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RESEARCH ARTICLE

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Post-inflammatory administration of *N*-acetylcysteine reduces inflammation and alters receptor levels in a cellular model of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a complex, multifactorial neurodegenerative disease with a prevalence of 1% over the age of 55. Neuropathological hallmarks of PD include the loss of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of Lewy bodies that contain a variety of proteins and lipids including alpha-synuclein (α-syn). Although the formation of α-syn occurs intracellularly, it can also be found in the extracellular space where it can be taken up by neighboring cells. Toll-like receptor 2 (TLR2) is an immune system receptor that has been shown to recognize extracellular α-syn and modulate its uptake by other cells. Lymphocyte-activation gene 3 (LAG3), an immune checkpoint receptor, has also been proposed to play a role in extracellular α-syn internalization; however, a recent study has disputed this role. Internalized α-syn can trigger expression and secretion of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-2, and IL-6 and induce neuroinflammation, apoptosis, and mitophagy that results in cellular death. In this study, we tested if *N*-acetylcysteine (NAC), an anti-inflammatory and anti-carcinogenic drug, can circumvent the detrimental effects of neuroinflammation and induce an anti-inflammatory response by modulating transcription and expression of TLR2 and LAG3 receptors. Cells overexpressing wild-type α-syn were treated with TNF-α to induce inflammation followed by NAC to inhibit the deleterious effects of TNF-α-induced inflammation and apoptosis. *SNCA* gene transcription and α-syn protein expression were validated by q-PCR and Western blot (WB), respectively. Cell viability was measured, and apoptosis was evaluated by WB and terminal deoxynucleotidyl transferase nick end labeling methods. Alterations in LAG3 and TLR2 receptor levels were evaluated by immunofluorescent labeling, WB, and q-PCR. TNF-α not only increased inflammation but also increased endogenous and overexpressed α-syn levels. NAC treatment decreased expression of TLR2 and increased transcription of LAG3 receptor and diminished

inflammation-mediated toxicity and cell death. Here, we demonstrate that NAC can reduce neuroinflammation that occurs as a result of alpha-synuclein overexpression, via a TLR2-associated pathway, making it a promising candidate for therapeutic intervention. Further studies are needed to elucidate molecular mechanisms and pathways related to neuroinflammation in PD and to develop possible new therapeutic approaches to slow the clinical progression of PD.

KEYWORDS

alpha-synuclein (α-syn), apoptosis, *N*-acetylcysteine (NAC), neuroinflammation, Parkinson's disease

1 | INTRODUCTION

Parkinson's disease (PD) is the most common age-related movement disorder and second most common neurodegenerative disorder after Alzheimer's disease.[1] Sporadic PD accounts for around 90% of all cases, whereas inherited PD cases represent less than 10% of all patients.[2]

Pathological hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of inclusion bodies that contain predominantly fibrillar alpha-synuclein (α-syn), ubiquitinated proteins, and lipids, called Lewy bodies.[3–5] PD is a progressive neurological disorder in which both non-motor and motor symptoms occur during the progress of the disease.[6] Generally, patients have some non-motor symptoms at early, prodromal stages of the disease with motor symptoms usually presenting when approximately 60%–80% of dopaminergic neurons are lost.[7]

Previous studies have highlighted various pathological mechanisms underlying PD. The most emphasized so far are; mitochondrial disorder, ubiquitin proteasome system dysfunction, change in calcium homeostasis and increased oxidative stress.[8] Although there is no single pathway describing the underlying mechanism of the disease, important mechanisms involved in the cellular pathology are the aggregation of misfolded α-syn protein with subsequent mitochondrial dysfunction and neuroinflammation.[9]

Misfolded α-syn protein is located in the cytosol or can bind to membrane structures, such as mitochondria, endoplasmic reticulum, and nucleus.[10,11] a-Syn also exists in the extracellular space and can pass from cell to cell[11] propagating pathology in a prion-like manner.[12,13] In addition to vesicular-associated release and uptake[8,14] extracellular α-syn can be internalized by neurons and glia cells (microglia, astrocytes, oligodendrocytes) by endocytosis.[13,15]

Numerous receptors have been studied in relation to endocytosis of α-syn.[16] One such receptor is Toll-like receptor 2 (TLR2), an immune system receptor associated with neuroinflammation. TLR2 is present in neurons and glia cells and has been shown to play a direct role in the endocytosis of both the oligomer and the fibrillar forms of α-syn.[17] In microglia cells, inflammatory cytokines are expressed and released from the cells via TLR2 activation. Triggered inflammation results in increased neurotoxicity and accelerated cell degeneration.[13,18] Inflammatory cytokines, especially tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, increase with inflammation, while IL-2 and IL-6 are also detected.[19] TNF-α is known to strongly induce reactive oxygen species production through mitochondria and can also induce apoptosis as an external apoptosis activator.[19] Similarly, when neuronal TLR2 is activated, there is an increase of inflammatory cytokine levels which leads to an increase in oxidative stress and also accumulation of endogenous α-syn.[17]

Another receptor that may play a role in the endocytosis of α-syn is Lymphocyte-activation gene 3 (LAG3). Previous studies demonstrated that extracellular protofibrillar forms of α-syn can bind to LAG-3 in neurons to facilitate uptake to the intracellular environment.[20] In the same study, the researchers did not detect LAG-3 receptors on astrocyte or microglial membranes leading them to the conclusion that LAG3 was important for the uptake of protofibrillar forms of α-syn into neurons. A subsequent study found no evidence for LAG3 expression in human neurons, although they confirmed that LAG3 interacts with α-synuclein fibril,[21] thus the role of LAG3 in α-syn internalization remains controversial.

N-acetylcysteine (also known as, *N*-acetyl-L-cysteine, or NAC) is a safe and well-tolerated FDA-approved medicine that has long been used in various clinical applications with only minor side effects.[22] NAC is the N-acetyl derivative of the amino acid L-cysteine. It is absorbed following oral administration and can cross the blood–brain barrier[23,24] where it has anti-apoptotic and anti-inflammatory effects in the central nervous system (CNS).[25] An increasing number of studies propose that NAC holds promise for the treatment of neurodegenerative diseases.[22]

Here, we tested the hypothesis that NAC treatment reduces in vitro apoptotic and neuroinflammatory responses by modulating transcription and expression of TLR2 and

LAG3 receptors. By modeling a neuroinflammatory microenvironment in a stable cell line expressing α-syn, we observed and compared the effects of inflammation and NAC on cell death ratio, apoptosis ratio, and changes in TLR2 and LAG3 receptors levels. α-Syn transcription and expression were validated by q-PCR and Western blot (WB), respectively, and cell viability and apoptosis were evaluated using a viability assay, WB, and terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) methods. Overall, we observed that NAC treatment diminishes inflammation-mediated toxicity and cell death by altering transcription and expression of TLR2 and LAG3 receptors. This study supports NAC as a promising candidate to reduce neuroinflammation that occurs in PD.

2 | METHODS

2.1 | Experimental model

A stable cell line over-expressing wild-type (WT) α-syn (H4 human neuroglioma cells) was used to model inflammation in PD. α-Syn over-expression is turned on or off by the absence [Tet (−) cells] or presence [Tet (+) cells] of tetracycline (Tet), respectively. All experiments were conducted with the four α-syn on groups and their syn-off counterparts. In this model, inflammation was triggered by incubating the cells with 75 ng/mL TNF-α. Cells were treated with 2 mM NAC to see the anti-inflammatory effects of the medicine.

