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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Beneficial effect of urolithin A on poly I:C-induced microglia activation

Yakum Benard Mingo

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Bert BRONE

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Senior internship - 2nd master BMW

Beneficial effect of urolithin A on poly I:C-induced microglia activation*

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*Running title: *UA attenuates poly I:C-induced microglia activation*

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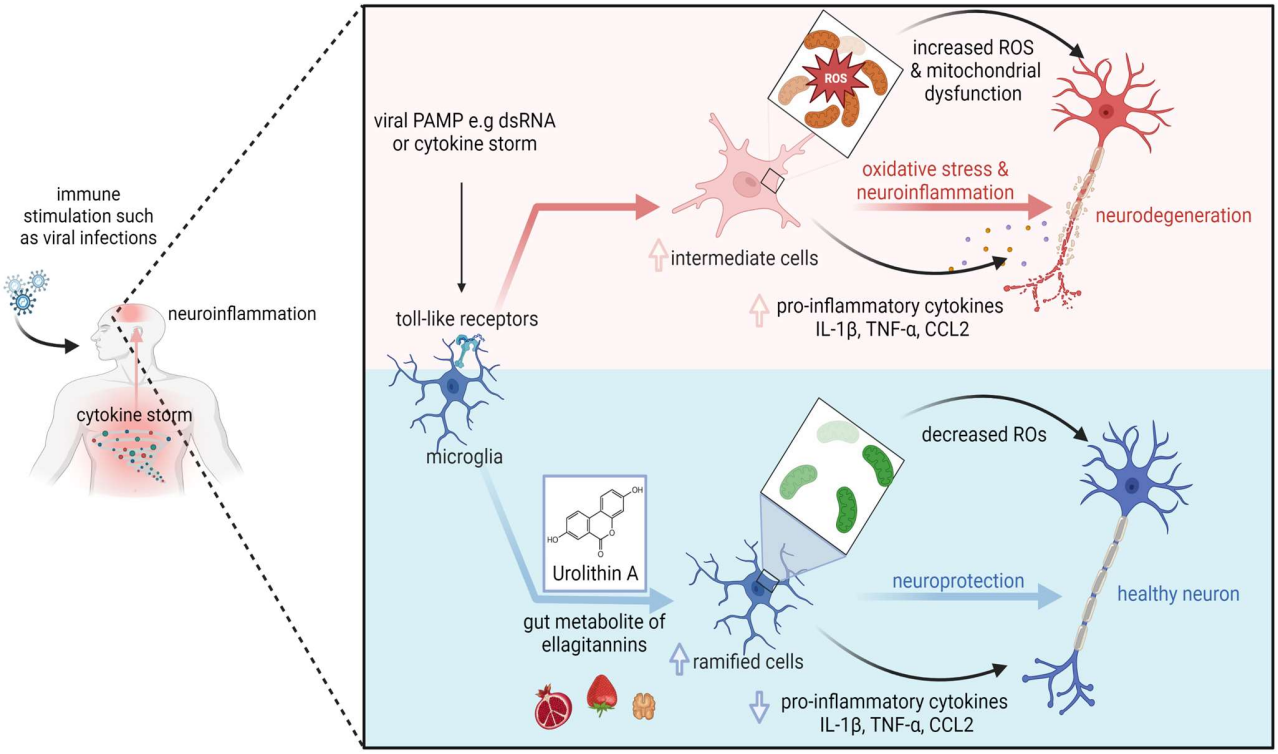
Keywords: Poly I:C, microglia activation, neuroinflammation, urolithin A



Graphical abstract: Immuno-stimulation with viral PAMP, e.g., dsRNA (mimicked by poly I:C in this study), triggers a shift in microglial morphology from the homeostatic ramified state to the pathogenic activated state, which constitutively releases proinflammatory mediators and ROS. Urolithin A attenuates microglial activation and may prevent neuroinflammation, thus protecting neurons.. *PAMP – pathogen-associated molecular patterns, dsRNA – double-stranded RNA, ROS – reactive oxygen species, IL-1β – interleukins one beta, TNF – tumor necrosis factor-alpha, CCL2 – C-C motif chemokine ligand 2*

