = 4$) at $p = 0.0117$; A53T 12 hour (-1.288 ± 0.354 , $n = 4$) versus A53T 24 hour (-0.49 ± 0.394 , $n = 8$) at $p = 0.0107$. This expression pattern was similar to those of TLR2 and TLR3, reflecting participation of NF- κ B in α -syn-induced inflammatory signal pathway. By contrast, WT α -syn upregulated NF- κ B expression only at early time-points (3 and 12 hour) (WT 12 hour (-1.043 ± 0.266 , $n = 4$) versus WT 24 hour (-0.553 ± 0.109 , $n = 4$) at $p = 0.0461$), which then declined later on, with no significant induction effect at 0.5–20 μ g/mL WT α -syn (Fig. 2a). In addition, the induction amplitude by A53T mutant was higher than WT α -syn at 8 and 20 μ g/mL (Fig. 2a), as statistically shown at Ct: WT-20 (-0.507 ± 0.102 , $n = 4$) versus A53T-20 (-1.530 ± 0.154 , $n = 4$) at $p = 0.001$.

α -Syn induced activation of cytokines in astrocytes

Cytokines, as effectors of inflammatory outcomes, contribute to a wide variety of neuroinflammatory disorders such as synucleinopathies, involving activation of glial cells and concomitant innate immune response. Expression and release of these cytokines is a pivotal immunological issue linked to neurotoxicity of extracellular space and neuronal injury. Accordingly, in this study, we determined α -syn-induced expression of cytokines IL-1 β , IFN- γ and IFN- β at mRNA or protein level in dose- and time-dependent manners.

Following stimulation of A53T mutant or WT α -syn, IL-1 β mRNA expression was significantly up-regulated in almost all treatment groups (Fig. 3a), displaying the typical induction patterns of PDEC and NTEC as evidenced in TLRs detection, and IFN- β expression was merely up-regulated by A53T mutant other than WT α -syn in most treatment groups (Fig. 3b), indicating involvement of IFN- β in A53T mutant α -syn-induced astrocyte activation. The significant difference at Ct ($p < 0.05$) between dose treatment groups was statistically shown as: **IL-1 β** (Fig. 3a): A53T 0.5 μ g/mL (A53T-0.5) (-3.737

± 0.534 , $n=7$) versus A53T-2 (-6.530 ± 0.903 , $n=7$) at $p=0.0269$; A53T-2 (-6.530 ± 0.903 , $n=7$) versus A53T-8 (-8.698 ± 0.637 , $n=5$) at $p=0.0428$; A53T-8 (-8.698 ± 0.637 , $n=5$) versus A53T-20 (-9.558 ± 0.354 , $n=4$) at $p=0.0394$; WT-0.5 (-4.553 ± 0.463 , $n=4$) versus WT-2 (-7.135 ± 0.432 , $n=4$) at $p=0.0013$; WT-2 (-7.151 ± 0.432 , $n=4$) versus WT-8 (-9.09 ± 0.438 , $n=4$) at $p=0.0381$; **IFN- β** (Fig. 3b): A53T-0.5 (-0.9901 ± 0.243 , $n=7$) versus A53T-2 (-1.462 ± 0.267 , $n=6$) at $p=0.0483$. The significant difference at Ct ($p < 0.05$) between time treatment groups was statistically shown as: **IL-1 β** (Fig. 3a): A53T 12 hour (-9.255 ± 0.586 , $n=6$) versus A53T 24 hour (-6.530 ± 0.903 , $n=7$) at $p < 0.0001$; A53T 24 hour (-6.530 ± 0.903 , $n=7$) versus A53T 48 hour (-4.637 ± 0.381 , $n=6$) at $p < 0.0001$; WT 12 hour (-9.415 ± 0.431 , $n=4$) versus WT 24 hour (-7.135 ± 0.432 , $n=4$) at $p=0.0006$; WT 24 hour (-7.135 ± 0.432 , $n=4$) versus WT 48 hour (-4.637 ± 0.290 , $n=4$) at $p=0.0492$; **IFN- β** (Fig. 3b): A53T 24 hour (-1.462 ± 0.267 , $n=6$) versus A53T 48 hour (-0.614 ± 0.195 , $n=5$) at $p=0.0472$; WT 3 hour (-0.383 ± 0.106 , $n=4$) versus WT 12 hour (-1.372 ± 0.591 , $n=4$) at $p=0.0358$; WT 12 hour (-1.372 ± 0.591 , $n=4$) versus WT 24 hour (-0.352 ± 0.209 , $n=4$) at $p=0.0295$. By contrast, IFN- γ expression was significantly down-regulated by both A53T mutant and WT α -syns with a dose-related effect and no time-related effect (Fig. 3c), indicating a negative correlation between IFN- γ expression and α -syn induction. The significant difference at Ct ($p < 0.05$) between treatment groups was statistically shown as (Fig. 3c): A53T-0.5 (0.196 ± 0.351 , $n=8$) versus A53T-8 (2.243 ± 0.581 , $n=6$) at $p < 0.0001$; A53T-0.5 (0.196 ± 0.351 , $n=8$) versus A53T-20 (1.824 ± 0.302 , $n=6$) at $p < 0.0001$; WT-0.5 (-0.895 ± 0.271 , $n=4$) versus WT-8 (2.365 ± 0.403 , $n=4$) at $p=0.0314$; WT 3 hour (0.495 ± 0.177 , $n=4$) versus WT 24 hour (1.463 ± 0.155 , $n=4$) at $p=0.0075$.