2.2 | Cell culture

H4 cells were maintained at 37°C in a 95% air/5% CO_2 humidified incubator in Opti-MEM (51985091; ThermoFisher) supplemented with 10% fetal bovine serum (10437028; ThermoFisher). Cells were passaged once a week and tested for mycoplasma contamination at regular intervals. To determine the appropriate doses of TNF-α and NAC, cells were treated with increasing doses of TNF-α and NAC separately. The doses for the applications were decided based on cell toxicity assay (Figure S1A,B). 75 ng/mL TNF-α (Recombinant Human TNF-α #570106; Biolegend) was applied in the medium and incubated for 24 h. NAC (A9165; Sigma-Aldrich) was dissolved in medium with a final concentration of 2 mM added to cells for 45 min of incubation. The media of NAC-treated groups were removed and replaced with fresh media after NAC treatment and incubated for another 24 h to observe the cell response. Cell morphology was observed using an Evos phase-contrast microscope each day (Invitrogen, Thermo Fisher Scientific). Experimental design and timeline are summarized in Figure 1A,B, respectively.

2.3 | Immunofluorescence

In order to confirm the expression of LAG3 and TLR2 receptors in H4 cells, immunofluorescent (IF) labeling was performed. Cells were cultured on 15-mm glass coverslips in 24-well plate without tetracycline for 72 h in accordance with experimental timeline. Next, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) for 15 min and blocked with 2% normal goat serum for 1 h at room temperature (RT). Cells were incubated overnight at 4°C with primary antibodies (Anti-Lag3 antibody, Anti-TLR2 antibody, α-synuclein antibody) prepared in PBS containing 1% BSA. The next day, cells were washed and treated with Alexa Fluor 488 and 568 secondary antibodies for 1 h at RT. All antibodies used in the study are described in Table 1. Coverslips were mounted on slides with Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield Mounting Medium with DAPI H-1200; Vector Laboratories) and cells were visualized using an Axio observer inverted microscope (Carl Zeiss). Fluorescence intensities of LAG3 and TLR2 receptors were evaluated and intensities were normalized to the total area with ImageJ[26] in 13 of the examined areas (Total 13 random areas in three set replicates).

2.4 | WB analyses

Western blot was performed for semi-quantitative analyses of α-syn, IL-1β, caspase-9, cleaved caspase-9, PARP, cleaved PARP, LAG3, and TLR2 in H4 cells. Cells were cultured on 6-cm petri dishes without tetracycline for 72 h in accordance with experimental timeline. Next, cells were washed with PBS, harvested with a scraper, and transferred to 1 mL centrifuge tubes. Total proteins were isolated by incubating H4 cells in lysis buffer supplemented with a protease inhibitor cocktail and halted by a phosphatase inhibitor cocktail. The protein concentration was determined using Pierce BCA Protein Assay kit (23225; ThermoFisher). Proteins (26 μg) were separated on Bis-Tris polyacrylamide gradient gels (NuPAGE Novex 4%–12% Bis-Tris Gel, Novex; ThermoFisher) and transferred to nitrocellulose membranes (Immobilon-P; Millipore). Then, membranes were blocked for 1 h at RT in 10% non-fat dried milk added TBS-T (500 mM NaCl, 20 mM Tris, 0.1% Tween 20, pH 7.4) solution. Subsequently, membranes were incubated overnight at 4°C with primary antibodies (Anti-Lag3 antibody,

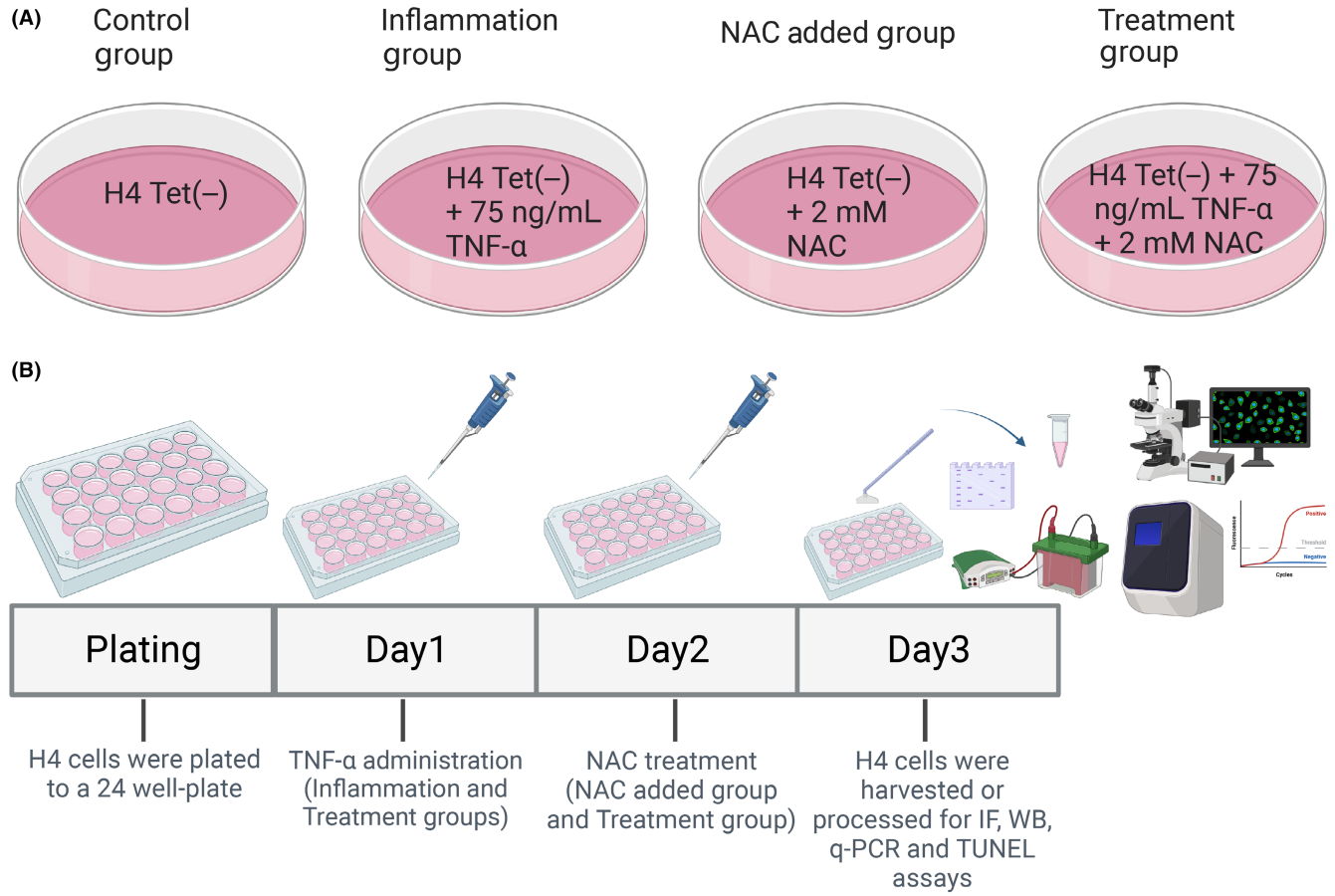

FIGURE 1 (A) Experiment setup. (B) Experiment timeline. All experiments were conducted with the four α-syn on groups described in (A) and their α-syn off counterparts (Created with BioRender.com). α-syn, alpha-synuclein.

