LRRK2 and WAVE2 regulate microglial-transition through distinct morphological phenotypes to induce neurotoxicity in a novel two-hit in vitro model of neurodegeneration

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Abstract
We report a novel in vitro classification system that tracks microglial activation state and their potential neurotoxicity. Mixed live-cell imaging was used to characterize transition through distinct morphological phenotypes, production of reactive oxygen species (ROS), formation of reactive microglial aggregates, and subsequent cytokine production. Transwell cultures were used to determine microglial migration (control and lipopolysaccharide (LPS) treated) to glutamate pre-stressed or healthy neurons. This two-hit paradigm was developed to model the vast evidence that neurodegenerative conditions, like Parkinson’s disease (PD), may stem from the collective impact of multiple environmental stressors.

We found that healthy neurons were resistant to microglial-mediated inflammation, whereas glutamate pre-stressed neurons were highly susceptible and in fact, appeared to recruit microglia. The LPS treated microglia progressed through distinct morphological states and expressed high levels of ROS and formed large cellular aggregates. Recent evidence implicates leucine-rich repeat kinase 2 (LRRK2) as an important player in the microglial inflammatory state, as well as in the genesis of PD. We found that inhibition of the LRRK2 signaling pathway using the kinase inhibitor cis-2,6-dimethyl-4-(6-(5-(1-methylcyclopropoxy)-1H-indazol-3-yl)pyrimidin-4-yl)morpholine (MLi2) or inhibition of the actin regulatory protein, Wiskott–Aldrich syndrome family Verprolin-homologous Protein-2 (WAVE2), stunted microglial activation and prevented neurotoxicity. Furthermore, inhibition of LRRK2 kinase activity reduced pro-inflammatory chemokines including MIP-2, CRG-2, and RANTES. These data together support the notion that LRRK2 and WAVE2 are important mediators of cytokine production and cytoskeletal rearrangement necessary for microglial-induced neurotoxicity. Furthermore, our model demonstrated unique microglial phenotypic changes that might be mechanistically important for better understanding neuron-microglial crosstalk.

Abbreviations: A, amoeboid; ABB, annexin binding buffer; ARP2/3, actin-related protein 2/3; bROS, BV2 reactive oxygen species; CM, complete media; CRG, CellRox Green; CRG-2, cytokine responsive gene 2; CTDR, Cell Tracker Deep Red; ctf, corrected total fluorescence; FAK, focal adhesion kinase; IGFBP, insulin-like growth factor binding protein 1; IL, interleukin; InD, integrated density; LPS, lipopolysaccharide; LRRK2, leucine-rich repeat kinase 2; MCP-1, monocyte chemotactant protein 1; MIP-2, macrophage inflammatory protein 2; ML2, 3(4-Pyrimidinyl) Indazole; nROS, neuronal reactive oxygen species; P, Passive; PD, Parkinson’s disease; PDL, poly-d-lysine; PI, propidium iodide; pLRRK2, phosphorylated LRRK2; PS, phosphatidylserine; pWAVE2, phosphorylated WAVE2; R, Reactive; RANTES, regulated on activation, normal T cell expressed and secreted; RNS, reactive nitrogen species; ROI, region of interest; ROS, reactive oxygen species; RSFM, reduced serum fluorobrite media; S, sampling; T, transition; TNFa, tumor necrosis factor-alpha; WAVE2, Wiskott-Aldrich syndrome protein family member 2; (SNpc), substantia nigra pars compacta.
1 | BACKGROUND

Microglia are highly plastic immunocompetent cells that act as rapidly responding sentinels protecting the central nervous system from infectious, traumatic, and other environmental stressors (Anderson et al., 2018; Wolf et al., 2017). However, microglia might also, in the long run, damage otherwise healthy brain tissue when faced with chronic activation and the adoption of a "hyperactive" pro-inflammatory phenotype. Although the "M1" (pro-inflammatory) and "M2" (anti-inflammatory) microglial phenotypes in vivo are well documented (Brown & Neher, 2014; Dwyer et al., 2020; Landrigan et al., 2020; Neher et al., 2013), these represent only the extremes and there likely exists a whole spectrum of differing microglial states contributing to the pathological processes in neurological disease.

Virtually all neurodegenerative diseases have some element of microglial-driven inflammation and in the case of Parkinson’s disease (PD), this has been linked to exposure to environmental toxins, aging, and certain genetic vulnerabilities (Goldman, 2014). In fact, progressive neurodegenerative conditions like PD or Alzheimer’s disease are believed to arise from a complex interaction between genetic background and exposure to a variety of environmental stressors or “hits” often referred to as a “multiple-hit hypothesis” (Cabezudo et al., 2020; Dwyer et al., 2020). Such “hits” can include pesticides (such as rotenone or paraquat), other chemical stressors (like MPTP or heavy metals), or microbial or pathogenic insults (such as certain viral or bacterial infections) that collectively produce neuronal injury (Patrick et al., 2019; Sadasivan et al., 2017). Mutations in leucine-rich repeat kinase 2 (LRRK2) are observed in PD and may promote neuronal vulnerability to environmental hits by virtue of enhanced microglial activation, inflammatory cytokine and chemokine production, and phagocytic and cytoskeletal changes (Cookson, 2015; Dwyer et al., 2020, 2020a; Gillardon et al., 2012; Lee et al., 2017; Subramaniam & Fedoroff, 2017). Importantly, LRRK2 is upregulated in activated microglia to facilitate vesicular sorting, mitochondrial fission, autophagy, and phagocytosis (Choi et al., 2015; Ho et al., 2018; K. S. Kim et al., 2018; Russo et al., 2015; Schapansky et al., 2014).

Activation of microglia with inflammatory stimuli, such as lipopolysaccharide (LPS), provokes LRRK2 phosphorylation, dimerization, localization to the plasma membrane, and co-localization with autophagosomes (Schapansky et al., 2014). These changes are coupled with microglial transition to an amoeboïd-like morphology, secretion of inflammatory cytokines, oxidative stressors, and increased phagocytic capacity (Moehle et al., 2012; Schapansky et al., 2014). Microglia with the G2019S LRRK2 mutation are associated with increased inflammatory states, while LRRK2 inhibition can reduce inflammation and neurotoxicity (Daher et al., 2014; Gillardon et al., 2012; B. Kim et al., 2012; Moehle et al., 2012). It is possible that the inflammatory aspects of LRRK2 might be regulated through the downstream factor, WAVE2 (a member of the WASP family of proteins; K. S. Kim et al., 2018; Marcogliese et al., 2017; Takenawa & Suetsugu, 2007). Specifically, LRRK2 phosphorylates WAVE2 at T96 and T470, the latter of which has been implicated in vitro dopaminergic cell death (K. S. Kim et al., 2018), and WAVE2 modulates proteins necessary for cytoskeletal rearrangement and phagocytosis (Kitamura et al., 2003; Suetsugu et al., 2006; Takenawa & Suetsugu, 2007).

Using a novel two-hit in vitro model, we found that mildly pre-stressed neurons recruited LPS-stimulated microglia allowing for localized crosstalk necessary and sufficient to induce neurotoxicity. Moreover, inhibition of LRRK2 kinase activity prevented this neurotoxicity and reduced the expression of chemokines. We characterized five distinct morphological states through which microglia progress, which we named Ready and Responsive states. Once in the Responsive state, microglia cells express high levels of ROS and associate with large, cellular aggregates of microglia. The mechanisms of microglia activation were LRRK2 and WAVE2 dependent and appeared to involve cytoskeletal rearrangement in neurotoxicity. These data not only have implications for PD but more broadly, neuron-microglial interactions in general and the characterization of progressive in vitro microglial phenotypes as pertains to pathogenic stress and neurodegeneration.