ABSTRACT

Neuroinflammation can be triggered by various stimuli, including viral infections. Viruses can directly invade the brain and infect neural cells or indirectly trigger a "cytokine storm" in the periphery that ultimately leads to microglial activation. While this initial activation of microglia cells is important for viral clearance, chronic activation leads to excessive inflammation and oxidative stress, which can cause neurodegeneration. Interestingly, recent studies have shown that certain viruses, such as influenza A virus and Epstein-Barr virus, are involved in the development of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Multiple sclerosis. Therefore, it is important to find therapeutic strategies against neuroinflammation triggered by viral infections. Here, the beneficial effect of urolithin A (UA) on microglial activation *in vitro* induced by a viral mimetic, poly I:C, was investigated. Immunocytochemistry was used to perform a comprehensive single-cell analysis of morphological changes in microglia. The findings show that poly I:C administration resulted in increased expression of microglial activation markers, CD68 and Iba-1, decreased microglial ramification, and a shift towards the intermediate phenotype, indicating microglial activation. Remarkably, UA treatment significantly prevented this poly I:C-induced microglial activation and restored the morphological changes. Furthermore, UA was able to reduce the release of proinflammatory mediators CCL2, TNF-α, and IL-1β, and showed a trend toward attenuation of cellular ROS production in poly I:C-treated cultures. Overall, this study suggests that UA may have therapeutic potential against virus-induced neuroinflammation and can be useful for future studies to prevent or treat neurodegenerative diseases by targeting the associated neuroinflammatory processes.

INTRODUCTION

Infectious diseases caused by pathogens such as viruses, bacteria, and parasites pose a significant health risk to the central nervous system (CNS) (1). In between, viral infections may pose the greatest potential threat to the CNS. Evidence is accumulating that both neurotropic and non-neurotropic viruses can cause acute and long-term neurological sequelae (2-7). These consequences include impairments in the structure and functions of neurons and glial cells, neurodegeneration, as well as cognitive decline, and behavioral changes. While the underlying causes of most neurodegenerative diseases remain elusive, recent studies suggest that neurological defects triggered by viral infections may contribute to the development or exacerbation of neurodegenerative diseases. For instance, *Bjornevik et al*. recently found that Epstein-Bar virus (EBV) infection in humans increases the risk of multiple sclerosis (MS) by 32-fold, making EBV one of the most important triggers for the development of MS (8). In addition, *Hosseini et al.* have previously shown that the non-neurotropic H3N2 influenza A virus exacerbates symptoms of Alzheimer's disease (AD) in the APP/PS1 mouse model (4). These studies highlight the important role that viruses play in the pathophysiology of neurodegenerative diseases. Moreover, these diseases affect more than 50 million people worldwide, and there are currently no effective cures (9). Therefore, there is a need for novel therapeutic approaches that target the underlying pathological mechanisms in the development of neurodegenerative diseases.

Microglia, the resident innate immune cells of the brain, make up about 10% of all cells in the brain (10). They perform multiple functions in neurodevelopment, synaptic pruning, CNS homeostasis, immune sensing, and protection against invading pathogens. Because of their vital roles in the CNS, microglial dysfunction has been shown to be an important factor in neurodegeneration (11). In the case of neurotropic viral infections, microglia recognize invading viral pathogen-associated molecular patterns (PAMPs) via PAMP recognition receptors (PRRs), such as Toll-like receptor 3 (TLR3) for viral double-stranded ribonucleic acid (dsRNA) (2, 12-14). In response, the ramified microglia retract their processes, proliferate, and enlarge into activated and amoeboid phenotypes, to mediate innate and adaptive immune responses (1, 2, 15, 16). In addition, microglial cells in the activated state may exhibit an intermediate morphology with partially retracted processes and produce pro- or anti-inflammatory cytokines depending on the microenvironment, whereas the amoeboid state

with fully retracted processes mainly engages in phagocytosis. Moreover, microglia cells can also adopt a state of activation during non-neurotropic viral infections. Indeed, excessive systemic inflammation induced by non-neurotropic viruses can cause disruption of the blood-brain barrier and trigger microglial priming via a "cytokine storm" without direct virus entry into the CNS (6, 17, 18).

Activated microglial cells can perform two opposing functions. Initial activation is essential for mediating inflammatory and cytotoxic processes required for the clearance of viral particles and virus-infected cells (6, 7). However, hyperactivation of microglia, such as in chronic infections, can inadvertently cause reactive microgliosis and lead to neurotoxicity. This neurotoxic effect is mediated through the excessive release of proinflammatory cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, and microglia/monocyte chemoattractant protein (CCL2) (2). CCL2, among other attractant proteins, recruits monocytes and other immune cells to aid in viral clearance and recovery, but this may exacerbate the existing inflammation.

Furthermore, viral infections have also been shown to trigger mitochondrial dysfunction in affected cells due to impaired mitophagy (19-21). Mitophagy is a selective form of autophagy in which damaged mitochondria are degraded and recycled for mitochondrial biogenesis. However, when disrupted, damaged mitochondria accumulate in cells, causing oxidative stress through increased release of reactive oxygen species (ROS) (9, 19). Long-term exposure of neurons to proinflammatory cytokines and excessive ROS leads to neuronal damage that can trigger or exacerbates neurodegeneration (1, 2, 22, 23). Therefore, modulation of these viral mechanisms in microglia may be a useful strategy to prevent viral-triggered neuroinflammation and subsequent neuronal damage.