Anti-TLR2 antibody, α-synuclein antibody, Anti-IL-1 beta antibody, TNF-α antibody, Caspase-9 antibody, PARP antibody, Anti-Actin antibody, Anti-Vinculin antibody; Table 1). Next morning, membranes were washed with TBS-T solution and incubated for 1 h at RT with HRP-conjugated secondary antibodies (Table 1). Proteins were detected using an enhanced chemiluminescent detection system (ECL, EMD Millipore) and imaged by ChemiDoc MP Imaging System (Bio-Rad). Membrane images were analyzed with ImageJ.[26] WB was performed in four replicates for each experiment group.

2.5 | Cytotoxicity assay

Toxilight-AK assay (ToxiLight BioAssay Kit, LT07-117; Lonza) was used to evaluate the effects of inflammation and NAC on cell survival. Cells were cultured in 24-well plate without tetracycline for 72 h in accordance with experimental timeline. Toxilight was applied per manufacturer's instructions to assess cell death every 24 h. ToxiLight AK reagent was added to each well and incubated at RT for 5 min, and luminescence was measured using an EnVision microplate reader (EnVision; PerkinElmer). Cell toxicity test was performed in four replicates including three repetitions for each group in each set.

2.6 | TUNEL assay

TUNEL is a frequently used immunochemical labeling method to detect excessive DNA breaks in cells or internucleosomal DNA fragmentation in apoptotic cells. The TUNEL evaluation was carried out using a TUNEL experimental kit (TUNEL Assay Kit-FITC ab66108; Abcam) according to the manufacturer's instructions. Cells were cultured on 6-cm petri dishes without tetracycline for 72 h in accordance with experimental timeline. Next, cells were washed with PBS, harvested by applying trypsin (Trypsin–EDTA [0.25%]—25200056; Gibco) and transferred to centrifuge tubes. For fixation, 1% PFA was added to each tube and incubated on ice for 15 min. Then, cells were centrifuged and washed twice with PBS. Five millilitre of 70% ethanol was added to

TABLE 1 Antibodies used for immunofluorescence (IF) and Western blot (WB).

	Source	Dilution
Antibody (primary)		
Lag3 (rabbit)	Abcam (ab209236)	1/200 (IF) 1/1000 (WB)
TLR2 (mouse)	Abcam (ab16894)	1/200 (IF) 1/1000 (WB)
α-Synuclein (mouse)	Biolegend (807806)	1/1000 (IF) 1/2000 (WB)
α-Synuclein (rabbit)	Santa Cruz (sc-7011)	1/200 (IF)
α-Synuclein (rabbit)	Cell Signaling (CST 2628)	1/400 (IF)
IL-1 beta (rabbit)	Abcam (ab9722)	1/1000 (WB)
TNF-α (rabbit)	Cell Signaling (CST 3707)	1/1000 (WB)
Caspase-9 (rabbit)	Cell Signaling (CST 9502)	1/1000 (WB)
PARP (rabbit)	Cell Signaling (CST 9542)	1/1000 (WB)
Actin (rabbit)	Sigma-Aldrich (A5060)	1/5000 (WB)
Actin (mouse)	Sigma-Aldrich (A5316)	1/5000 (WB)
Vinculin (mouse)	Sigma-Aldrich (V9131)	1/5000 (WB)
Antibody (secondary)		
Alexa Fluor 488 (goat anti-mouse)	Thermo Fisher Scientific (A11001)	1/500 (IF)
Alexa Fluor 488 (goat anti-rabbit)	Thermo Fisher Scientific (A11008)	1/500 (IF)
Alexa Flour 568 (goat anti-mouse)	Thermo Fisher Scientific (A11004)	1/500 (IF)
Alexa Fluor 568 (goat anti-rabbit)	Thermo Fisher Scientific (A11011)	1/500 (IF)
Goat anti-mouse HRP	Bio-Rad (101005)	1/5000 (WB)
Goat anti-rabbit HRP	Southern Biotech (4010-05)	1/5000 (WB)

Abbreviations: IL, interleukin; LAG3, lymphocyte-activation gene 3; TLR2, Toll-like receptor 2; TNF-α, tumor necrosis factor alpha.

the cells and kept on ice for 30 min. After centrifuge, the ethanol was removed, and cells were washed with wash buffer. Subsequently, cells were incubated with 50 µL of staining solution at 37°C for 1 h on a shaker. Next, cells were rinsed with the rinse buffer and centrifuged. Then, the pellet was suspended by adding 0.5 mL propidium iodide (PI)/RNase solution and incubated in the dark at RT for 30 min. 50–100 µL from the cell suspension was dripped onto the slides, covered with coverslips, and left in a dark box at RT overnight. The next day, cells were visualized using an Axio observer inverted microscope (Carl Zeiss) with ZEN software. Nuclei were labeled with PI in red and apoptotic cells with FITC-dUTP in green. TUNEL assay was performed in three sets of replicate experiments and cells were counted in 20 different random fields under the microscope for each group by a blinded researcher. The apoptotic index was calculated as the percentage of FITC-dUTP positive cells divided by the total number of cells in the microscopic fields.

2.7 | TaqMan qPCR

To assess the expression of α-syn, LAG3 and TLR2 mRNA, qPCR was performed using TaqMan method. Cells were cultured on 6-cm petri dishes without tetracycline for 72 h in accordance with experimental timeline then harvested and transferred to 1 mL centrifuge tubes. qPCR was performed in four sets including three repetitions for each group in each set (total 16 analyses) and analyzed by using QuantStudio™ Real-Time PCR Software (Version 1.3). One set was excluded from final TLR2 analysis for being considered outlier data after statistical evaluation with ROUT method on Graph Pad Prism 8.0.0 software (3 sets, 4 repetitions, 12 analyses for TLR2 expression).

2.7.1 | RNA isolation

Total RNA from the cells was extracted by using TRIzol (Invitrogen) according to the manufacturer's instructions. Quantity and purity of RNAs were measured with NanoDrop spectrometer (Nanodrop 2000; Thermo Fisher Scientific) at 260/280 and 230/260 nm wavelengths.

2.7.2 | cDNA synthesis and qPCR analysis

cDNA was synthesized using Taqman™ III Universal PCR mastermix(4305719;Applied Biosystems, Life Technologies). The reactions in 96-well plates were performed with cDNA

Thermal cycler (Eppendorf Mastercycler Pro) under 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and ∞ at 4°C. TaqMan probes used were HPRT1 (Hs02800695_m1; Thermo Fisher) as a housekeeping gene, SNCA (Hs00240906_m1; Thermo Fisher), LAG3 (Hs00958444_g1; Thermo Fisher), and TLR2 (Hs00152932_m1).

qPCR reactions were run with the following cycling parameters: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min by real-time PCR instrument (QuantStudio 7; Applied Biosystems). The means of the groups were calculated and compared using QuantStudio™ Real-Time PCR Software (Version 1.3) and ΔΔCt (comparative Ct) method.

2.8 | Statistical analysis

All data were analyzed using Graph Pad Prism 8.0.0 software and analysis was conducted between all four groups α-syn on and a separate analysis for all four groups α-syn off. Statistical significance between groups was determined by one-way analysis of variance with Tukey's multiple comparisons test and unpaired *t*-test was used for pairwise comparisons. Results presented as mean ± standard error of the mean within 95% confidence interval, and $p < 0.05$ values were considered statistically significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

3 | RESULTS

3.1 | TNF-α treatment alters endogenous and over-expressed α-syn levels, inflammation, apoptosis, and receptor expression

We created an inflammatory PD model using a previously described inducible cell model of human α-syn overexpression[27,28] regulated by the presence [Tet (+) cells/α-syn off] or absence [Tet (−)/α-syn on] of tetracycline. IF (Figure S2A) and WB (Figure S2B) results showed that α-syn over-expression was successfully suppressed when α-syn was off. Consistently, qPCR (Figure S2C) results confirmed that α-syn transcription was reduced in the α-syn off groups and only endogenous α-syn transcription maintained in the cells. Considering that α-syn off groups do not model PD and did not show effects regarding TNF-α and NAC treatment (data not shown), subsequent data focus on syn-on groups only.