2 | METHODS

Reagents were purchased from Sigma-Aldrich unless noted otherwise.

2.1 | Differentiation and injury of SH-SY5Y cells

For all experiments (unless noted otherwise), SH-SY5Y cells (ATCC) were plated at 1 × 10⁵ cells/ml in complete media (CM: 10% fetal bovine serum [FBS], 1% penicillin/streptomycin, 89% high glucose DMEM) and allowed to adhere for 24 h. Retinoic acid (RA: 10 μM in CM) was added to SH-SY5Y cultures every other day for a total of 7 days for differentiation. After SH-SY5Y cells were differentiated, they were treated with 100 mM glutamate in reduced serum FluoroBrite DMEM Media (RSFM; 1% FBS, 1% penicillin/streptomycin, and high glucose FluoroBrite DMEM [Thermo Fisher Scientific]) or RSFM only for 24 h. This concentration of glutamate was determined from a dose-response we conducted, wherein 100 mM glutamate induced a reversible state of distress/injury with minimal to no cell death and minimal reactive oxygen species (ROS) production (Figures 1i and S1b). Furthermore, this glutamate dose was based on findings from previous reports (Terziöglu Bebitoğlu et al., 2020). All single-channel bright-field images of cultures in CM were captured on a Zeiss Axio Observer D1 inverted microscope with an N-Achroplan 10×/0.25NA Ph1 M27 objective and AxioCamMR3 monochrome camera.
FIGURE 1  Defining neuronal injury in the transwell coculture model. BV2 cells or primary microglia are seeded onto transwell inserts and then inserted into 24-well plates with differentiated SH-SY5Y cells grown on coated glass coverslips. The secreted factor model uses a 0.4 μm pore size to exclude microglia cell migration (a) while the migration model uses an 8 μm pore size to promote microglia cell migration (b). The state of neuronal distress was categorized into four levels. Healthy neurons were phosphatidylserine (PS) and propidium iodide (PI) negative (c), low injury neurons exhibited low levels of PS expression (d), high injury neurons exhibited high levels of PS expression (e), and dead neurons were positive for PI (f). Levels of ROS in SH-SY5Y cells exposed to 100 mM glutamate (i) are slightly elevated compared to those cultured in media-only (g) or 50 mM glutamate (h). We compared the ROS induced by 100 mM glutamate to that induced by exposure to 1 mM H₂O₂ for 1 h (l). Glutamate-induced ROS over 24-h (i) is comparable to baseline ROS levels within the first 15 min of H₂O₂ exposure (j, k). Therefore, pre-stressed SH-SY5Y cells used in the two-hit model were treated with 100 mM glutamate and exhibit low levels of injury. In microscopy images: Blue (Hoescht dye; nuclei), Green (PS), Red (PI), Scale bar = 20 μm (c-f); 10 μm (g-l)
2.2 Secreted factor (supernatant) monoculture survival model and experimental design

SH-SY5Y cells were plated on poly-d-lysine (PDL; 10 μg/ml) and laminin (1.8 μg/ml) coated 96-well plates at 5 × 10^4 cells/ml in CM, differentiated, and pre-stressed with 100 mM glutamate. BV2 cells (gift from David Park lab) were separately plated on PDL treated 12-well plates at 1 × 10^5 cells/ml in CM for 24 h. BV2 cells were then treated with 100 ng/ml LPS in RSFM or RSFM-only and incubated for a further 48 h. BV2 supernatants were collected and centrifuged at 1200 rpm for 5 min to remove cellular debris. The glutamate treatment was removed from the SH-SY5Y cells and the BV2 supernatants were then added to the SH-SY5Y cultures and allowed to incubate for 48 h. In short, there were four experimental conditions where SH-SY5Y cells were exposed to BV2 supernatants: (1) control SH-SY5Y cells exposed to LPS-stimulated BV2 supernatants, (2) pre-stressed SH-SY5Y cells exposed to stimulated BV2 supernatants, (3) control SH-SY5Y cells exposed to resting BV2 supernatants, and (4) pre-stressed SH-SY5Y cells exposed to resting BV2 supernatants. Control or pre-stressed SH-SY5Y cells were also exposed to RSFM or 100 ng/ml LPS in RSFM.

Cell survival was measured using the PrestoBlue survival assay (Thermo Fisher Scientific). Briefly, 10% PrestoBlue reagent was added to each experimental well and incubated for 1 h at 37°C and 5% CO₂. Absorbance was measured using a Molecular Devices SpectraMax 190 with SoftMax Pro version 5.3 at 570 nm with reference at 600 nm. Due to the variability inherent to this assay method, all experimental conditions were performed with 8 internal replicates. Minimal mean absorbance values at 570/600 nm representing viable, confluent cultures were validated based on bright-field microscopy observations of all eight biological replicates of a given condition. This value was used as the baseline absorbance mean ratio representing no cell death in the interpretation of the Presto Blue data.

2.3 Primary cortical microglia isolation

Primary microglia were isolated from G2019S knock-in (G2019SKi; on a C57Bl/6 background) mice or wild-type (WT) littermate controls. Cortical tissue was dissected from 5 to 10 P1-P2 mice (1 litter) and placed in ice-cold HBSS and manually dissociated using surgical blades, followed by chemical dissociation with TrypLE (Thermo Fisher Scientific) for 30 min at 37°C. The dissociated tissue was centrifuged at 1200 rpm for 5 min and resuspended in 10 ml of CM. Following trituration, the cell suspension was filtered through a 70 μm cell strainer to remove debris. The filtered cell suspension was then plated in a PDL-coated T75 flask. Media was changed after 2–3 days and then allowed to incubate for 14–21 days. On the day of microglia harvesting, the cells were placed on an orbital shaker at 300 rpm for 2–4 h at room temperature (RT). Microglia in suspension after shaking were collected and put on ice. The astrocyte layer was dissociated from the flask by incubating with 0.08% Trypsin, 0.6 mM CaCl₂, and 0.16 M EDTA at 37°C for 30 min. Following incubation, the astrocyte layer is removed, and the isolated microglia are then added to the remaining microglial monolayer and allowed to propagate.

2.4 Two-hit transwell coculture model and experimental design

SH-SY5Y cells were plated on PDL and laminin-coated glass coverslips in 24-well plates, differentiated, and pre-stressed with glutamate. Then, BV2 or primary microglial cells were separately plated on PDL treated 0.4 μm (for Secreted Factor Coculture Assays) or 8 μm (for Migration Coculture Assays) Corning transwell inserts (Thermo Fisher Scientific) at 1 × 10^5 cells/ml in CM. BV2 (or primary microglial) cells were then treated with LPS (100 ng/ml or 1 μg/ml) or LPS with MLi2 (100 nM; LRRK2 inhibitor; Tocris Biotechnology) in RSFM and incubated for 24 h. Note, this concentration of MLi2 corresponds to the MLi2_high concentration used in subsequent live cell experiments. BV2 (or primary microglia) cells in transwell inserts were washed in CM and then added to the SH-SY5Y wells and cocultured for 24 h (Figure 1a,b). Transwell inserts were removed and the state of neuronal health was determined by phosphatidylserine (PS) expression and propidium iodide (PI) incorporation.