Western blotting analysis (Fig. 3e) showed that IL-1 β protein level was reduced at 8 and 20 $\mu\text{g/mL}$ in A53T mutant α -syn dose-related induction (Fig. 3f), an inconsistent induction pattern with PDEC at its mRNA level. The significant difference at grayscale ($p < 0.05$) between dose treatment groups was statistically shown as (Fig. 3f): control (0.967 ± 0.127 , $n=3$) versus A53T-8 (0.454 ± 0.157 , $n=3$) at $p=0.0248$; control (0.967 ± 0.127 , $n=3$) versus A53T-20 (0.421 ± 0.161 , $n=3$) at $p=0.0216$. This abnormal phenomenon may be attributed to a potential exhaustive secretion or release of IL-1 β under high-dose α -syn stimulation although no endotoxin or intrinsic toxicity of A53T mutant is detected by manufacturer. At time-point of 3 hour, IL-1 β level was obviously enhanced (control (0.967 ± 0.127 , $n=3$) versus A53T 3 hour (1.549 ± 0.194 , $n=3$) at $p=0.0429$) and then declined later on, a consistent pattern with NTEC at its mRNA level (Fig. 3f), indicating that α -syn exerts a powerful stimulation action at early time-points and subsequent exhaustive release may occur with time extension. Likewise, IFN- γ protein was detected at 19 kDa with a weak signal (Fig. 3e), showing that its protein levels were significantly reduced in almost all treatment groups compared to the control protein level, but at 48 hour, the protein level was somewhat reversed (Fig. 3g). The significant difference at grayscale ($p < 0.05$) between dose treatment groups was statistically shown as (Fig. 3g): control (0.395 ± 0.0724 , $n=3$) versus A53T-2 (0.136 ± 0.057 , $n=3$) at $p=0.0358$; control (0.395 ± 0.0724 , $n=3$) versus A53T-8 (0.146 ± 0.0472 , $n=3$) at $p=0.0403$; control (0.395 ± 0.0724 , $n=3$) versus A53T 3 hour (0.117 ± 0.0442) at $p=0.0429$. Furthermore, extracellular release profile of IFN- γ was detected by ELISA: release level was significantly reduced at 20 $\mu\text{g/mL}$ of A53T mutant or