To determine the effect of TNF-α, cells overexpressing α-syn were treated with 75 ng/mL TNF-α for 24 h. Strikingly, cell adhesion and cell density were decreased (Figure 2A) while SNCA transcription significantly increased in the presence of TNF-α (Figure 2B). Similarly, elevated inflammation is detected with increased levels of IL-1β with WB after cells were treated with TNF-α (Figure 2C). Apoptosis rates were compared using an apoptotic index determined by assessment of DNA damage via TUNEL assay and levels of Caspase 9 and PARP via WB. Cleaved PARP/PARP protein levels (Figure 2D) and apoptotic index (Figure 2E) were significantly increased by 75 ng/mL TNF-α-treated cells, while there was no significant change in cleaved caspase9/caspase9 protein levels. Lastly, we evaluated receptor expression by fluorescent labeling, WB assay, and qPCR. IF labeling showed no significant difference in LAG3 receptor levels (Figure 3A) while fluorescent density of TLR2 was significantly higher in TNF-α treated cells (Figure 3B), although this increase was not detected via WB (Figure 3C,D) or qPCR (Figure 3E).

3.2 | NAC administration reduces apoptosis and cytotoxicity

To observe anti-inflammatory and anti-apoptotic effects of NAC, cells overexpressing alpha-synuclein were treated with 2 mM NAC for 45 min and then incubated with fresh media for another 24 h. After NAC treatment, cell adhesion and density in the flasks were slightly increased, compared to control group (Figure 4A). As a result of Toxilight-AK assay, cytotoxicity was significantly decreased in NAC treated cells when cells are overexpressing α-syn (α-syn on) (Figure 4B). Similarly, WB revealed Cleaved Caspase9/Caspase9 levels were significantly decreased in NAC-treated cells (Figure 4C), whereas no significant change was observed by TUNEL assay (Figure 4D). Additionally, no change was detected in the expression levels of LAG3 or TLR2 by IF (Figure S3A,B) and WB assays (Figure S3C,D). However interestingly, LAG3 transcription was significantly higher in NAC-added group (G6) (Figure 4E).

3.3 | NAC treatment relieves inflammation and α-syn induced alterations

In order to see the effects of NAC treatment after TNF-α-induced inflammation, TNF-α-treated cells were compared with TNF-α + NAC treated cells. Cell adhesion and density were slightly increased when NAC was included compared to TNF-α only treated cells (Figure 5A). The anti-inflammatory effect of NAC was demonstrated by significantly decreased IL-1β protein levels by WB assay (Figure 5B). The results of cytotoxicity test, WB assay, and TUNEL assay showed increased cell survival in NAC-treated cells. With the cytotoxicity assay, cells overexpressing

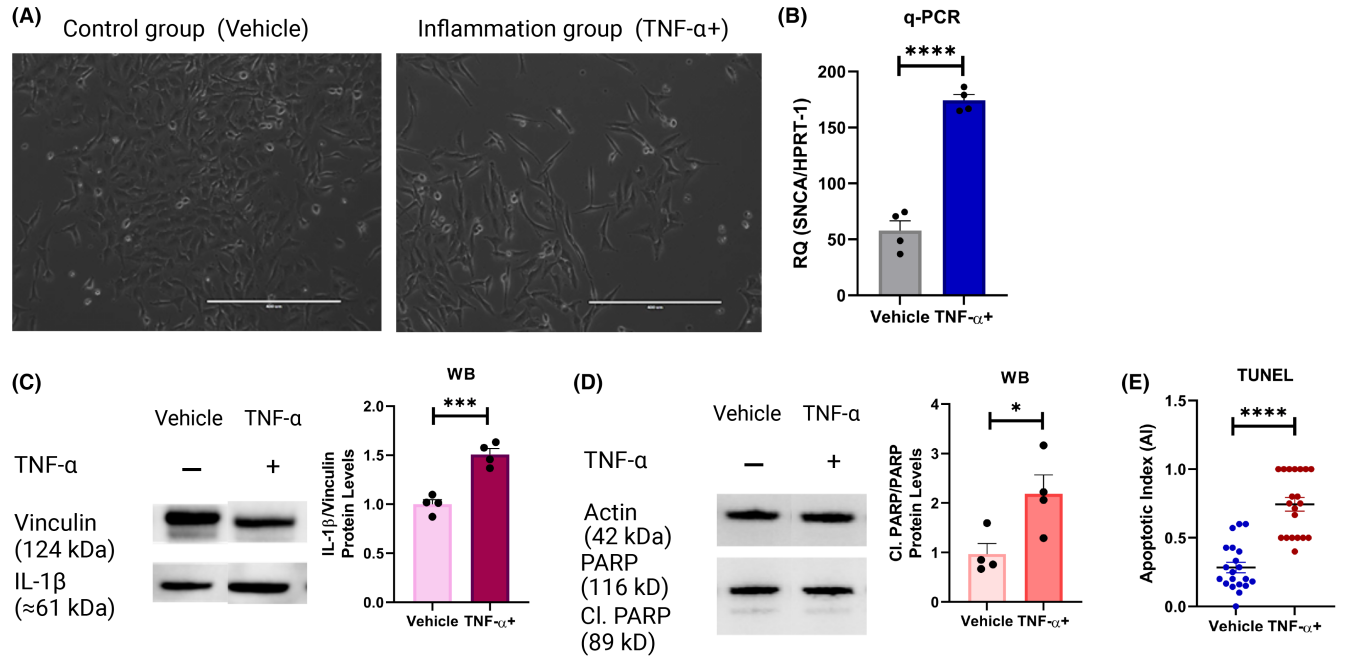

FIGURE 2 TNF-α treatment alters α-syn transcription, inflammation, and apoptosis in H4 human neuroglioma cell line. (A) Evos microscopy images of α-syn in control and TNF-α-treated cells. Scale bar = 400 μm. (B) q-PCR of SNCA transcription in control and TNF-α-treated cells, $n = 4$ (four repetitions for each group in each set, 16 analyses). (C) Representative Western blot of IL-1β and vinculin in cell lysates from control and TNF-α-treated cells, and Western blot quantitation, $n = 4$. (D) Representative Western blot and quantitation of PARP, cleaved PARP and Actin levels in cell lysates from control and TNF-α treated cells, $n = 4$. (E) Apoptotic index from TUNEL staining of apoptosis rates in control and TNF-α-treated cells, $n = 20$ (three sets, 20 different fields). Error bars represent the mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. (Predicted molecular weight of IL-1β is 31 kDa, detected at ≈61 kDa). α-syn, alpha-synuclein; IL, interleukin; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.

α-syn were found to be more responsive than cells where tet was added to turn α-syn overexpression off to the anti-inflammatory treatment after the inflammation is triggered by TNF-α. Results of Toxilight cytotoxicity assay indicated that cytotoxicity was significantly decreased in NAC-treated group compared to TNF-α added group (Figure 5B). This result supported by significantly decreased protein levels of Cleaved PARP/PARP in WB assay in NAC-treated group (Figure 5C) and of the apoptotic index in the TUNEL assay (Figure 5D). The analyses of fluorescent labeling and WB assay showed no significant difference for LAG3 receptor (Figure 6A,C), while TLR2 expression was significantly decreased in NAC-treated group (Figure 6B,D). Although transcription levels of both receptors showed no significant difference between these groups, LAG3 transcription levels seemed slightly higher and TLR2 transcription levels seemed slightly lower in NAC-treated group compared to TNF-α-added group (Figure 6E).