2.5 Characterization of neuronal health (PS and PI)

Annexin V-488 (Thermo Fisher Scientific) was used to detect PS expression. SH-SY5Y cells were first washed with a solution of annexin binding buffer in phosphate-buffered saline (PBS; ABB). Thereafter, the cells were incubated with 10 ng/ml of PI (Thermo Fisher Scientific) and 1:20 dilution of stock annexin V-488 solution in ABB for 15 min at 37°C. Cells were then further washed with PBS, fixed with 2% paraformaldehyde (PFA) for 15 min at RT, and counterstained with hoescht dye. Following subsequent washes, SH-SY5Y cells were mounted using gelvatol and imaged using a Zeiss Axio-observer Z1 inverted epifluorescence microscope with Zen Blue 2.3 Software (camera = AxioCam MRm CCD monochrome; objective = plan-aposochromat 40×/0.95NA Corr M27; excitation Filters = DAPI, FITC, and CY3).

For each cell in an image, the state of neuronal health was categorized as follows: healthy neurons were PS and PI negative, low injury neurons exhibited low levels of PS expression (mean cell fluorescence intensity range 10–35, max single-pixel intensity < 50), high injury neurons (mean cell fluorescence intensity range > 50) exhibited high levels of PS expression, and dead neurons were PI-positive (Figure 1c–f). Total cell counts and dead neurons were quantified using Fiji ImageJ software (version 2.0.0-rc-69/1.52n). For this analysis, images were opened in Fiji and the channels were split into single-channel black and white 8-bit images. The DAPI (hoescht dye) channel was used to identify and record each nucleus as an ROI. This ROI was then applied to the Cy3 (PI) channel. Total cell number per image and the total number of dead neurons were quantified using this approach. Finally, the RGB channels were merged and PI-negative nuclei were manually categorized into healthy, low injury, or high injury neurons based on PS expression (FITC channel). For all images included in Figure 2, a 50-rolling ball radius background correction was applied to the DAPI channel before merging the three channel images.
Figure 2
(See caption on next page)
2.6 Proportional-odds logistic regression for neurotoxicity in the two-hit transwell model

We modeled the effect of our experimental conditions on neurotoxicity in the secreted factor and migration transwell assay with a specialized regression model. We classified the neurotoxic state into one of four ordered levels: healthy, low injury, high injury, and dead. To account for ordering, we used a proportional-odds logistic regression model. These models take into account the ordering of the outcome and are more powerful than a generic multinomial model which can account for multiple outcomes but does not take advantage of their relative ordering. Our regression model also included random effects. Random effects account for outcomes that may covary within some levels of nonexperimental independent variables. In our experimental design, several sample images were taken within and between single well(s) on a plate. In turn, these wells shared a common plate. We would expect each plate and well to share micro-environmental characteristics. Thus, we modeled these as a nested random effect. Models that include both traditional, fixed effects, and random effects are called mixed effect models. Most completely, we performed a mixed-effects, proportional-odds, logistic regression model using the ordinal package (version ordinal_2019.4-25) of R (version 3.6.1).

For the first three conditions, BV2 cells (or primary microglial $1 \times 10^5$ cells/ml) were prepared in the corresponding concentration of LPS alone or together with MLi2 in RSFM and added directly to SH-SY5Y cultures. For the CK-869 condition, BV2 (or primary microglial) cells were plated in 12-well plates at $1 \times 10^5$ cells/ml with LPS and CK-869 in RSFM and incubated at 37°C, 5% CO₂ for 2 h, before coculture with SH-SY5Y cells. The cells were then washed in PBS, resuspended in $1 \mu$g/ml LPS in RSFM at $1 \times 10^5$cells/ml, and then added directly to SH-SY5Y cells. The pretreatment with CK-869 was necessary because direct application of CK-869 on SH-SY5Y cells was toxic (Figure S1f).

At the start of coculture, CellRox Green (CRG: 2.5 mM; Thermo Fisher Scientific) was immediately added to the cultures and incubated for 30 min before imaging. Live cell imaging was performed using a GE DeltaVision Elite on Olympus Xi-71 inverted epifluorescence microscope (Objective = Olympus 60×/1.42NA Plan Apo N; Camera = Scientific CMOS Camera) with environmental chamber control (37°C, 5% CO₂, with humidity control) using SoftWorx imaging software (version 7.0). Three-channel images (excitation filters: FITC, CY5, and POL) were captured every 5 min for 6 h, with Ultimate Focus every four time points.

2.7 Live cell oxidative stress and neurotoxicity model and experimental design

SH-SY5Y cells were plated in PDL and laminin-coated Ibidi eight-well chamber slides at $2 \times 10^5$ cells/ml in CM, differentiated, and then exposed to glutamate (as in transwell experiments). To fluorescently label SH-SY5Y cells, the glutamate treatment was removed and replaced with 2.5 μM CellTracker Deep Red (Thermo Fisher Scientific) for 30 min at 37°C, followed by PBS wash, before coculture with BV2 or primary microglial cells.

For live-cell experiments, there were four experimental conditions: LPS (1 μg/ml LPS in RSFM), MLi2low (1 μg/ml LPS and 10 nM MLi2 in RSFM), MLi2high (1 μg/ml LPS and 100 nM MLi2 in RSFM), and CK-869 (1 μg/ml LPS and 100 μM CK-869 in RSFM). A previous study using cultured macrophages reported that 50 nM of MLi2 was suppressed LRRK2 kinase activity, as indicated by levels of the phosphorylated LRRK2 protein (pLRRK2; J. Kim et al., 2019).

For live-cell video consisted of three color channels (brightfield, green, and red) and 61 time points. For our quantitative analysis, we focused on the following time points: 60, 110, 130, 175, 205, 235, 280, and 360 min. Images were opened in Fiji with each color channel split. Bright-field images were used to manually identify: (1) BV2 cells, (2) SH-SY5Y cells, (3) BV2 morphological state, (4) number of BV2 cells in an aggregate, and (5) whether a particular BV2 cell is in physical contact with an SH-SY5Y cell. BV2 and SH-SY5Y cell identity was maintained between channels and time points using the ROI manager. Although the morphological characteristics of BV2 and SH-SY5Y cells are easy to distinguish using bright field microscopy, we verified the identification of SH-SY5Y cells using the red channel (CTDR). The morphological state of BV2 cells was categorized as P (assive), T(ransition), S(ampling), R(active), or A(moeboid), quantitated, and a descriptive analysis of ROS production in each state was performed. These distinct morphological states/categories were
arrived upon after assessment of over 500 BV2 cells during 6 h of live-cell imaging and confirmed in primary microglia (Figure 5a,b).

The size of microglial cell aggregates at each timepoint was manually counted using the bright-field channel of the live-cell videos. A microglial aggregate is defined as the number of microglia in a cluster where each microglia is in direct cell–cell contact with at least one other microglia in the cluster. Each microglia counted was assigned an identifier number and the size of its aggregate was recorded. If a microglial cell was not associated with another microglial cell, its aggregate size was 1. For any microglia that were associated with one or more microglial cells, the aggregate size was the number of microglia in that cluster. We compared the aggregation sizes under multiple experimental conditions (LPS, LPS + 10 nM MLi2, LPS + 100 nM MLi2, and LPS + CK-869) using Tukey’s Honest Significant Difference (THSD) statistical test, as it corrects for multiple comparisons in an efficient way by respecting the ordered magnitudes of the compared values.

