INTRODUCTION

Parkinson’s disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and a subset of Alzheimer’s disease (AD) are collectively referred to as synucleinopathies and characterized by abnormal deposition of α-synuclein (α-syn) in disease-affected brain regions [1, 2]. α-syn is a neuronal protein localized in presynaptic terminals that have been shown to be involved in synaptic vesicle trafficking and synaptic plasticity [3, 4]. Under certain conditions, a small amount of α-syn can be released into the extracellular space via unconventional exocytosis [5-7]. Neuron-released α-syn can then stimulate neurotoxic responses in neighboring neurons and glia by interacting with cell surface receptors [8-11]. In microglia, α-syn induces a pro-inflammatory phenotype characterized by enhanced cell migration, oxidative stress, nitric oxide production, and release of cytokines and chemokines [12-15]. The microglial receptors proposed to recognize extracellular α-syn are toll-like receptor 2 (TLR2), TLR4, cluster of differentiation 36 (CD36), macrophage-1 antigen (MAC1), nicotinamide adenine dinucleotide phosphate oxidase 2 (Nox2), and β1-integrin [16-21]. Neurons, on the other hand, may interact with extracellular α-syn via TLR2 and lymphocyte activation gene 3 (LAG3) to modulate autophagy and α-syn transmission, eventually leading to abnormal deposition of α-syn and neuronal death [9, 22, 23]. As such, targeted regulation of these receptors may have beneficial therapeutic effects by alleviating α-syn-mediated toxicity.

TLRs are a family of membrane-bound pattern recognition receptors (PRRs) responsible for an innate immune response. Upon recognizing their specific pathogens, TLRs engage in an assortment of intracellular signaling pathways that can induce the expression of inflammatory genes to regulate the host’s immune response [24]. TLRs are primarily expressed by innate immune cells such as monocytes/macrophages and dendritic cells, but they can also be found on adaptive immune and non-immune cells [25]. In the central nervous system, microglia, astrocytes, neurons, and oligodendrocytes are all known to express certain types of TLRs.
Although TLRs have been extensively studied in the innate immune system, recent studies have also suggested a critical role for TLRs in neurodegenerative diseases such as synucleinopathies and AD [27-29]. Here, we will explore α-syn-induced microglial and neuronal TLR2 activation and discuss TLR2 as a potential therapeutic target for synucleinopathies.

**PATHOLOGICAL INTERACTION OF MICROGLIAL TLR2 AND EXTRACELLULAR α-SYNUCLEIN**

In 2013, we demonstrated the pathological role of microglial TLR2 in synucleinopathies [30]. To investigate the microglial response to neuron-released α-syn, we introduced culture media obtained from differentiated human neuroblastoma SH-SY5Y cells (dSY5Y) overexpressing either wildtype human α-syn (αSCM) or β-galactosidase (LZCM, control conditioned media) to rat primary microglia. Cells treated with αSCM displayed increased amoeboid morphology, cell proliferation, and nitric oxide production, indicating microglial activation. However, αSCM contains not only neuron-released α-syn but also other cellular byproducts. To evaluate whether microglial activation by αSCM depends on the presence of α-syn in the conditioned medium, we serially and selectively eliminated α-syn from αSCM and observed a concomitant decrease in microglial activation. Total depletion of α-syn from the conditioned media completely abolished the microglial response to αSCM. Microglial activation was conversely increased by treatment of α-syn purified from αSCM in a dose-dependent manner.

To gain a comprehensive mechanistic understanding for α-syn-induced microglia activation, we then analyzed the early (6 hr) and late (24 hr) responses in the gene transcriptome of microglia exposed to neuron-released α-syn. Jak-STAT, cytokine-cytokine receptor, leukocyte transendothelial migration, and nitric oxide production, indicating microglial activation. However, αSCM contains not only neuron-released α-syn but also other cellular byproducts. To evaluate whether microglial activation by αSCM depends on the presence of α-syn in the conditioned medium, we serially and selectively eliminated α-syn from αSCM and observed a concomitant decrease in microglial activation. Total depletion of α-syn from the conditioned media completely abolished the microglial response to αSCM. Microglial activation was conversely increased by treatment of α-syn purified from αSCM in a dose-dependent manner.