4 | DISCUSSION

In this study, we assessed the response of a neuroglioma (H4) cell line overexpressing WT α-syn protein to TNF-α-induced inflammation and rescue by NAC anti-inflammatory treatment. Importantly, neuroglioma cells originate from astrocytes, which play an important role in neuroinflammatory processes. Here with TNF-α administration, we observed an increased inflammation and α-syn levels. After NAC treatment, expression of TLR2 was decreased and inflammation-mediated toxicity and cell death were diminished successfully.

TNF-α is a well-known pro-apoptotic and pro-inflammatory cytokine[29] with an active role in both physiological and neuroinflammatory conditions in the CNS.[30] In healthy CNS, TNF-α has regulatory functions of important physiological processes such as synaptic plasticity,[31] learning and memory,[32,33] sleep,[34] and food and water intake.[35] Under pathological conditions, astrocytes and microglial cells synthesize and secrete significant amounts of TNF-α, an important component of neuroinflammatory neurodegeneration.[36,37] Moreover, high levels of TNF-α have been correlated with traumatic brain injury,[38] ischemia,[39] Alzheimer's disease,[40] PD,[41,42] multiple sclerosis,[43] and amyotrophic lateral sclerosis.[44]

TNF-α binds to two receptors located on the cell membrane, TNFR-1 and TNFR-2. When TNF-α binds to TNFR-1, extrinsic apoptosis pathway is activated through Caspase 8, Caspase 3, and PARP in the cells. Whereas, TNFR-2 is associated with inflammation and survival pathways in

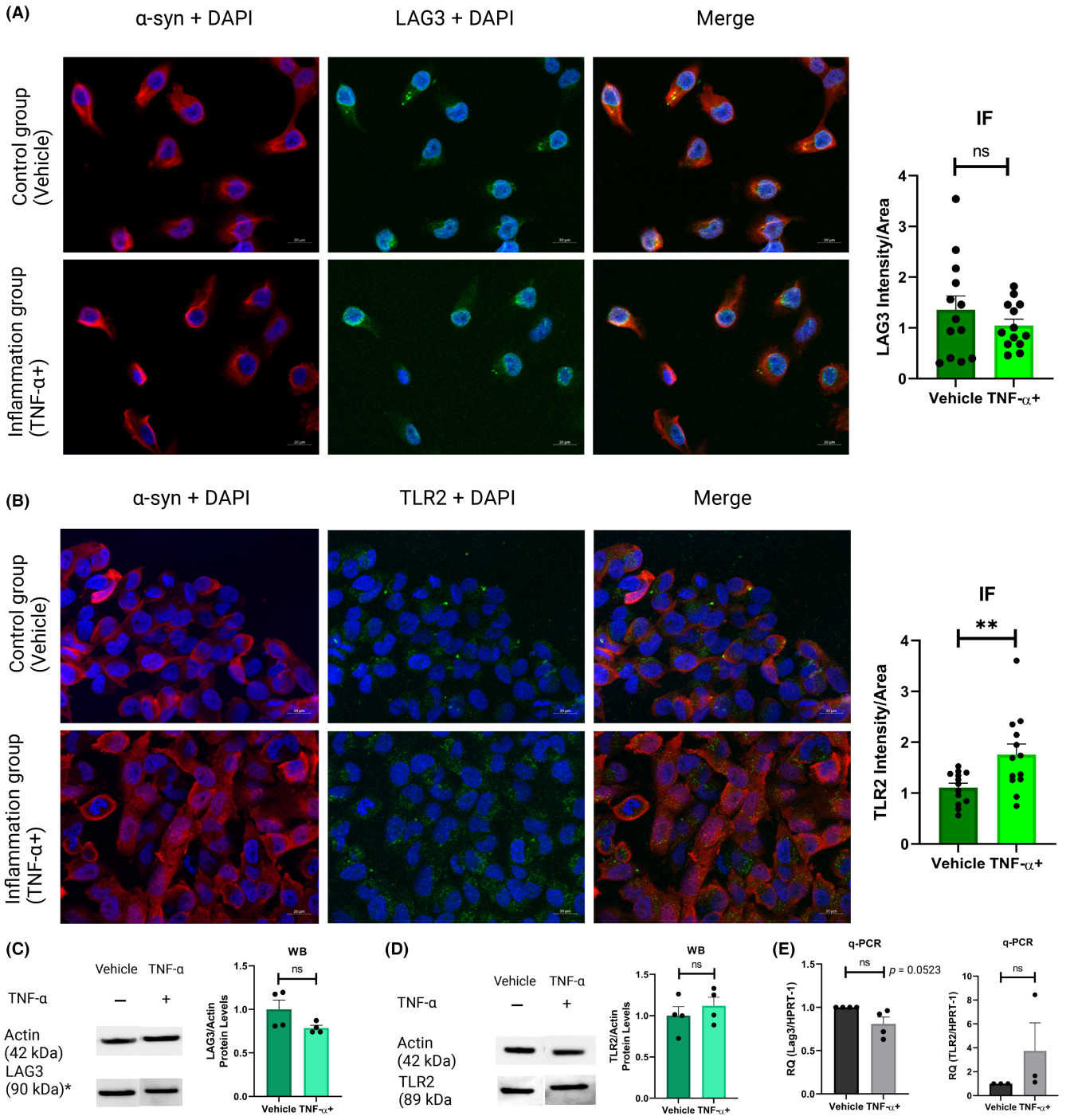

FIGURE 3 TNF-α treatment alters LAG3 and TLR2 expression in H4 human neuroglioma cell line. (A) Fluorescence microscopy images of control and TNF-α-treated cells. Nuclei in blue, α-syn in red, LAG3 in green. Scale bar = 20 μm. IF intensity quantification reflecting LAG3 intensity/area, $n = 13$ areas (three sets, 13 different fields). (B) Fluorescence microscopy images of control and TNF-α-treated cells. Nuclei in blue, α-syn in red, TLR2 in green. Scale bar = 20 μm. IF intensity quantification reflecting the TLR2 intensity/area, $n = 13$ areas (three sets, 13 different fields). (C) Representative Western blot and quantification of LAG3 and Actin in cell lysates from control and TNF-α-treated cells, $n = 4$. (D) Representative Western blot and quantification of TLR2 and Actin in cell lysates from control and TNF-α-treated cells, $n = 4$. (E) q-PCR quantitation of transcript levels of LAG3 and TLR2 in control and TNF-α-treated cells, LAG3 $n = 4$ (four repetitions for each group in each set, 16 analyses), TLR2 $n = 3$ (four repetitions for each group in each set, 12 analyses). Error bars represent the mean ± SEM, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. (Predicted molecular weight of LAG3 is 57 kDa, detected at ≈90 kDa). α-syn, alpha-synuclein; IF, immunofluorescent; IL, interleukin; LAG3, lymphocyte-activation gene 3; TLR2, Toll-like receptor 2; TNF-α, tumor necrosis factor alpha.