The green channel (CRG) was used for quantitating the fluorescence of ROS in BV2 cells and SH-SY5Y cells. All data were collected from black and white single-channel 8-bit images. For each selected time point, cells were manually identified in the brightfield channel and saved to the ROI manager. This ROI was then applied to the green channel where the following measurements were obtained: area, mean gray value, min & max gray value, and integrated density. Corrected total cell fluorescence was calculated using the following equation: Integrated Density = (Area of selected cell × Mean fluorescence of background readings). All ROS levels are reported as mean corrected total fluorescence (ctf). To determine the significance of reduced ROS levels when LRRK2 or WAVE2 are inhibited, we performed a Tukey HSD multiple comparisons of the means statistical test.

2.9 | Cytokine array

SH-SY5Y cells were plated in 12-well plates at 1 × 10^5 cells/ml in CM. Cells were differentiated with 10 μM RA for 7 days and pre-stressed with 100 mM glutamate for 24 h. BV2 cells were activated with 1 μg/ml LPS or 1 μg/ml LPS and 100 nM MLi2 and added to pre-stressed SH-SY5Y cells as per the live-cell experimental protocol. At 175 min of coculture, supernatants, and cell lysates were collected. The supernatants were centrifuged at 1800 rpm for 5 min to remove cells from the suspension and then frozen at −20°C until used for Abcam Mouse Cytokine Antibody Array assay. Lysis buffer (from Abcam Kit) with halt protease inhibitor was added to each well of the cocultures and cells were scraped and pipetted to lyse cells. Cell lysates were vortexed and then incubated at 4°C for 15 min. Lysates were vortexed again and then centrifuged at 13,800 rpm for 10 min at 4°C and then stored at −20°C until use.

The cytokine detection was performed as described in the product manual for the Abcam Mouse Cytokine Antibody Array (see Figure S2a,b for a map of the Cytokine C3 and C4 membranes). Briefly, supernatants and lysates were thawed and centrifuged for 3 min at 10,000 rpm to remove particles. Lysates were diluted at 1:5 in blocking buffer. Membranes were incubated in a blocking buffer for 30 min at RT and then incubated in cell supernatants or lysates for 2 h at RT. Membranes were incubated in Biotinylated Antibody Conjugate Cocktail C3 or C4 for 2 h at RT and HRP-Conjugated Streptavidin for 2 h at RT. Wash steps were performed after each 2 h incubation step. Following the final wash step, detection buffers were added for 2 min at RT and then imaged using the Syngene G:Box gel documentation system and GeneSys software (version 1.6.9.0) for chemiluminescence detection (see Figure S2c,d for original blots).

Densitometry data for each membrane was obtained using Fiji. First, an ROI for the positive control of one membrane was imported into the ROI manager. Then, this ROI was duplicated for each spot on the first row of a membrane representing a cytokine sample (14 samples across for C3 membranes and 12 samples across for C4 membranes). Integrated density was calculated for each ROI on that row using the Analyze-Measure feature. The ROI used for the first row was then used for each subsequent row of the membrane, analyzing the integrated density (InD) of each spot one row at a time. We used a conservative background subtraction method by subtracting the highest value blank or negative InD obtained on a membrane from each sample InD value. All membranes were normalized to the LPS-lysate membrane using the equations: 

\[ X(Ny) = X(y)P_1/P_y \]

where X(Ny) is the normalized InD for a given cytokine, X(y) is the mean InD for that cytokine, P_1 is the mean InD for the positive control for the LPS-lysate membrane, and P_y is the mean InD for the positive control on the membrane where InD is being normalized. Normalized InD values were then converted to percent of P_1. Heatmaps were generated using the Python package seaborn (version 0.9.0). For proteins to be included in our results, they had to meet the following criteria: (1) the mean expression value of the protein was greater than 10%InD, (2) there was a difference in expression between the LPS and MLi2 conditions within the same sample type (i.e., supernatants or lysates), (3) the difference between the minimum percent InD value in one condition was greater than 10%InD above the maximum of the second condition. While there are other proteins that may be of interest represented in the generated heatmaps, they were not evaluated for inclusion in this analysis.

3 | RESULTS

We hypothesized that the reversibly distressed state of a neuron, combined with over-activated microglia, leads to neuronal oxidative stress and potentially phagoptosis that might be relevant for neurodegeneration in general and PD in particular. Therefore, we developed a novel two-hit model, whereby we stimulate BV2 cells with LPS and pre-stress differentiated SH-SY5Y with sub-toxic levels of glutamate. To test our hypothesis, we characterized the (1) direct effects of BV2 supernatants on SH-SY5Y cells, (2) effects of microglial-derived ROS on neuronal ROS, (3) distinct morphological changes of activated BV2 cells, (4) formation of, and ROS reactivity in, large cellular aggregates of activated BV2 cells, (5) expression of
inflammatory and anti-inflammatory proteins, and (5) potential for premature phagocytosis of neurons by activated microglia (primary phagoptosis). We also, in some studies, utilized primary microglia from G2019S LRRK2 mutants (and WT littermates) to assess whether this mutation further augments toxicity.

3.1 | Effects of BV2 microglial cells on neurotoxicity

3.1.1 | Activated BV2 cell secretions are insufficient to induce neurotoxicity

Microglia may secrete inflammatory cytokines and/or reactive oxygen and nitrogen (ROS and RNS) species to mediate neurotoxicity. To determine if activated BV2 cells secrete stable, soluble factors sufficient to induce neurotoxicity, we exposed healthy, SH-SY5Y cells to supernatants collected from LPS-stimulated and non-stimulated BV2 cells. However, following 48 h of supernatant exposure, there was no evidence of any neurotoxicity (Figure S3, gray bars).

To further assess whether pre-stressed neurons are more susceptible to BV2 secretions than control neurons, we exposed SH-SY5Y cells to sub-toxic levels of glutamate before BV2 supernatant exposure. In this two-hit model, BV2 supernatants once again did not induce neurotoxicity in pre-stressed SH-SY5Y cells (Figure S3, black bars). Therefore, we conclude that LPS stimulation of BV2 mononuclei is insufficient to induce SH-SY5Y neurotoxicity through secreted soluble factors.

3.1.2 | Microglial-induced neurotoxicity requires close cell–cell proximity, mild neuronal distress, and appeared to involve LRRK2 kinase functioning

To determine if crosstalk between BV2 cells and SH-SY5Y cells is necessary and sufficient to induce neurotoxicity, we designed a two-hit transwell coculture model. To this end, resting (non-stimulated) or LPS stimulated BV2 cells were plated in 0.4 μm transwell chambers (for secreted factor analysis, as small pores do not allow cell passage) or 8 μm transwell chambers (for migration and cell–cell proximity analysis, as pores are large enough to allow cell passage) and cocultured with control or pre-stressed SH-SY5Y cells (Figure 1a,b).