WT α -syn, or reduced at 3 and 12 hour by WT α -syn other than A53T mutant (Fig. 3d), indicating a specific correlation of IFN- γ release and WT α -syn induction. The significant difference at protein release ($p < 0.05$) between treatment groups was statistically shown as (Fig. 3d): control (3.936 ± 1.961 , $n = 10$) versus A53T-20 (0.4682 ± 1.457 , $n = 5$) at $p = 0.0376$; control (3.936 ± 1.961 , $n = 10$) versus WT-2 (2.104 ± 0.707 , $n = 4$) at $p = 0.0418$; WT-2 (2.104 ± 0.707 , $n = 4$) versus WT-20 (1.556 ± 0.354 , $n = 4$) at $p = 0.0474$; WT 3 hour (0.713 ± 0.224 , $n = 4$) versus A53T 3 hour (3.225 ± 0.994 , $n = 5$) at $p = 0.0464$; WT 12 hour (1.458 ± 0.356) versus A53T 12 hour (2.547 ± 0.942) at $p = 0.0417$.

Collectively, A53T mutant or WT α -syn potentiated expressions of cytokines IL-1 β and IFN- β at mRNA level to various degrees, generally showing two types of induction patterns, i.e., PDEC and NTEC. However, high dose A53T mutant α -syn reduced intracellular protein level of IL-1 β , indicating a possible exhaustive effect by α -syn, and both A53T mutant and WT α -syns suppressed protein expression and release of IFN- γ as evidenced by qRT-PCR, western blotting and ELISA, strongly indicating a possible negative correlation of α -syn with IFN- γ other than IFN- β in murine astrocyte activation.

TLR2 blockage suppressed α -syn-induced activation of innate immunity

To explore the role of TLRs in α -syn-induced activation of innate immunity, we chose TLR2 antagonist (CPT22, 5 μ g/mL) and TLR4 antagonist (C34, 5 μ g/mL) to coinubate with A53T mutant α -syn (2 μ g/mL) and then determined expression profiles of inflammatory pathway components. Additive application of CPT22 significantly downregulated mRNA expressions of TLR3 (Fig. 4a) and IL-1 β (Fig. 4e), along with compensatory upregulation of TLR2 (Fig. 4a) or NF- κ B expression (Fig. 4c), indicating an interaction existing between TLR2 and TLR3. By contrast, C34 had no significant effect on TLR2 or TLR3 expression (Fig. 4b) but upregulated NF- κ B (Fig. 4d) and IL-1 β (Fig. 4f) expressions, a different regulation pattern from CPT22, indicating a functional difference between TLR2 and TLR4 in α -syn induction process. The significant difference at Ct ($p < 0.05$) between treatment groups was statistically shown as: **TLR2** (Fig. 4a): A53T (-1.406 ± 0.272 , $n = 8$) versus A53T/CPT22 (-1.881 ± 0.353 , $n = 7$) at $p = 0.0056$; **TLR3** (Fig. 4a): A53T (-0.6791 ± 0.223 , $n = 11$) versus A53T/CPT22 (-0.095 ± 0.353 , $n = 6$); **NF- κ B** (Fig. 4c,d): A53T 3 hour (-1.405 ± 0.676 , $n = 4$) versus A53T/CPT22 3 hour (-2.675 ± 0.318 , $n = 4$) at $p = 0.0027$; A53T 12 hour (-1.288 ± 0.354 , $n = 4$) versus A53T/CPT22 12 hour (-2.955 ± 0.262 , $n = 4$) at $p = 0.0023$; A53T 3 hour (-1.405 ± 0.676 , $n = 4$) versus A53T/C34 3 hour (-2.728 ± 0.317 , $n = 4$) at $p = 0.0133$; A53T 12 hour (-1.288 ± 0.354 , $n = 4$) versus A53T/C34 12 hour (-2.185 ± 0.318 , $n = 4$) at $p = 0.0492$; **IL-1 β** (Fig. 4e,f): A53T 12 hour (-9.255 ± 0.586 , $n = 5$) versus A53T/CPT22 12 hour (-6.615 ± 0.530 , $n = 4$) at $p = 0.006$; A53T 24 hour (-6.538 ± 0.903 , $n = 7$) versus A53T/CPT22 24 hour (-4.029 ± 1.189 , $n = 8$) at $p = < 0.0001$; A53T 12 hour (-9.255 ± 0.586 , $n = 5$) versus A53T/C34 12 hour (-10.145 ± 0.332 , $n = 4$) at $p = 0.0395$. Taken together, blockage of TLR2 other than TLR4 suppressed α -syn-induced activation of innate immunity by downregulating TLR3 and IL-1 β expressions, reflecting that TLR2 and TLR4 differently mediate α -syn-induced innate immunity, and TLR2 or TLR4 blockage does not inhibit NF- κ B activity. However, the interaction mechanism of α -syn, TLRs and cytokines is complex and needs to be further investigated elsewhere.