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In a subsequent study, we crossed Thr2+/− mice with a synucleinopathy mouse model expressing human A53T α-syn (A53T+ Thr2+/−) to examine whether TLR2 activity is associated with overall synucleinopathy pathology in vivo [43]. In accordance with our previous findings, genetic depletion of TLR2 significantly reduced astrogliosis, microgliosis, and neuronal loss in A53T+ Thr2+ mice. Remarkably, however, we also found a decrease in the neuronal α-syn pathology of A53T+ Thr2+/− mice without alteration in A53T−/−.
TLR2 in Synucleinopathies

α-syn gene expression. Although TLRs are typically associated with immune cells, multiple studies have proposed that neurons also express TLR2 [22, 44, 45]. Therefore, we verified the expression of TLR2 in primary mouse neurons and human neural progenitor cells. Interestingly, we also observed neuronal TLR2 expression in A53T mice. These findings suggested a potential role for neuronal TLR2 in synucleinopathies.

To assess this role, we stimulated α-syn-overexpressing neuronal cells with the TLR2-specific agonist, pam3CSK4. Strikingly, stimulation of TLR2 significantly increased the intracellular accumulation of high molecular weight α-syn aggregates, with cytotoxicity. On the other hand, lentiviral vector-mediated knockdown of TLR2 in these cells normalized α-syn accumulation to that of control levels regardless of whether the agonist was introduced. Having observed that TLR2 was associated with α-syn deposition in vitro, we next knocked down TLR2 expression in a synucleinopathy mouse model, Line 61 (Thy1-human-α-syn). This popular model mimics the neuropathological and functional aspects of synucleinopathy, including α-syn pathology and neuroinflammation. Delivery of a TLR2-knockdown lentiviral vector not only significantly decreased both neuroinflammation and neuronal accumulation of α-syn, but also improved behavioral deficits.

We next sought to understand the signaling mechanism by which TLR2 affects neuronal α-syn accumulation. Given that α-syn mRNA levels were unaffected by genetic depletion of TLR2 and, in general, TLRs are associated with pathogen clearance, we hypothesized that neuronal α-syn deposition might be associated with cellular protein homeostasis mechanisms such as autophagy [46, 47]. To verify this idea, we monitored proteostasis markers in TLR2-stimulated neurons and demonstrated that activation of TLR2 increased the accumulation of not only high molecular weight α-syn aggregates but also sequestosome 1 (p62/SQSTM1) and puncta formation of microtubule-associated proteins 1A/1B light chain 3B (LC3), which are indicators of autophagy [48]. Treatment of rapamycin, an autophagy inducer, significantly reduced TLR2-mediated α-syn accumulation. Notably, co-treating neurons with TLR2 agonist and autophagy inhibitor bafilomycin A1 did not produce an additive effect on α-syn accumulation, which suggests that the two drugs act along the same pathway.

The mammalian target of rapamycin (mTOR) is a key modulator of autophagy [49]. Given that rapamycin reversed the effects of TLR2 agonist on α-syn pathology, we investigated the role of mTOR signaling in TLR2-mediated neuronal α-syn deposition. Interestingly, activation of TLR2 in α-syn-overexpressing dSY5Y cells increased the inhibitory phosphorylation of mTOR as well as the phosphorylation of AKT, an mTOR negative regulator. As