To verify the effects of LPS stimulated microglia on pre-stressed SH-SY5Y cells, we repeated the 8 μm transwell chamber migration assay with LPS stimulated primary cortical microglia (rather than BV2 cells). We found that WT, LPS stimulated, primary microglia did indeed induce SH-SY5Y injury as evident from PS flip and PI incorporation (Figure 2p–solid line), albeit to a somewhat lesser extent than that seen with BV2 cells. Surprisingly, LPS stimulated primary microglia from LRRK2 G2019S knock-in overexpressor mice induced less neurotoxicity compared to WT microglia (Figure 2q–dashed line). This is at odds with the MLi2 data, wherein LRRK2 kinase inhibition was protective. As will be discussed later, this unexpected finding might stem from the G2019S mutation reducing microglial motility in this specific model.
3.2 Early mechanisms of BV2 activation and subsequent ROS accumulation in SH-SY5Y cells

The transwell migration assay demonstrated the potent neurotoxic capabilities of LPS-stimulated microglial cells following a 24-h incubation period. We were next interested in elucidating the morphological changes and ROS production associated with the early stages of activation of BV2 cells during coculture. Therefore, we expanded our two-hit model where we cultured LPS-stimulated BV2 cells and pre-stressed SH-SY5Y cells as mixed (rather than separate as in the previous experiments) cultures. Hence, we assessed concomitant BV2 and neuronal ROS production, as well as BV2 morphological changes over the first 6 h of coculture via live-cell microscopy.

3.2.1 Early ROS accumulation in BV2 cells is LRRK2-independent but is WAVE2-dependent: Live-cell microscopy

To determine if BV2 cells in the two-hit model induce an oxidative burst as an early inflammatory signaling event, we used live-cell microscopy to measure the changes in ROS over the first 6 h of coculture. MLi2 (LRRK2 kinase inhibitor; Figure S5) and CK-869 (an inhibitor of the WAVE2 regulatory pathway via Arp2/3) were used to determine whether LRRK2 or WAVE2, respectively, can influence ROS production. LPS-stimulated BV2 cells showed minimal to no ROS production (barely detectable, less than 300 ctf) within the first 60 min of coculture with pre-stressed SH-SY5Y cells (Figure 3a). By 175 min of coculture, ROS production begins to increase in BV2 cells with mean total cell fluorescence now clearly detectable (~500 ctf). BV2 ROS continued to increase until ROS production peaked (mean fluorescence greater than 1500 ctf) by 360 min. While there was no significant effect of the MLi2 LRRK2 kinase inhibitor on ROS production ($p = 0.230$ for $M_{\text{Lii}}$low and $p = 0.0821$ for $M_{\text{Lii}}$high), the Arp2/3 inhibitor (downstream WAVE2 target), CK-869, did significantly decrease ($p = 0.0001$) mean ROS produced by BV2 cells (Figure 3). Indeed, CK-869 reduced the overall levels of ROS (Figure 3c), as well as the rate of ROS accumulation compared to the LPS-only treated BV2 cells (Figure 3a, Video S1).

3.2.2 Early ROS accumulation in SH-SY5Y cells is dependent on both LRRK2 and WAVE2 signaling in BV2 cells: Live-cell microscopy

Reactive oxygen and nitrogen species produced in stimulated microglia may be toxic to nearby neurons. Therefore, we assessed the accumulation of oxidative stress in glutamate pre-stressed SH-SY5Y cells when exposed to LPS stimulated BV2 cells. In this model, SH-SY5Y ROS (neuronal ROS: nROS) levels were relatively undetectable through the
first 130 min (Figure 4). However, nROS levels began to rise after 130 min and continue to do so over the 360 min of coculture. By 175 min, nROS were significantly elevated to 879 ctf, but at this time, the ML2 and CK-869 inhibitors significantly blunted the elevation of nROS levels (mean values below 550 ctf; ML2low \( p = 0.001 \), ML2high \( p = 0.041 \), CK-869 \( p = 0.001 \); Figure 4; Video S2). By 360 min, maximum nROS levels were observed (greater than 1750 ctf) in SH-SYSY cells. Maximum ROS levels were not reduced by ML2 induced LRRK2 kinase inhibition, but when WAVE2 function was inhibited by CK-869, the nROS levels were significantly reduced to almost baseline levels, with mean nROS at 463 ctf (\( p = 0.001 \); Figure 4).

3.2.3 | Both BV2 cells and primary microglia undergo specific morphological changes characteristic of increased activation states

Microglia are known to cycle through obvious changes in morphology that correspond with shifts from “resting or baseline surveillance” to activated pro-inflammatory states (often referred to as M1), or sometimes (depending upon the microenvironment and stimulus present) to more anti-inflammatory and reparatory (often called M2) states. In reality, there are probably many intermediate variations in these states, in which cell morphology and inflammatory secretagogues together dictate physiological outcomes and whether neurotoxicity might ensue. We have previously utilized a rating scale to capture such microglial morphological changes in vivo (Littlejohn et al., 2011; Mangano & Hayley, 2009). Presently, we sought to characterize the in vitro morphological changes in BV2 microglial cells when stimulated with LPS and exposed to glutamate pre-stressed neurons in our two-hit model. We characterized five distinct in vitro stages of BV2 cellular activation (Figures 5 and 6): (1) spherical shaped microglia with no detectable ROS (median ctf < 300), termed passive (P), (2) irregular shaped microglia with no detectable ROS (median ctf < 300), termed transition (T), (3) microglia extending extensive filopodia with detectable ROS (300 < media ctf < 1000), termed sampling (S), (4) microglia showing extensive membrane blebbing without vesicular budding and high ROS (ctf > 1000), termed reactive (R), and finally, (5) spherical microglia exhibiting high levels of ROS (ctf > 1000), termed amoeboid (A) (Figure 5a). We termed the P and T phenotypes as microglial ready states (presumably reflecting some early surveillance states) and S, R, and A ones as microglial response states (presumably reflecting some activation states that are actively engaged in defensive strategies). Through our live-cell microscopic analysis, we observed a clear orderly progression through these morphological states, such that once a BV2 cell reaches the S phenotype, it will invariably progress through to the R and A stages as it increases ROS production (Figure 3a; Video S3). Primary microglia paralleled the BV2 cell changes, in that they progressed through the exact same morphological stages as they increased ROS production (Figure 5b).

Next, we sought to determine if inhibiting LRRK2 or WAVE2 would alter the proportion of Ready (P and T) and Response (S, R, and A) microglial phenotypes over time. In this regard, the LPS-stimulated BV2 microglial cells were in a predominantly Ready state for the first 110 min of coculture. However, by 130 min, two-hit activation of BV2 cells results in an approximate 1:1 ratio of Ready to Response phenotypes and this ratio was stable throughout the 6 h of coculture (Figure 5c, LPS). Further, inhibition of LRRK2 kinase and WAVE2 signaling did shift this ratio in favor of Ready phenotypes (Figure 5c, ML2, and CK-869), suggesting the importance of LRRK2-WAVE2 in sculpting the microglial progression through responsive phenotypes.

3.2.4 | Higher ROS levels are observed in active BV2 morphological states

We characterized the ROS levels in BV2 cells of each morphological phenotype to confirm whether the oxidative burst is associated with the distinct morphological activation states. The BV2 cells of all morphological states and varying ROS levels were found in all conditions (Figure 6a). As predicted, we found that ROS levels in Response microglial phenotypes were elevated, when compared to those in Ready microglial phenotypes for all experimental conditions (Figure 6c). The highest levels of ROS were detected in the Reactive and Amoeboid states (median ROS levels > 1000 ctf). Finally, WAVE2 inhibition (via CK-869) reduced the ROS levels in the Response cell states (Figure 6d–f), supporting a connection between the WAVE2 mediated morphological changes and the production of soluble oxidative stress factors.