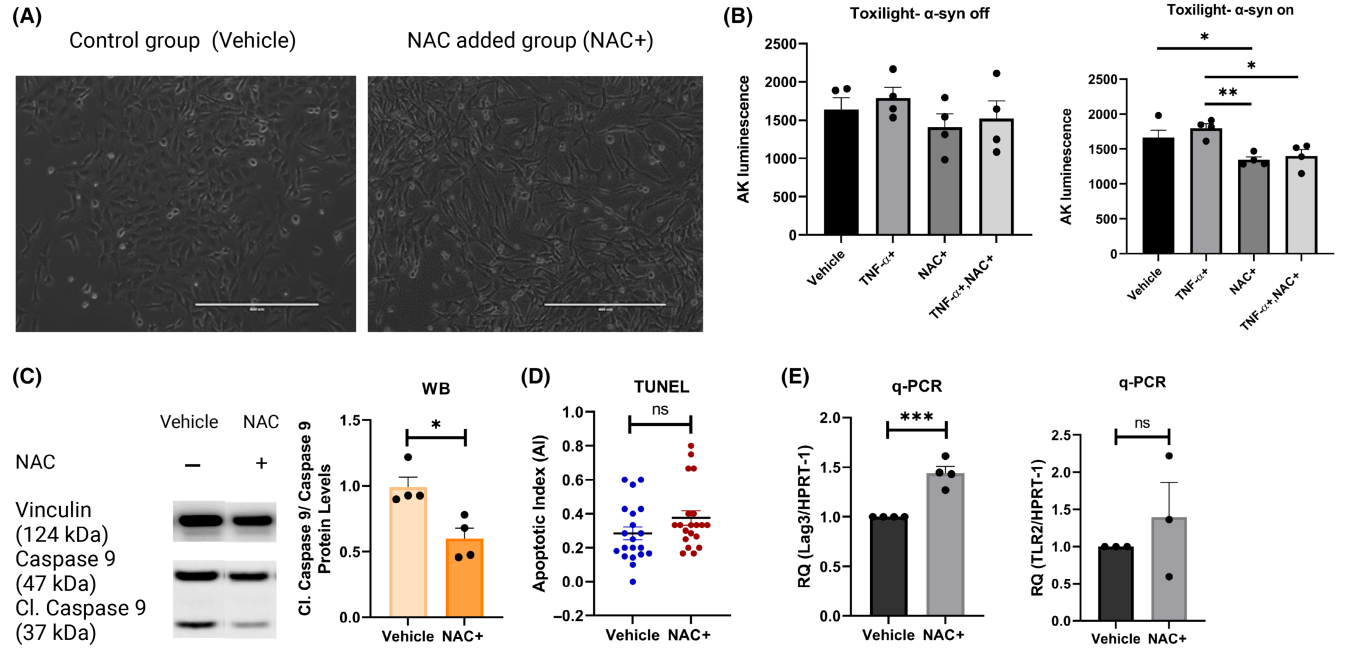

FIGURE 4 NAC administration reduces apoptosis and cytotoxicity in H4 human neuroglioma expressing α-synuclein. (A) Evos microscopy images of control and NAC-treated cells. Scale bar = 400 μm. (B) AK luminescence measurements reflecting cell toxicity and death rates. Results for α-syn off groups on experiment Day 3 showed no significant changes when α-syn over-expression is decreased. Whereas, in α-syn over-expressing groups, NAC effects on cell toxicity and death were prominent in NAC-treated cells on Day 3. $n = 4$ (three repetitions for each group in each set). (C) Representative Western blot and quantitation of caspase 9, cleaved caspase 9 and vinculin in cell lysates from control and NAC-treated cells, $n = 4$. (D) TUNEL assay analyses via calculated apoptotic index of apoptosis rates in control and NAC-treated group, $n = 20$ (three sets, 20 different fields). (E) q-PCR analyses of LAG3 and TLR2 receptor transcript levels in control and NAC-treated groups, LAG3 $n = 4$ (four repetitions for each group in each set, 16 analyses), TLR2 $n = 3$ (four repetitions for each group in each set, 12 analyses). Error bars represent the mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. α-syn, alpha-synuclein; LAG3, lymphocyte-activation gene 3; NAC, *N*-acetylcysteine; TUNEL, terminal deoxynucleotidyl transferase nick end labeling; TLR2, Toll-like receptor 2.

the cells.[29] In this study, inflammation was successfully triggered by adding TNF-α to cells. Consistent with previous studies, we found levels of α-syn transcription and expression were increased with TNF-α added,[45–47] indicating a correlation between the increase in inflammation and α-syn levels. Cell toxicity and apoptosis evoked by TNF-α also increased. Overall, our results demonstrate that TNF-α induces both inflammation and cell death in this model.

NAC is a precursor of L-cysteine and is a potent antioxidant with anti-inflammatory and anti-carcinogenic effects by regulating cystine glutamate anti-porter activity and glutathione biosynthesis in cells.[48,49] NAC can decrease proinflammatory cytokines, especially TNF-α, IL-1, and IL-6 in the early stages of the immune response.[50] In addition, NAC can inhibit the activation of the nuclear factor kappa B, a pathway related to inflammation.[22,51] In this study, we aimed to observe the effect of NAC on inflammation and cell survival after TNF-α administration and possible effects on α-syn levels. As expected, our results indicate that NAC does not induce inflammation and effectively reduces inflammation initiated by TNF-α. Notably, we observed that effect of NAC on toxicity and cell death is related with increased α-syn levels.

It is well known that oxidative stress initiates intrinsic apoptosis pathway in cells through mitochondria.[52] Consistent with NAC being a strong antioxidant and preventing the accumulation of molecules that cause oxidative stress, we observed a significant decrease in the levels of Caspase 9, which represents the intrinsic pathway, in cells receiving NAC, but not in cells receiving TNF-α. Meanwhile, levels of PARP, which represents TNF-α mediated extrinsic pathway, decreased in cells that received NAC after TNF-α treatment. These findings are supported by cell toxicity and apoptosis experiments.

These results answered two main questions of the present study: "Is there a decrease in inflammation and apoptosis after NAC treatment?", and "Does cell survival increase after NAC treatment?" In our neuroinflammation/PD in vitro model, it was shown that inflammation could be initiated by TNF-α and NAC treatment successfully diminished inflammation. Additionally, we showed that cell survival increased, and TNF-α-related apoptosis decreased after NAC treatment.

Next, we investigated possible pathways altered by NAC treatment. For this, we focused on TLR2 and LAG3 receptors that play a part in neuroinflammation. TLRs are