Discussion

Despite much progress made in recent years, the underlying mechanism that α -syn triggers onset of several synucleinopathies such as AD, PD and MSA, remains to be fully elucidated. Chronic neuroinflammation and activated astrocyte and microglia are the common feature of these diseases (Harms et al., 2013; Rivest, 2009), and dysfunctional α -syn, as an endogenous immunogenic substance or cell debris like DAMPs or SASP, may be a pivotal risk factor to exacerbate such a neuroinflammatory process (Barcia et al., 2012). Among multiple isoforms of α -syn, A53T mutant α -syn is proven to be neurotoxic, but there is not enough evidence for WT α -syn neurotoxicity. In this study, we demonstrated that both A53T mutant and WT α -syns induced astrocyte-mediated innate immune response, which is presented by activation of canonical inflammatory pathway components such as TLRs 1–4 and NF- κ B and upregulated expression of IL-1 β and IFN- β . However, IFN- γ , as an important effector or regulator cytokine in inflammatory response, was unexpectedly downregulated at its mRNA, protein and protein release levels. Additive application of TLR2 antagonist (CPT22) partly inhibited α -syn-induced expressions of TLR3 and IL-1 β , whereas TLR4 antagonist (C34) did not do so. These results suggest that α -syn is a TLRs-mediated powerful immunogenic agent and can activate canonical inflammatory signal pathway to regulate innate immunity, with A53T mutant having a stronger induction effect than WT α -syn.

Both astrocytes and microglia are the important players of immune activation and neuroinflammation in neurodegenerative pathogenesis (Barcia et al., 2012; Desplats et al., 2009; Hirsch & Hunot, 2009; Ma et al., 2013), and contribute to formation of inflammatory microenvironment responsible for adjacent neuronal damage. While research predominantly focuses on microglia involvement in synucleinopathies (Roodveldt et al., 2013), however, α -syn-induced astrocyte activation is still not fully investigated or only supported by limited literature. For example, α -syn released from neuron is directly transferred to astrocytes by sequential exocytosis and endocytosis and induces inflammatory response (Lee et al., 2010), and its mutated isoforms (A30P, E46K, and A53T) have more stimulation effect than WT α -syn in release of IL-6 and ICAM-1 and up-regulation of mitogen-activated protein kinase (MAPK) pathway in astrocytes and astrocytoma cells (Klegeris et al., 2006). Astrocyte activation degree *in vivo* is inversely proportional to distance to α -syn inclusions, along with widespread neuroinflammation in α -syn-injected forebrain (Radford et al., 2015). In astrocytoma cell culture, co-incubation of α -syn and IL-1 β potentiates protein expression but not RNA expression of CXCL10 (a proinflammatory chemokine), highlighting the importance of α -syn in modulating inflammatory events in astroglia (Tousi et al., 2012). In primary astrocytes, A53T mutant α -syn triggers neuroinflammatory response by upregulating expression of GFAP, cyclooxygenase-2 (COX-2) and inducible nitric oxide (NO) synthase (iNOS), and by increasing production of TNF- α and IL-1 β (Yu et al., 2018). This study, for the first time, reported in-depth characterization of astrocyte-mediated innate immune response induced by A53T mutant and WT α -syn, suggesting the importance of astrocyte activation in mediating neuroinflammation in synucleinopathies (Roodveldt et al., 2010).