In the current study, UA was used as a potential neuroprotective agent to prevent microglial activation associated with viral infections. Viral infection was mimicked by the administration of polyinosine:polycytidylic acid, a synthetic TLR3-agonist that mimics dsRNA viral nucleic acids. Urolithins (A-D) are metabolites of intestinal bacteria from foods rich in ellagic acids and ellagitannins, such as pomegranate, berries, and nuts (24). Recent studies have reported the beneficial properties of Urolithin A (UA) on aging and various diseases by attenuating inflammation and improving mitochondrial function. Notably, UA prolongs the lifespan of *Caenorhabditis elegans* and has recently been shown to improve cognitive impairment and prevent neuronal apoptosis in an APP/PSI mouse model of AD (25, 26). Furthermore, UA was recently shown to limit lipopolysaccharide (LPS)-induced microglial activation and reduce motor deficits in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease (27). However, the potential beneficial role of UA in the pathology of viral infections is still unknown. Here, we hypothesized that UA rescues poly I:C-induced microglial activation and attenuates excessive inflammation and oxidative stress in an *in vitro* model consisting of a co-culture of neurons, astrocytes, and microglia.

To investigate this hypothesis, a comprehensive single-cell analysis of microglial cells was first performed using structured illumination microscopy (SIM) with ApoTome. The results showed that UA was able to prevent poly I:C-induced microglial activation and proliferation and limit the characteristic morphological shift of microglia from ramified to activated intermediate phenotype. In addition, UA suppressed the production of proinflammatory molecules (CCL2, TNF-α, and IL-1β) and showed a tendency to induce mitochondrial biogenesis by attenuating cellular ROS production in poly I:C-stimulated cultures. Overall, this study provides further insight into the beneficial potential of urolithin A against neuroinflammatory processes induced by viral infections and can be useful for future studies on therapeutic strategies against various neurological complications associated with viral infections.

EXPERIMENTAL PROCEDURES

Animals – The animals used in this study were wild-type C57BL/6J mice and genetically modified CX3CR1$^{eGFP/+}$ mice expressing the enhanced green fluorescent protein (eGFP) in the CX3CR1 promoter, specific in microglial cells in the brain. Murine brains were collected for the preparation of primary neural cultures. All experiments were performed *in vitro,* and animal ethical clearance was not required. For culture preparations, all

animals were handled carefully according to the guidelines of the Ethics Committee of TU Braunschweig and the authorities (LAVES, Oldenburg, Germany) according to the national guidelines of the animal welfare law in Germany.

Preparation of primary embryonic hippocampal culture – C57BL/6J mice at embryonic day 17.5 (E17.5) were decapitated, and the hippocampi were carefully separated from the cortices and dissociated in 1X trypsin/EDTA solution (Sigma, T3924) for 25 min at 37°C. 7×10^4 cells per well were plated in a 24-well plate and cultured for 21 days in Neurobasal-A medium (Invitrogen, 10888-022), supplemented with 10X N2 (self-made), B27 (Invitrogen, 17504-001), and L-glutamate (Invitrogen, 25030-024), to obtain a dissociated culture of primary neurons and astrocytes.

Preparation of co-culture of microglia, neurons, and astrocytes – The co-culture model was adapted from *Goshi et al.* (28). Briefly, primary neural cultures were first prepared, and microglia were isolated at DIV 14 and plated onto the primary embryonic hippocampal culture containing neurons and astrocytes at DIV 21. For this purpose, the cerebral cortices of C57BL/6J or CX3CR1$^{eGFP/+}$ mice were harvested after the first to fourth postnatal day in cold 1X HBSS medium (Gibco, 14185-045) and centrifuged at 2000 rcf for 5 min at 4°C. The purified neural cells were cultured for 14 days in DMEM medium (Gibco, 6195-026) containing 10% fetal calf serum (FCS) (Capricorn-scientific, FCS-62A) and 1% penicillin/streptomycin (Gibco, 15070-063), keeping only glial cells. Microglia were then isolated by shaking in a shaker incubator at 200 rpm at 37°C for 3 h. 3×10^4 microglia were plated onto the embryonic hippocampal culture per well and allowed to adhere overnight. The co-cultures were treated 48 h after microglia plating.

Treatment of co-cultures – A synthetic TLR3-agonist, polyinosinic:polycytidylic acid (poly I:C) was used to mimic TLR3 stimulation of cells during dsRNA viral infections. 14 DIV microglia were added to co-cultures, and 48 h later (21 DIV) treated with 50 µg/ml poly I:C (Merck KGaA, 42424-50-0) and different concentrations of UA (10 µM and 30 µM) (MedChem Express, HY-100599). All reagents were prepared in Neurobasal-A medium (Invitrogen, 10888-022), supplemented with 10× N2 (self-made), B27 (Invitrogen, 17504-001), and L-glutamate (Invitrogen, 25030-024). The medium was added to the control groups. At 24h after treatment, cell supernatants were collected for cytokine analysis, and the coverslips were processed for immunocytochemistry.