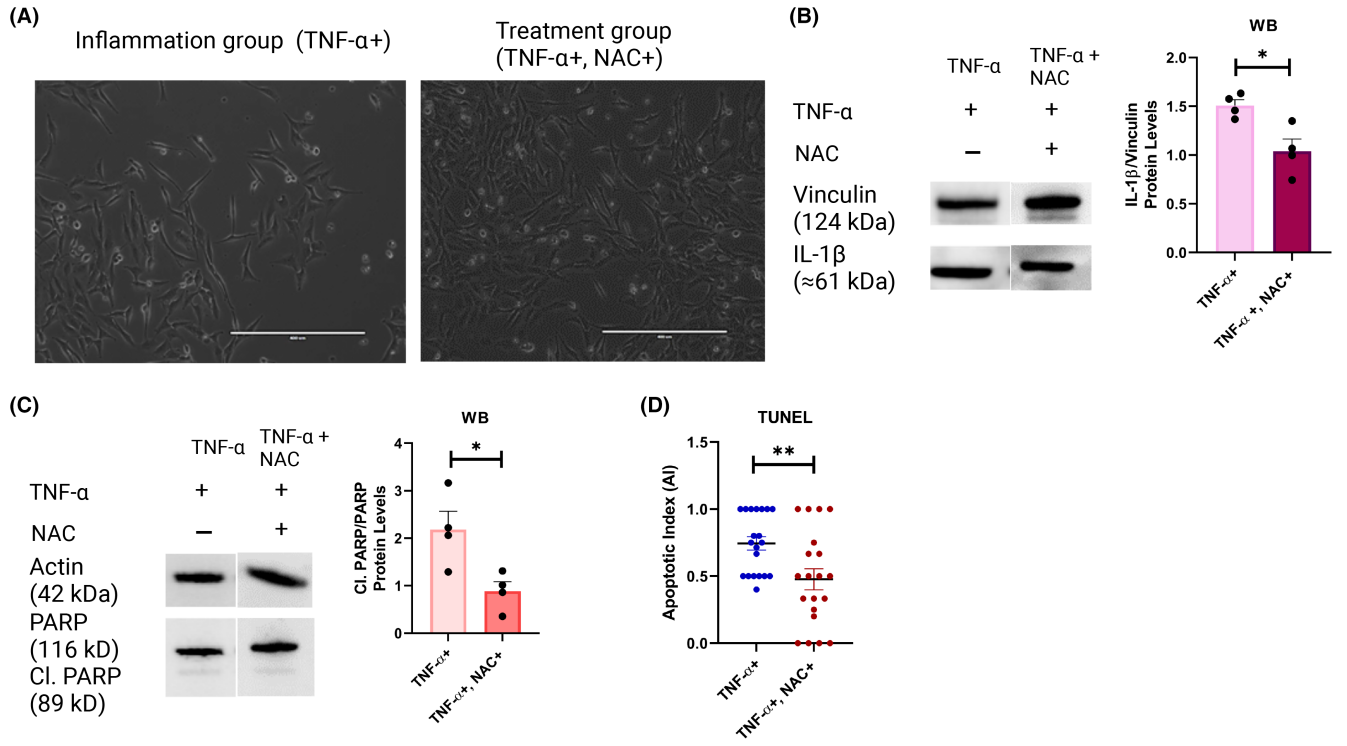

FIGURE 5 NAC treatment relieves inflammation, apoptosis, and cytotoxicity in H4 human neuroglioma cells treated with TNF-α. (A) Evos microscopy images of TNF-α and TNF-α + NAC-treated cells. Scale bar = 400 μm. (B) Representative Western blot and quantitation of IL-1β and vinculin in cell lysates from TNF-α and TNF-α + NAC-treated groups, $n = 4$. (C) Representative Western blot and quantitation of PARP, cleaved PARP, and Actin in cell lysates comparing TNF-α and TNF-α + NAC-treated groups, $n = 4$. (D) TUNEL assay analyses via calculated apoptotic index reflecting the comparison of apoptosis rates of TNF-α added groups and TNF-α + NAC treated groups, $n = 20$ (three sets, 20 different fields). Error bars represent the mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. (Predicted molecular weight of IL-1β is 31 kDa, detected at ≈61 kDa). IL, interleukin; LAG3, lymphocyte-activation gene 3; NAC, *N*-acetylcysteine; TUNEL, terminal deoxynucleotidyl transferase nick end labeling; TNF-α, tumor necrosis factor alpha.

one of the most important components of the innate immune system. TLR2 (CD282) is a transmembrane protein located in the cell membrane, encoded by the TLR2 gene in humans.[53] TLR2 is prominently expressed in neurons, microglia cells, Schwann cells, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, B cells, and T cells.[54,55] TLRs are of great importance in the neuroinflammation process, which is involved in the emergence and progression of neurodegenerative diseases including PD.[55] TLRs located on the cell membrane of neurons and glia cells recognize extracellular α-syn protein and trigger an inflammatory response.[53,56] α-Syn activation of TLR2 activates the NF-kB signaling pathway in the cell. As a result of NF-kB translocation to the nucleus, transcription and expression of inflammatory cytokines are initiated and an inflammatory response occurs.[57,58] Accordingly, TLR2 expression increased not only in the cells of the CNS but also in monocytes of PD patients indicating that inflammation is not limited to the CNS in PD.[59]

Lymphocyte-activation gene 3 (LAG3 or CD223) is a transmembrane protein located on the cell surface, acting as an immune checkpoint receptor belonging to the immunoglobulin superfamily and encoded by the LAG3 gene in humans.[60] It was first identified in the peripheral immune system in natural killer cells, CD4+ and CD8+ T cells, B cells, and dendritic cells. In the CNS, it has been shown to be expressed in microglia cells and neurons.[61] Although there are studies on the importance of LAG3 in autoimmune diseases[62] and cancer,[63] knowledge on its functions in the CNS and its role in neurodegenerative diseases is limited. Recently, it has been proposed that LAG3 may regulate the "prion-like" transition of α-syn preformed fibrils and α-syn protein during neuroinflammation in PD.[20,64] However, a recent study suggested that human neurons do not express LAG3 receptor, and LAG3 does not take a significant role in alpha-synucleinopathies.[21] On the other hand, a clinical study with PD patients showed that serum soluble LAG3 (sLAG3) levels were significantly higher in patients with tremor compared to control groups. In the same study, inflammatory cytokine levels were found to be positively correlated to LAG3 levels in patients with non-motor and motor symptoms.[65] These findings raise a question on whether serum sLAG3 could be used as a potential biomarker in PD.