3.2.5 | BV2 aggregate formation is reduced when LRRK2 or WAVE2 are inhibited

Neurodegenerative diseases, notably PD, are typically characterized by abnormal protein clumping (e.g., α-synuclein-rich Lewy bodies), which is also associated with the aggregation of various inflammatory cells, including microglia (Huang et al., 2009; S. Kim et al., 2009). Although these microglia could potentially be beneficial in clearing such plaques, they can also damage adjacent dopamine neurons through secretion of neurotoxins or phagocytosis of distressed, but still live, neurons (phagoptosis). We were interested in understanding the potential role of large BV2 cellular aggregates in these two mechanisms of neurotoxicity. Therefore, we focused on the ability of LPS stimulated BV2 cells to form aggregates with other BV2 cells and then, the likelihood of these aggregates to associate with SH-SYSY...
Within the first hour of coculture, LPS-stimulated BV2 cells associated with each other to form cellular aggregates ranging from 2 to 16 cells per aggregate (Figure 7). The majority of BV2 cells are associated with 1–3 other cells in a dynamic manner, wherein the cells will often leave the aggregate to move to other areas within the culture environment. Very large aggregates of six or more BV2 cells tended to maintain their size throughout the 6 h of coculture, although the morphological states of the cells in the aggregates changed over time (Figure 7c). Inhibition of LRRK2 or WAVE2 significantly decreased the stability of the very large BV2 aggregates (MLI2_low p = 6.83*10^{-7}, MLI2_high p = 0.00013, CK-869 p = 2.97*10^{-6}), whereby most cells in these conditions were reduced to aggregate sizes of 2–4 cells (Figure 7).

We further assessed the likelihood of BV2 aggregates to make cell–cell contact with SH-SY5Y cells. In this regard, we found that the large aggregates of LPS-stimulated BV2 cells were most often found in close proximity to SH-SY5Y cells, but without actually making cell–cell contact (Figure 7b). Again, inhibition of LRRK2 or WAVE2, using MLI2 or CK-869, prevented the formation of these large, cellular aggregates. Although the BV2 morphology varied within any particular aggregate, Response phenotypes predominated in the large LPS-stimulated aggregates (Figure 7c), however, the presence of Response phenotypes in aggregates was less prominent when the LRRK2 or WAVE2 inhibitors were applied (Figure 7c). These data are consistent with BV2 microglia adopting aggregate clusters when reaching their more active Response phenotypes and that LRRK2-WAVE2 again may modulate these transitional states.

### 3.3 Potential cytokine targets in LRRK2-mediated neurotoxicity

Our data suggest that the neurotoxic effects of LPS-stimulated BV2 microglial cells on glutamate pre-stressed SH-SY5Y cells may be mediated by LRRK2, potentially through the downstream activation of WAVE2. To identify potential cytokine targets, we lastly assessed the expression of a panel of 96 cytokines and chemokines at 175 min in the two-hit live-cell model. We then
FIGURE 5 Microglial transition through ready and response activation states. BV2 (a) and primary (b) microglial cells progress through five distinct morphological states further grouped into Ready and Response states. Ready states include (P) Passive and (T) Transition states while Response states include (S) Scanning, (R) Reactive, and (A) Amoeboid states. When LPS-activated BV2 cells are cocultured with pre-stressed SH-SY5Y cells, 50% of BV2 cells in culture are in a Responsive state by 130 min (c, LPS). When LRRK2 is inhibited, the progression to 50% Responsive phenotype is delayed (c, MLi2). This reduction in transition to Responsive phenotypes is greatest when WAVE2 is inhibited (c, CK-869). Data are mean percent ± standard deviation. Scale bar = 20 μm
compared this expression profile to that seen when LRRK2 kinases activity was inhibited by MLi2 in both cell lysates and supernatants (allowing for discrimination of cell-associated and secreted cytokines). Hence, we collected lysate and supernatant from BV2-SH-SY5Y co-cultures that received both of the LPS and glutamate hits either alone or in the presence of MLi2. Of the 96 proteins initially assessed, 34 remained in our analysis based on our inclusion criteria (see Section 2; Figure S2). In the cellular lysates, it was found that LRRK2 kinase inhibition resulted in the downregulation of 11 cytokines (Figure S2). In cellular supernatants, MLi2 mediated LRRK2 kinase inhibition resulted in variations of 16 cytokine proteins (Figure S2). Of these proteins, changes in MIP-2 (CXCL-2), RANTES (CCL5), CRG-2 (CXCL10, IP-10), IGFBP-3, IGFBP-5, LIX (CXCL5), and MCP-1 (CCL2) were the most robust that were observed in this two-hit live-cell model (Figure S5). In particular, MLi2 reduced cell-associated lysate levels of RANTES (CCL5), CRG-2 (CXCL10, IP-10), IGFBP-3, and MIP-2, while supernatant levels of the soluble cytokines MCP-1 (CCL2), MIP-2 (CXCL-2), IGFBP-3, and LIX (CXCL5), were reduced (Figure S5). Curiously, IGFBP-5 supernatant levels were actually increased by the MLi2 treatment. It is particularly noteworthy that, besides the two insulin-like growth factors (IGFs; IGFBP-3 and -5), these proteins are all actually chemokines, from the subset of cytokines particularly involved in the regulation of chemotaxis. This suggests that, in our model, the LRRK2 kinase activity might have been more aligned with cellular migration and aggregation, rather than pro-inflammatory or oxidative functions.

4 | DISCUSSION

LRRK2 mutations associated with PD are known to drive microglial activation (Cookson, 2015; Gillardon et al., 2012; K. S. Kim et al., 2018; Subramaniam & Federoff, 2017), leading to the production of inflammatory factors, ROS, and increased microglial phagocytic capacity (De Virgilio et al., 2016; González et al., 2015; Subramaniam & Federoff, 2017). Yet, microglial activation alone rarely leads to substantial dopaminergic toxicity nor reproduces motor symptoms (Littiejohn et al., 2011; Mangan & Hayley, 2009; Rudyk et al., 2019). This is consistent with the multi-hit hypothesis, wherein multiple insults are required for pathology associated with LRRK2 mutations of variable penetrance and age-dependent expressivity (Martin et al., 2016). The present study used a novel two-hit in vitro model, as depicted in Figure 8, whereby we report (1) the requirement for a second pathological factor in microglia-induced neurotoxicity, (2)
requirement of microglial migration to distressed neurons, (3) distinct morphological changes in microglia as they transition from physiological to pathological phenotypes, (4) steady ROS accumulation as microglia progress to more active states, (4) the tendency of activated microglia to form stable, cytokine secreting, aggregates, and (5) the role of LRRK2 and WAVE2 as downstream mediators of microglia-induced neurotoxicity.