TLRs are known as a family of transmembrane proteins to recognize extracellular immunogenic molecules and directly trigger innate immune pathways, leading to subsequent inflammatory response (Kawai & Akira, 2010). Although correlation of TLRs activation and α -syn stimulation has been described (Fellner et al., 2013), whether TLRs mediate α -syn-induced astrocyte activation and innate inflammatory response is still uncertain. In microglia culture, misfolded α -syn (50 ng/mL, 24 hours) up-regulates TLR2 and TLR3 expressions, with no effect on NF- κ B and TNF- α (Béraud et al., 2011), but in spinal cord injury, TLR2 rather than TLR3 or TLR4 mediates microglia activation by α -syn (Qian et al., 2015); Application of specific TLR agonists Pam3Csk4 and single-stranded RNA40 in microglia confirms differential effects of cell surface TLR1/2 and intracellular TLR7 on α -syn-induced innate immunity, indicating TLR stimulation affects α -syn-induced response (Roodveldt et al., 2013); In PD brain pathology *in vivo*, different α -syn aggregates trigger inflammatory response through activation of TLR2, suggesting an association of α -syn cytotoxicity with its intrinsic structure (Gustot et al., 2015); α -Syn released from neuron into extracellular space is tested as an endogenous agonist for TLR2 to activate microglia-mediated inflammatory response (Kim et al., 2013; Roodveldt et al., 2013). In this study, we demonstrated that A53T mutant or WT α -syn induced activation of TLRs 1–4 in a PDEC or NTEC pattern, and also observed the induction pattern difference between cell surface TLR2 and intracellular TLR3, reflecting a possible biological implication: under α -syn stimulation, TLR2 mediates extracellular inflammatory pathway, while TLR3 mediates intracellular inflammatory pathway via α -syn endocytosis (Watson et al., 2012). Moreover, additive application of antagonists indicates that TLR2, TLR3 and TLR4 are differently involved in α -syn-induced inflammatory process. Our result that blockage of α -syn stimulation by TLR2 antagonist downregulated TLR3 expression is also consistent with the data from transgenic mice and *in vitro* models, which demonstrates that inactivation of TLR2 increases clearance of neuronal α -syn by activation of autophagy and reduces intracellular accumulation of α -syn (a TLR3 stimulator), and vice versa (Kim et al., 2015). Likewise, TLR4 knockout technique shows participation of TLR4 in activation of microglia and astrocytes induced by WT α -syn (0.7 μ M, 72 hour), but with no influence on uptake of α -syn in astrocytes other than microglia (Fellner et al., 2013; Rannikko et al., 2015), indicating lack of direct involvement of TLR4 in intracellular accumulation of α -syn linked to TLR3 activation, as observed in our study.

NF- κ B, a pleiotropic regulator of target genes controlling physiological function and pathological process of neurodegeneration, plays a critical role in inflammatory pathway activation and production of cytokines/chemokines by glial cells (Lanzillotta et al., 2015). Its family members such as Rel-A (p65), Rel-B, c-Rel, NF- κ B1 (p50/p105) and NF- κ B2 (p52/p100) can interact to form a cardinal controller of multiple signal pathways and participate in brain programming of many disease processes. Association of α -syn with NF- κ B activation has been demonstrated. For instance, in murine microglia (BV2), exposure of α -syn causes migration of NF- κ B p65 to nucleus (Couch et al., 2011), and however, this is not observed in TLR4 knockout astrocytes (Rannikko et al., 2015). Likewise, Reynolds and colleagues have shown that exposure of aggregated and nitrated α -syn modestly increases expression of all NF- κ B subunits in microglia with a peak at as early as 4 hour post-stimulation (Reynolds et al., 2008), a similar pattern to what we observed in primary murine astrocytes in this study.

These data support our results that A53T mutant α -syn significantly upregulated NF- κ B expression at its high dose (8 and 20 μ g/mL) and early time-points (3 and 12 hour), indicating a potential role of NF- κ B pathway activation in α -syn induction of astrocyte-mediated neuroinflammation.