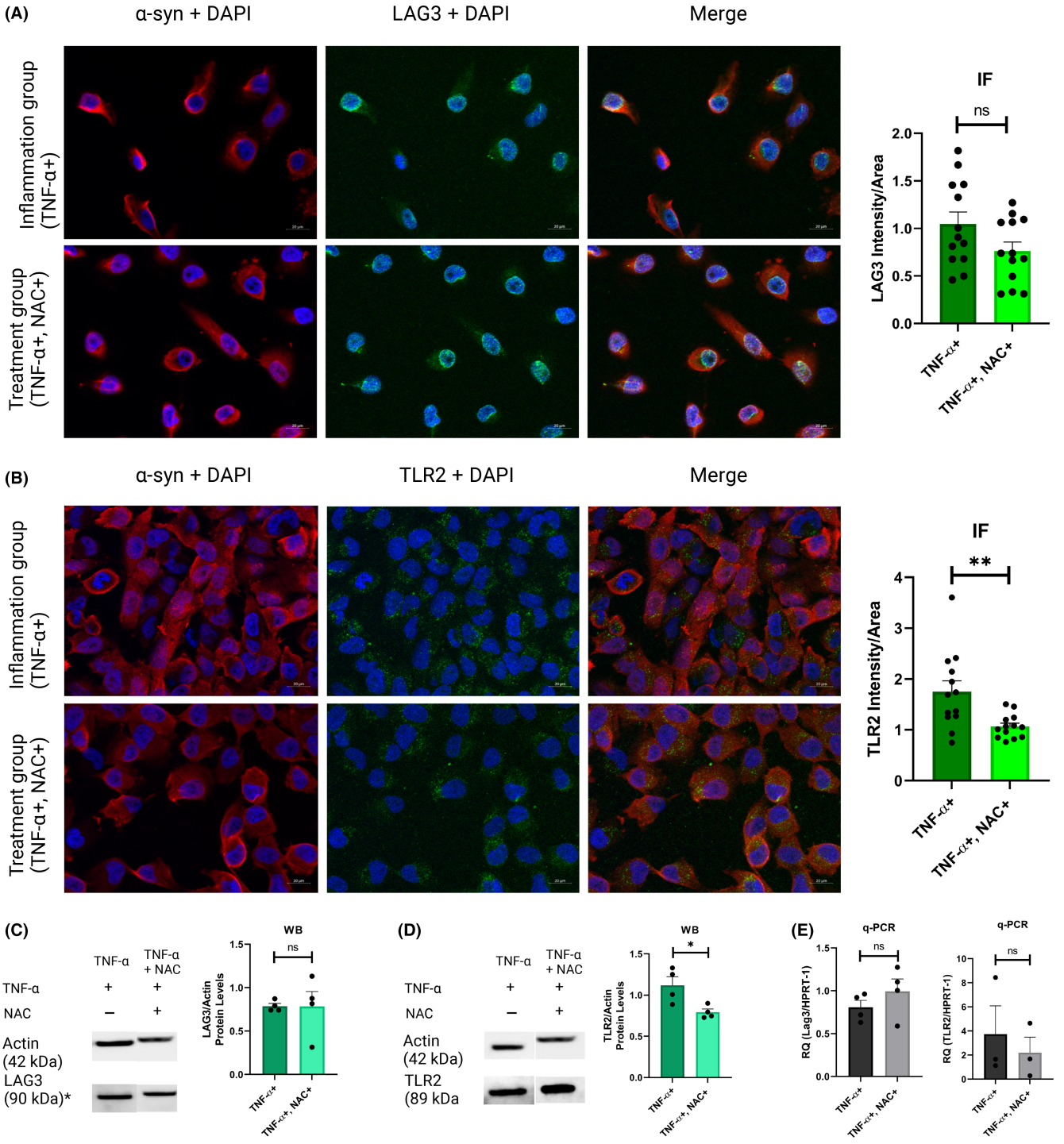

FIGURE 6 NAC treatment relieves inflammation and inflammation-associated alterations in an H4 human neuroglioma cell line overexpressing α-synuclein. (A) Fluorescence microscopy images of LAG3 in TNF-α and TNF-α + NAC-treated cells. Nuclei in blue, α-syn in red, LAG3 in green. Scale bar = 20 μm. IF analyses reflecting the LAG3 intensity/area, $n = 13$ (three sets, 13 different fields). (B) Fluorescence microscopy images of TLR2 in TNF-α and TNF-α + NAC-treated cells. Nuclei in blue, α-syn in red, TLR2 in green. Scale bar = 50 μm. IF analyses reflecting the TLR2 intensity/area, $n = 13$ (three sets, 13 different fields). (C) Representative Western blot and quantitation of LAG3, TLR2 and Actin in cell lysates from TNF-α and TNF-α + NAC-treated groups, $n = 4$. (D) Representative Western blot and quantitation of TLR2 and Actin in cell lysates from TNF-α and TNF-α + NAC-treated cells, $n = 4$. (E) q-PCR analyses reflecting the alterations in LAG3 and TLR2 transcript levels between TNF-α and TNF-α + NAC-treated cells, LAG3 $n = 4$ (four repetitions for each group in each set, 16 analyses), TLR2 $n = 3$ (four repetitions for each group in each set, 12 analyses). Error bars represent the mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. (Predicted molecular weight of LAG3 is 57 kDa, detected at ≈90 kDa). α-syn, alpha-synuclein; IF, immunofluorescent; LAG3, lymphocyte-activation gene 3; NAC, *N*-acetylcysteine; TLR2, Toll-like receptor 2; TNF-α, tumor necrosis factor alpha.

LAG3 is expressed in key cells involved in neuroinflammation and alterations in LAG3 levels have been detected in PD patients. However, it is still unknown if LAG3 is expressed in astrocytes or if it is related to neuroinflammation in PD. We hypothesized that LAG3 is a major regulator of neuroinflammation in this astrocyte derived cell line, and that LAG3 levels can be altered by TNF-α and NAC treatments. Contrary to what we expected, LAG3 transcription levels were seemed lower after administration of TNF-α. Interestingly, when cells treated with only NAC, LAG3 transcript levels were increased significantly.

By contrast, there was no significant change in LAG3 protein after TNF-α-induced inflammation. The possible role of LAG3 receptor in neuroinflammation and its significance in the CNS is unknown.[61] Inflammation evokes the activation of ADAM10 and ADAM17 metalloproteinases located on the cell membrane, leading to cleavage of LAG3 receptor and release of sLAG3.[62,66] Here, we initially assumed that a similar pathway might be activated to cause a decrease in LAG3 after inflammation. This assumption is also supported by a clinical study that detected increased serum sLAG3 levels in PD patients.[65] Considering that NAC administration causes a significant increase in LAG3 transcription levels, it is possible that activation of ADAM10 and ADAM17 would be inhibited by NAC. Here, we suggest that increased levels of LAG3 result from cleavage of LAG3 receptor from the membranes by metalloproteinases and/or cellular recovery is induced due to the anti-inflammatory effect of NAC.

On the other hand, TLR2 expression levels were higher in TNF-α-treated groups and lower in the groups treated with NAC after TNF-α. TLR2 plays a role in inflammation through the NF-kB signaling pathway, while NAC exerts an anti-inflammatory effect by inhibiting this pathway.[51] In our neuroinflammation/PD neuroglioma cell model shows, for the first time, that expression of TLR2 in astrocyte-derived neuroglioma cells is altered by inflammatory and anti-inflammatory conditions. However, no significant difference in TLR2 transcription levels was observed possibly due to low transcription levels of TLR2 in astrocytes.[67]

Currently, there is no cure for PD. Various medical and surgical treatments are provided to slow down the progression of the disease,[68,69] unfortunately there are limitations related to surgery[70,71] and long-term use of pharmacological treatments.[72] As an anti-inflammatory, anti-apoptotic, and antioxidant drug, NAC has an advantage of having less side effects than other anti-inflammatory drugs and immunomodulators and being well tolerated by patients. It is also an inexpensive and easy-access medicine. There are some clinical studies on NAC treatment in PD patients (https://clinicaltrials.gov/). While some of these studies have been completed, some of them are still ongoing.

5 | CONCLUSION

In conclusion, this study was carried out in vitro and might not exactly reflect all the parameters of the in vivo neuroinflammation. Due to this limitation, the anti-inflammatory and anti-apoptotic effects of NAC in neuroinflammation and its connection with TLR2 and LAG3 receptors were observed in a neuroinflammation model of PD created with H4 cells. As a result of this study, the anti-inflammatory and anti-apoptotic effects of NAC on cells were successfully demonstrated and it was concluded that NAC could play a role in slowing the progression of the disease by increasing cell survival. While TLR2 receptor responded to NAC as expected at the level of expression and transcription, unexpected alterations were observed in the LAG3 receptor. NAC may be a promising candidate to slow down and to recover from the neuroinflammation that occurs in PD. Further pharmacokinetic studies are necessary to evaluate the in vivo dose adjustments based on our in vitro results. Additionally, more studies are needed to elucidate molecular mechanisms underlying the disease, molecular pathways related to neuroinflammation and to develop possible new therapeutic approaches to slow down the clinical progression of PD.

AUTHOR CONTRIBUTIONS

Zeynep Bengisu Kaya contributed to data acquisition, analysis, interpretation, and drafted the manuscript; Elif Karakoc contributed to data interpretation and revised the manuscript; Pamela J. McLean contributed to analysis, interpretation, and critically revised the manuscript; Esen Saka contributed to conception and design; Pergin Atilla contributed to conception, design, and interpretation. All authors proofread and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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