4.1 Microglial-induced neurotoxicity requires close cell–cell proximity to mildly distressed neurons and LRRK2 signaling

Distressed neurons have elevated levels of Ca²⁺ and ROS (Brown & Neher, 2014; Neher et al., 2011, 2013), making them more susceptible to microglial-derived factors. Specifically, microglia-derived
Proposed cellular and molecular mechanisms of microglia-mediated neurotoxicity. Ready state microglia (Passive and Transition) transition to Response state (Scanning, Reactive, and Amoeboid) microglia following an external stimulus. Neurons exposed to sub-toxic insults recruit Response state microglia as they form large aggregates of microglial cells proximal to pre-stressed neurons. Microglial aggregates secrete glutamate, ROS, cytokines, and chemokines which further stimulate microglia and induce oxidative stress in neurons (a). The mechanisms regulating the phenomena in this two-hit model of PD are still under investigation, but it is clear that LRRK2 and WAVE2 play key roles in ROS and cytokine/chemokine production as well as cytoskeletal rearrangement critical for microglia to achieve their full inflammatory and pathological function. We propose a novel molecular pathway emphasizing the role of neuronal-microglia cross-talk in LRRK2 and WAVE2 mediated microglia-induced neurotoxicity (b). In this model, neurons are exposed to an external insult leading to mildly elevated calcium and ROS levels. Concurrently, microglia are activated by an external stimulus, such as LPS. The LPS receptor TLR4 induces phosphorylation of LRRK2 (pLRRK2). pLRRK2 induces (1) phosphorylation of WAVE2 and its subsequent induction of cytoskeletal rearrangement, (2) induction of an oxidative burst, and (3) production of cytokines, chemokines, and glutamate. Pre-stressed neurons are in a state of reversible, mild distress, but due to the already elevated calcium and ROS, these cells are more susceptible to the microglial secretions. Hence, pre-stressed neurons will quickly induce extracellular phosphatidylserine (PS) expression and potentially apoptosis in response to activated microglia. Microglia respond to PS flip in neurons by secreting the opsonizing protein MFG-E8 (milk fat globule E8 protein). MFG-E8 binds to integrin receptors on microglia further activating WAVE2-mediated cytoskeletal rearrangement, and potentially phagoptosis.
glutamate may further depolarize neurons, while microglia-derived pro-oxidants may be directly taken up by neurons and initiate damage. These events could lead to further elevation of intracellular Ca$^{2+}$ and production of superoxide and peroxy nitrite leading to irreversible extracellular PS expression, apoptosis, and phagocytosis (Brown & Neher, 2014; Fricker et al., 2018; Neher et al., 2011, 2013; Figure 8b). While LPS-stimulated microglia secrete numerous soluble factors that can impact neuronal survival (Barger et al., 2007; Bozic et al., 2015; Riester et al., 2019), we found that supernatants from LPS-stimulated BV2 microglial cells were not sufficient to induce toxicity to SH-SY5Y neuronal cells. That said, it may be possible to see some degree of neurotoxicity with secreted factors if culture time was further extended to 48 h, as seen with atrazine-induced microglia activation (Ma et al., 2015). Nevertheless, we posit that microglia secrete sufficient concentrations of cytokines and ROS to induce neurotoxicity only when in close proximity to distressed, but not healthy, neurons. Indeed, neuronal toxicity was only achieved when LPS stimulation of microglia was coupled with low-dose glutamate-induced neuronal pre-stress. Furthermore, activated BV2 microglia were only toxic to neurons when plated on transwell inserts with large enough pores (8 μm) to allow cell migration, as opposed to transwells with 0.4 μm pores that were too small for cell migration. Hence, direct migration of microglia to SH-SY5Y neuronal cells was necessary to induce pathology. The present study not only confirms the ability of distressed neurons to recruit activated microglia, but also the requirement for close microglial-neuronal proximity, which might be mediated by chemokines.

We assessed the importance of LRRK2 signaling in microglial-neuronal interactions due to its implicated role in regulating microglial inflammatory states (Choi et al., 2015; K. S. Kim et al., 2018; Lee et al., 2017). Indeed, when activated BV2 cells were treated with MLI2, a specific LRRK2 kinase inhibitor, neuronal injury was prevented. Furthermore, when we analyzed lysates and supernatants from microglial-neuronal mixed cultures, we observed an ML2-induced reduction in the pro-inflammatory chemokines in both biological compartments, these included CRG-2 (CXCL10), MIP-2 (CXCL2), RANTES (CCL5), LIX (CXCL5), and MCP-1 (CCL2; Figure S5). This suggests that LRRK2 regulates these chemokines, which in turn, likely mediated microglial chemotaxis and interactions with neurons. The presently used MLI2 dose of 100 nM should be very specific and largely reduce LRRK2 phosphorylation in microglia, as 50 nM of the inhibitor previously significantly suppressed LRRK2 kinase activity (pLRRK2), without change in overall protein levels in macrophages (U. Kim et al., 2019). Moreover, MLI-2 was exceptionally potent and selective for LRRK2 (compared to a broad range of kinases and ion channel receptors) in kinase and radioligand competition binding assays (Fell et al., 2015).

Importantly, the effects observed in BV2 cells were replicated in primary microglia, with parallel morphological and neurotoxic effects observed in both cell types. This indicates both primary and BV2 microglia may be effectively used to assess neurotoxicity in our two-hit model. As the G2019S LRRK2 mutation is associated with familial and sporadic PD (Gaig et al., 2009; Martin et al., 2016; Pchelina et al., 2006), we predicted that primary microglia from LRRK2 G2019S knock-in mice would show enhanced neurotoxic effects. Curiously, instead, the opposite occurred wherein the G2019S mutation actually diminished the neurotoxic effect. It is possible that this unexpected outcome was due to the requirement of microglial migration in our model. In fact, LRRK2 normally inhibits focal adhesion kinase (FAK), resulting in decreased microglial motility (Choi et al., 2015). The effects of LRRK2 on FAK are mediated through the same domain that is affected by the G2019S mutation (Choi et al., 2015). In effect, the primary microglia overexpressing G2019S may exhibit reduced motility, hence impeding their migration into the neuronal chamber and subsequent neurotoxicity. Other processes, such as protein scaffolding-mediated events, that have been reported for LRRK2 could help explain the unexpected outcome of the G2019S mutation.

4.1.1 Early ROS levels in cocultured BV2 and SH-SY5Y cells is modulated by WAVE2

We sought to address the timing of the oxidative burst in the early stages of microglial activation using a two-hit mixed culture live-cell model. The LPS induced production of ROS in BV2 microglial cells (bROS) was initiated between 130 and 175 min of culture and steadily increased over time. A robust suppression of ROS accumulation in BV2 microglia was apparent when ARP2/3 (which is part of the WAVE2 actin regulatory pathway) was inhibited by CK-869. Specifically, microglia in the highly activated Response phenotypes exhibited reduced ROS levels with inhibition of WAVE2 signaling. This is consistent with the evidence that WAVE2 is (1) upregulated in microglia with G2019S mutations, (2) regulates cytoskeletal realignment, (3) induces lamellipodia formation, and (4) mediates phagocytosis (K. S. Kim et al., 2018; Suetsugu et al., 2006; Takenawa & Suetsugu, 2007). Therefore, we posit that WAVE2 is a novel mediator of bROS through indirect regulation of cytoskeletal changes needed for microglia to achieve a fully amoeboid phenotype.

Concurrent with elevated bROS, neuronal ROS (nROS) was also increased in the BV2 microglia- SH-SY5Y neuron co-cultures. Based on the timing of nROS production, we believe that this is in response to the production of inflammatory cytokines, glutamate, and possibly other factors by activated microglia (Barger et al., 2007; Bozic et al., 2015; Riester et al., 2019). LRRK2 kinase inhibition delayed the nROS accumulation, but not the overall levels achieved in the SH-SY5Y neuronal cells.