Proinflammatory cytokines are deleterious effectors of inflammatory response, determining immune activation status and cytotoxic outcomes. This process involves a range of pro-inflammatory and cytotoxic molecules such as TNF- α , IL-6, IL-1 β , superoxide, nitric oxide, reactive oxygen species and excitatory amino acids, and several anti-inflammatory cytokines such as IL-10, IL-13, IL-14 and transforming growth factor- β (Kettenmann et al., 2011; Vieira et al., 2015). Accumulation of α -syn is a core feature of PD brain and leads to microglia and astrocyte activation and inflammatory cytokine/chemokine production. For example, in mouse microglia (BV-2), conditioned medium from SH-SY5Y cells over-expressing A53T mutant or WT α -syn, induces transcription and release of pro-inflammatory cytokines (Alvarez-Erviti et al., 2011). In α -syn endocytosis study in astrocytes, intracellular α -syn induces gene expression profile of pro-inflammatory cytokines and chemokines such as CXCL10, CCL2 and IL-6, which is correlated with the extent of α -syn accumulation (Farina et al., 2007). Similar to our results in Western blot analysis, A53T mutant or WT α -syn (1–5 μ g/mL, 20 hours) does not induce production of TNF- α , IL-1 β and IFN- γ in primary glial cell culture by ELISA (Roodveldt et al., 2010). Importantly, in α -syn-transgenic mouse model, mRNA expressions of cytokines such as TNF- α , IL-1 β and IL-6 are upregulated at 1, 6 and 12 month, with maximal expression at 1 month which subsequently reduces to the minimal at 12 month (Su et al., 2009), a temporal pattern with much longer time intervals similar to the NTEC observed in our study. In addition, we also identified the pattern of PDEC in α -syn-induced mRNA expression of IL-1 β , IFN- β and TNF- α (similar data not shown). These temporal and dose induction patterns suggest that persistent release and accumulation of α -syn might be required for initiation and maintenance of inflammatory response in the synucleinopathy (Alvarez-Erviti et al., 2011).

Moreover, we are the first to confirm the dose-related inhibitory effect of α -syn (A53T mutant or WT) on IFN- γ expression and production in primary murine astrocytes. This unexpected finding deserves further discussion and explanation: 1) We simultaneously observed upregulation of IFN- β , a cousin molecule of IFN- γ in innate immunity, providing a positive control. This result may also suggest differential roles of IFN- γ and IFN- β in α -syn-induced astrocyte activation; 2) A spatio-temporal differential distribution of IFN- γ expression has been observed in the brain. For example, a mutually exclusive distribution of α -syn and IFN- γ , i.e., their strong negative correlation is observed in the healthy substantia nigra, whereas in the PD substantia nigra, their co-expression or positive correlation is only detected (Liscovitch & French, 2014). Likewise, IFN- γ high expression is mainly distributed in microglia from PD monkey substantia nigra, whereas TNF- α is highly expressed in astrocytes but not in microglia (Barcia et al., 2012); 3) The negative association between IFN- γ and other cytokines has also been demonstrated: co-exposure of IFN- γ with IL-1 β or TNF- α leads to an inhibitory effect of IL-1 β on IFN- γ expression in astrocytes and production of nitric oxide in microglia (Tichauer et al., 2007); and 4) IFN- γ , primarily as a Th1-type proinflammatory cytokine, has also been shown to possess anti-inflammatory

property at least in transgenic mouse model of experimental autoimmune encephalomyelitis (Mangalam, AK, et al. 2014). Possibly in a highly inflammatory environment of synucleinopathy, IFN- γ could play an anti-inflammatory role, and by suppressing its expression, α -syn and its A53T mutant could further promote neuroinflammation. Collectively, the immune mechanism by which α -syn induces astrocyte activation and subsequent neuroinflammation is likely complex and our observation may provide an evidence for a novel immune molecule interaction mode that deserves further investigation.