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Fig. 1. Model of pathological TLR2 activation by neuron-released α-synuclein in neurons, astrocytes, and microglia. Under disease conditions, neurons release pathogenic α-syn into the extracellular space where they can interact with TLR2 on neighboring cells. In microglia and astrocytes, α-syn activates a TLR2 signaling cascade that induces a pro-inflammatory response, thereby generating a neurotoxic environment. α-syn can also interact with neuronal TLR2 to induce neurotoxic α-syn deposition by impairing autophagy. As such, TLR2 immunotherapy is a promising therapeutic strategy to prevent α-syn-mediated glial activation and cell-to-cell transmission of α-syn aggregates, ultimately ameliorating neurotoxic conditions in the synucleinopathy brain.
further evidence, stimulation of TLR2 increased the accumulation of p62/SQSTM1 and α-syn in human neural precursor cells over-expressing α-syn. In addition, treatment with an AKT inhibitor or lentiviral knockdown of TLR2 was sufficient to reverse these effects, suggesting that neuronal TLR2 activation by α-syn promotes intracellular α-syn deposition through an AKT/mTOR-dependent inhibition of autophagy. These findings were supported by Dzamko et al, who described the induction of neuronal TLR2 in PD patient brains [50]. Dzamko et al also verified that TLR2 activation disrupts neuronal autophagy and results in α-syn accumulation. As such, neuronal TLR2 might also be a viable therapeutic target for synucleinopathies (Fig. 1).

TLR2 IN CELL-TO-CELL TRANSMISSION

The cell-to-cell transmission of α-syn has also been proposed to play a critical role in synucleinopathy pathogenesis [9, 51, 52]. To assess whether TLR2 is associated with α-syn transmission, we employed a live-cell monitoring system consisting of neuronal donor cells expressing α-syn conjugated to the amino-terminus of the fluorescent protein Venus and neuronal recipient cells expressing α-syn conjugated to the carboxy-terminus of Venus [53, 54]. Intriguingly, induction of TLR2 through overexpression or an agonist increased the cytotoxic neuron-to-neuron transmission of α-syn. However, functional or genetic inhibition of TLR2 suppressed this transmission. These results indicate that TLR2 plays a critical role in the neuron-to-neuron transmission of α-syn aggregates. In addition to neuron-to-neuron transmission, α-syn may also be transferred to neighboring glial cells [8]. Although astrocytes do not express α-syn, we observed significant α-syn accumulation in the astrocytes of both synucleinopathy patients and mouse models [8, 55]. We thus also verified that astrocytic α-syn aggregates originated from neurons in a TLR2-dependent manner [8].

TLR2 IMMUNOTHERAPY IN SYNUCLEINOPATHY MOUSE MODEL

With evidence to support that extracellular α-syn pathologically activates TLR2 in synucleinopathies, we shifted our focus to the therapeutic potential of modulating TLR2 activity [56]. We first verified that TLR2 was present and elevated in the neurons and microglia of synucleinopathy patients and animal models. Then, we administered a TLR2 functional blocking antibody (T2.5) into a synucleinopathy mouse model mimicking the neuropathological and functional aspects of the disease [56]. As expected, blockade of TLR2 successfully diminished α-syn deposition in neurons, especially that of triton-insoluble high molecular weight α-syn aggregates. Treatment with T2.5 also reduced astrogliosis and microgliosis as well as decreased the expression of pro-inflammatory cytokines such as tumor necrosis factors alpha (TNFa) and interleukin 6 (IL-6). Remarkably, T2.5 administration significantly ameliorated neuronal loss in synucleinopathy mice, and this neuroprotective effect was reflected in the improvement of behavioral deficits in the model such as hyperactivity.

Based on these data and our previous findings, we propose three mechanisms by which functional inhibition of TLR2 could alleviate α-syn pathology, inflammation, and neurodegeneration in models of synucleinopathy. First, functional inhibition of TLR2 reduces glial activations and subsequent neuroinflammation [30]. Second, TLR2 blockade relieves the suppression of neuronal autophagy to decrease abnormal deposition of α-syn [43]. Finally, neutralization of TLR2 may inhibit neuron-to-neuron and neuron-to-glia α-syn transmission (Fig. 1).

CONCLUSIONS

TLR2 is involved in the neuropathogenesis of synucleinopathy in in vitro and in vivo models via i) the induction of pro-inflammatory glial activation, ii) autophagy-mediated neuronal α-syn accumulation, and iii) pathogenic neuron-to-neuron and neuron-to-glial α-syn transmission (Fig. 1). We further showed that functional modulation of TLR2 can ameliorate the neuropathogenesis and behavioral deficits in synucleinopathy mouse models. Therefore, we propose that targeting TLR2 is a promising immunotherapeutic strategy for synucleinopathies.

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