It is possible that inhibition of neuronal LRRK2 may negatively affect neurite stability and/or nROS (Feng et al., 2018; Marte et al., 2019; Parisiadou et al., 2014), but since LPS and ML2 treatment of SH-SY5Y neuronal cells had no effect on neuronal cell survival or morphology, this is unlikely in our model (Figure S1). Instead, elevated nROS at later times (360 min) maybe promoted by the clustering of activated microglia around SH-SY5Y neurons. Inhibition of WAVE2 signaling can also affect nROS production but since microglia were exposed to the ARP2/3 inhibitor before coculture, the
effects of WAVE2 inhibition were specifically related to microglia and not due to any direct effect on neurons. This is consistent with the upregulation of microglial, but not neuronal WAVE2 observed in G2019S LRRK2 mice (K. S. Kim et al., 2018). Besides inhibiting nROS production, the CK-B69, ARP2/3 antagonist, also promoted a less active morphological phenotype and diminished microglial aggregation (Figures 5-7), further supporting the importance of the known role of WAVE2 in lamellipodia formation affecting microglial activation state (K. S. Kim et al., 2018; Suetsugu et al., 2006; Takenawa & Suetsugu, 2007).

4.2 | Unique progression of microglia through distinct phenotypic states as they form large cellular aggregates

Microglial activation states have traditionally been classified (albeit not exclusively) into M1 (classical activation) and M2 (alternative activation) phenotypes (Boche et al., 2013; Michelucci et al., 2009). M1 microglia are pro-inflammatory with an amoeboid morphology and inflammatory cytokine profile, reflecting the functional phenotype of classically activated macrophages underlying an innate immune response to pathogens (Boche et al., 2013). The M2 microglia are thought to provide tissue repair, synaptic pruning, and general homeostasis and often release anti-inflammatory factors (Tang & Le, 2016). M1 and M2 were once thought to be distinct fate-determined phenotypes, but it is more likely that these are dynamic phenotypes adopted as microglia respond to their environment.

As the end-state M1/M2 classification is likely an oversimplification of the dynamic nature of microglia, there is a need for a more comprehensive nomenclature of phenotypes. A variety of morphological changes occur as microglia transition between functional states, with the two best-characterized morphologies being ramified and amoeboid and are often associated with a resting or active phenotype, respectively (Fernández-Arjona et al., 2019). A computational approach to image analysis using lacunarity, cell circularity, and convex hull span ratio revealed at least four distinct morphological categories of microglial states in vivo (Fernández-Arjona et al., 2019). These categories are consistent with a progression from a scanning to reactive phenotype that correlated with changes in cytokine production (Fernández-Arjona et al., 2019).

The classification of microglial morphologies in vitro is poorly characterized, with these cells rarely displaying a fully ramified, or classically defined resting phenotype. We sought to characterize the dynamic transition of activation states in our two-hit toxin model using live-cell microscopy. Our morphological and ROS analyses revealed five distinct morphological phenotypes in both BV2 and primary cortical microglia consistent with those described in vivo (Fernández-Arjona et al., 2019). We divide these five phenotypes into two groups of activation states: Ready and Response. Ready state cells, termed (P) passive and (T) transition, are available to respond to cellular cues but are not actively scanning their microenvironment. Response state cells, termed (S) scanning, (R) reactive, and (A) amoeboid, are actively scanning and responding to their microenvironment. We posit that the P and T phenotypes are the in vitro equivalent to the resting, ramified microglia found in vivo. The S phenotype is consistent with the slightly less ramified but highly motile microglia, whereas the R phenotype was characterized by extensive membrane blebbing and high ROS levels. Finally, the highest ROS levels and most extreme inflammatory morphology were evident in microglia that transitioned to the A phenotype, reminiscent of an end state classical M1 phenotype. We found that once a microglial cell reached the S phenotype, they invariably progressed on through to R and eventually A states. This is important as it suggests that the phenotypic progression may be set once some threshold has been reached. What we do not know is whether these cells transition back to less responsive phenotypes at much later times.

Besides their changes in morphology and ROS levels, the formation of large (>6 cells) clusters of Response state microglial aggregates was essential for microglial-mediated neurotoxicity. These aggregates were stable after 6 h and found to be proximal to distressed SH-SY5Y neuronal cells. We posit that LRRK2 upregulated chemokines, such as MIP-2 and RANTES, to mediate microglial migration and aggregation proximal to neurons, such as that reported to be associated with microglial aggregation in an in vitro model of Alzheimer’s disease (Huang et al., 2009).

Major cytoskeletal changes were necessary for microglia to cluster into aggregates and develop extensive, dynamic filopodia and the membrane blebbing necessary for Response phenotypes. We propose that cytoskeletal changes in microglia are mediated by LRRK2 and WAVE2 signaling (working model depicted in Figure 8; Cao et al., 2015; K. S. Kim et al., 2018; Sudhaharan et al., 2016; Suetsugu et al., 2006). In this pathway, (1) LPS, via TLR4 activation, induces LRRK2 phosphorylation (pLRRK2; Schapansky et al., 2014), (2) pLRRK2, in turn, induces phosphorylation of WAVE2 (K. S. Kim et al., 2018), and finally, (3) WAVE2 directly or indirectly induces actin cytoskeletal rearrangement through ARP2/3 and other downstream factors (e.g., Rac1, small GTPases, and Cdc42; Kitamura et al., 2003; Suetsugu et al., 2006; Takenawa & Suetsugu, 2007). In support of this hypothesis, we observed changes in the ratio of Ready to Response microglia when LRRK2 and WAVE2 signaling was inhibited. Indeed, the activation of BV2 microglial cells resulted in a stable 1:1 ratio of Ready to Response phenotypes, whereas inhibition of LRRK2 kinase and WAVE2 signaling shifted this ratio clearly in favor of Ready phenotypes and this occurred in a dose-dependent manner.

5 | CONCLUSIONS

Microglial-induced neurotoxicity requires a second, pathological factor that pushes neurons into a reversibly distressed state. Once in this state, distress signals from neurons likely initiate microglial recruitment, stimulating microglial progression through distinct morphological phenotypes from Ready to Response states via LRRK2 and WAVE2 signaling (Figure 8). During this transition, microglial cells
induce an oxidative burst, followed by inflammatory chemokine/cytokine release. Such inflammatory soluble factors further stimulate microglia to form large cellular aggregates that cross-talk with neurons, ultimately leading to neurotoxicity.

The present in vitro model parallels our previous in vivo findings, wherein re-treatment of mice with LPS sensitized the microglial response and loss of SNc neurons upon subsequent exposure to a toxicant, such as paraquat (Litteljohn et al., 2011; Mangano & Hayley, 2009). We further also delineate the nature of microglia states in vitro and indicate their importance in migrating to the vicinity of neurons in coculture. Of course, these data are not only mechanistically important for better understanding neuron-microglial crosstalk and its role in neurodegenerative conditions, like PD but also our model paves the way for a new framework for describing in vitro microglial phenotypic states.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

ETHICS STATEMENT
All procedures used in this paper have been approved and comply with regulations of the Animal Care Committee of Carleton University and the Canadian Council of Animal Care.

AUTHOR CONTRIBUTIONS
Barbara M. Fenner and Shawn P. Hayley designed and conceptualized the study. Barbara M. Fenner and Natalie Prowse designed and performed experiments. Barbara M. Fenner collected and assembled the data. Mark E. Fenner generated all code for data analysis and visualization. Barbara M. Fenner, Mark E. Fenner, and Shawn P. Hayley validated, analyzed, and interpreted the data. The manuscript was written by Barbara M. Fenner and Shawn P. Hayley and revised by Mark E. Fenner. The authors read and approved the manuscript. All authors read and approved the final manuscript.

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