Conclusions

As shown in Graphical Abstract Figure (Kawai & Akira, 2010), our data collectively confirm that A53T mutant or WT α -syn upregulated mRNA expression of the canonical inflammatory pathway components such as TLRs 1–4, NF- κ B and cytokines IL-1 β , IFN- β . Our in-depth analyses showed two types of induction patterns, i.e., PDEC and NTEC and a possible receptor interaction between TLR2 and TLR3 by antagonizing TLR2 and TLR4. In this process, however, IFN- γ expression was significantly downregulated at mRNA, protein or protein release level, indicating a complex interaction of these inflammatory molecules. Therefore, we conclude that α -syn activates astrocyte-mediated innate immunity, suppresses IFN- γ expression and shows its immunogenic attribution, with A53T mutant having a stronger induction effect than WT α -syn. These findings may unravel a potential neurotoxic mechanism in synucleinopathies: a persistent release and accumulation of α -syn is required for maintenance of innate immunity activation, and cell type-dominant expression of α -syn, IFN- γ , IL-1 β and TNF- α and their interaction in extracellular space might provide a novel immunological mechanism underlying inflammatory pathogenesis of neurodegenerative diseases.

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Abbreviations

AD	Alzheimer's disease
ANOVA	analysis of variance
C34	TLR4-IN-C34
CPT22	CU-CPT22
DAMPs	damage-associated molecular patterns
DMEM	Dulbecco's modified Eagle medium
ELISA	enzyme-linked immunoabsorbent assay
FBS	fetal bovine serum

GAPDH	glyceraldehyde phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
IFN-γ	interferon- γ
IL-1β	interleukin-1 β
LSD	fisher's least significant differences
NF-κB	nuclear factor-kappa B
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PD	Parkinson's disease
qRT-PCR	quantitative real-time polymerase chain reaction
SASP	senescence-associated secretory phenotype
TLR	toll-like receptor
TNF-α	tumor necrosis factor- α

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Figure 1.

The expression profiles of TLR2 and TLR3 induced by A53T mutant or wild-type (WT) α -syn in primary astrocytes. **a.** A representative image showing GFAP-immunolabeled astrocytes, with a heterogeneous population accounting for 94% (93.96±4.03%) of total cells, Scale Bar: 50 μ m. **b.** The paralleling immunostaining for IgG equivalent to GFAP antibody, showing no immune-positive cells detected. Scale Bar: 50 μ m. **c,d.** mRNA expressions of TLR2 and TLR3 were significantly up-regulated by A53T mutant or WT α -syn, as shown with a p value ($p < 0.05$) between treatment groups, displaying two types of induction patterns: positive dose-effect correlation and negative time-effect correlation. **e.** Western blotting analysis for TLR2 (140 kDa), TLR3 (104 kDa), and β -actin (45 kDa), a representative of three experiments. The signal size was identified according to manufacturer's instruction, and β -actin was used as reference control. **f,g.** Densitometric analyses of western blotting (ImageJ software), indicating that TLR2 and TLR3 protein level (grayscale) were significantly enhanced at 20 μ g/mL and at 0.5–20 μ g/mL, respectively, compared to control level ($p < 0.05$). In time dependency, the maximal level of TLR3 was detected at 12 hour ($p < 0.05$ compared to 3 hour level or control level) and then declined to an appropriate level. The above data are presented by scatter charts showing sample distribution, sample number (n), means (–) and p values (significant difference specified at p

< 0.05, by ANOVA, Margin means or LSD). Dose treatment at 2 µg/mL (24 hour) is the same condition as time treatment at 24 hour (2 µg/mL).

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Figure 2.

The mRNA expression profiles of NF- κ B, TLR1 and TLR4. **a.** A53T mutant α -syn moderately upregulated NF- κ B expression with a statistical significance ($p < 0.05$) between high dose (8 and 20 μ g/mL) and low dose (0.5 and 2 μ g/mL) groups or between early (3 and 12 hour) and later (24 and 48 hour) time-point groups. A53T mutant had a stronger induction effect than WT α -syn, displaying an induction tendency of positive dose-effect correlation or negative time-effect correlation. **b,c.** TLR1 and TLR4 mRNA expressions were upregulated by A53T mutant α -syn, as shown with p values ($p < 0.05$) between treatment groups, displaying an induction tendency of positive dose-effect correlation. The above data are presented with scatter charts showing sample distribution, sample number (n), means (–) and p values (significant difference specified at $p < 0.05$, by ANOVA, Margin means or LSD). Dose treatment at 2 μ g/mL (24 hour) is the same condition as time treatment at 24 hour (2 μ g/mL).

Figure 3.

The expression profiles of cytokines induced by A53T mutant or wild-type (WT) α-syn. **a,b.** mRNA expressions of IL-1β and IFN-β was significantly upregulated, as shown with a p value ($p < 0.05$) between treatment groups, displaying two types of induction patterns, i.e., positive dosage-effect correlation and negative time-effect correlation, with an atypical pattern for IFN-β. **c.** IFN-γ mRNA expression was significantly downregulated, as shown with a p value ($p < 0.05$) between treatment groups, and its minimal level was detected at high concentration (8 or 20 μg/mL) and early time-point (3 or 12 hour). **d.** Protein release profile of IFN-γ by ELISA, demonstrating that IFN-γ supernatant levels were significantly reduced at high concentrations (8 and 20 μg/mL) and early time-points (3 and 12 hour), as shown with a p value ($p < 0.05$) between control and treatment groups, with WT α-syn having more reduction amplitude than A53T mutant. **e.** Western blotting analysis for IL-1β (75 kDa), IFN-γ (19 kDa) and β-actin (45 kDa), a representative of three experiments. The signal sizes were identified according to manufacturer's instruction and β-actin was used as reference control. **f.** Densitometric analysis (ImageJ software) of IL-1β for panel e. Expression level (grayscale) of IL-1β was significantly enhanced by A53T mutant α-syn at 3 hour, and then reduced at 12 and 24 hour, a similar pattern to its mRNA expression, as

shown with a p value ($p < 0.05$) between control and treatment groups. However, at 8 and 20 $\mu\text{g/mL}$, IL-1 β expression was significantly downregulated, a different pattern from its mRNA expression, which may be associated with a potential exhaustive release of IL-1 β . **g.** Densitometric analysis (ImageJ software) of IFN- γ for panel e. Expression level (grayscale) of IFN- γ was significantly reduced by A53T mutant α -syn at 0.5–20 $\mu\text{g/mL}$ or early time-points (3 and 12 hour), a similar pattern to its mRNA expression, as shown with a p value ($p < 0.05$) between control and treatment groups. The above data are presented with scatter charts showing sample distribution, sample number (n), means ($-$) and p values (significant difference specified at $p < 0.05$, by ANOVA, Margin means or LSD). Dose treatment at 2 $\mu\text{g/mL}$ (24 hour) is the same condition as time treatment at 24 hour (2 $\mu\text{g/mL}$).

Figure 4.

Additive application of TLR2 and TLR4 antagonists. **a,b.** TLR2 antagonist (CPT22, 5 $\mu\text{g/mL}$) significantly downregulated mRNA expression of TLR3 induced by A53T mutant $\alpha\text{-syn}$ (2 $\mu\text{g/mL}$, 24 hour), along with a compensatory upregulation of TLR2, but TLR4 antagonist (C34, 5 $\mu\text{g/mL}$) had no extra induction effect on TLR2 and TLR3. Each comparison was shown with a p value ($p < 0.05$) between treatment groups. **c,d.** Both CPT22 and C34 significantly upregulated NF- κB mRNA expression induced by A53T mutant $\alpha\text{-syn}$, with a p value ($p < 0.05$) between treatment groups, indicating an activation state of inflammatory pathway in $\alpha\text{-syn}$ induction. **e,f.** In A53T mutant $\alpha\text{-syn}$ stimulation, IL-1 β mRNA expression was significantly downregulated by CPT22 at 12 and 24 hour, but was significantly upregulated by C34 at 12 hour, indicating a functional difference between TLR2 and TLR4 in $\alpha\text{-syn}$ induction. The above data are presented with scatter charts showing sample distribution, sample number (n), means (—) and p values (significant difference specified at $p < 0.05$, by ANOVA, Margin means or LSD). Dose treatment at 2 $\mu\text{g/mL}$ (24 hour) is the same condition as time treatment at 24 hour (2 $\mu\text{g/mL}$).