Immunocytochemistry – The cells were fixed in cold 4% paraformaldehyde (PFA) for 15 to 20 min at room temperature and washed 3 times with 1X PBS for 5 min each. Afterward, the cells were permeabilized with 0.2% Triton X-100 (AppliChem, A4975,0100) for 30 min on a shaker and blocked in 1% bovine serum albumin (BSA) for 5 min to prevent non-specific binding. The cells were then stained with primary antibodies and incubated in a humidity box for 1 h at 28°C. The primary antibodies used were mouse anti-Iba-1 microglia marker (1:1000, SySy, 234011), rat anti-CD68 microglial activation marker (1:1000, BioRad, MCA1957), rabbit anti-Ki67 proliferation marker (1:500, Thermofisher, MA5-14520), and rabbit anti-COX4 inner-mitochondrial marker (1:500, SySy, 298002). The plates were washed 3 times and blocked again with 1% BSA for 5 min. Next, the plates were incubated in a humidity box for 30 min at 28°C with Cy2-AffiniPure Goat anti-Mouse IgG (H + L) (115-225-146), Cy5-AffiniPure Goat anti-Rat IgG (H + L) (112-175-167), and Cy3-AffiniPure Goat anti-Rabbit IgG (H + L) (111-165-144) secondary antibodies (1:500, Jackson ImmunoResearch Laboratories). Finally, nuclear DNA was counter-stained with 1% DAPI for 5 min at room temperature and washed with one time with milliQ for 5 min. The coverslips were then mounted and imaged.

Fluorescence microscopy – Immunostained images of single-cell microglia were acquired by SIM technique using ApoTome microscope (Axiocam 705 Imager.M2, Zeiss; Jena, Germany). Multiple optical z-sections of each cell were acquired at 0.25µm intervals using the 63X oil immersion objective and deconvoluted with the ApoTome image function. Fluorescence intensities were analyzed using ImageJ (Fiji, version 2.9.0).

Morphological analysis – Changes in microglia morphology were analyzed as previously described (29). The images were first pre-processed by conversion of the green channel (Iba-1 or eGFP) to 8-bit grayscale images and then binarized to obtain black and white images by applying a selected auto-threshold (huang dark) in ImageJ. Next, the images were manually processed to

remove pixels belonging to neighboring cells or artifacts in the background and add some pixels to join processes belonging to the selected cell. This step was performed from the view of the original image. The branching data were obtained using MorphData macro, which utilizes the Skeletonize plugin (30). The different morphological parameters (cell circularity, cell area, convex hull area, fractal dimension, lacunarity, density, and roughness) were analyzed using the FracLac plugin (31). Next, the data from individual cells were combined into a single summary file using Anaconda (Spyder IDE software version 5.2.2) (30). Finally, a three-component principal component analysis (PCA) was performed using Anaconda. The optimal number of clusters was selected by the elbow method, followed by K-Means clustering (sklearn version 1.0.2), to cluster the cells by the degree of dissimilarity of morphological parameters (fractal dimension, lacunarity, density, span ratio major_minor, convex hull area, convex hull perimeter, convex hull circularity, diameter bounding circle, mean radius, max span across convex hull, max_min radii, cell area, cell perimeter, roughness, cell circularity) through dimensionality reduction.

Enzyme-linked immunosorbent assay – Cell supernatants were collected 24 h after treatment with poly I:C and UA and stored at – 20ºC until use. Mouse TNF-alpha DuoSet (DY410), Mouse IL-1beta/IL-1F2 DuoSet (DY401), Mouse IL-10 DuoSet (DY417), and Mouse CCL2/JE/MCP-1 DuoSet (DY479) ELISA kits (R&D SYSTEMS Duoset Ancillary reagent kit 2) were used to determine the levels of cytokines (TNF-α, IL-1β, IL-10), and chemokine (CCL2) in the cell supernatants respectively. Briefly, 100µl of cytokine-specific capture antibodies were coated in medium-binding 96-well microplates and incubated overnight at room temperature. The plates were washed three times with 0.05% v/v Tween 20 in PBS wash buffer (R&D systems, Cat. WA126) and blocked in 300µl 1% BSA in PBS reagent diluent (R&D systems, Cat. DY995) for 1 h at room temperature. After washing again, 100µl of samples and standards were added and incubated for 2 h at room temperature. Thereafter, plates were washed three times and incubated with 100µl of detection antibodies for 2 h at room temperature. Plates were rinsed, and 100µl of streptavidin-HRP was added per well and incubated for 20 min at room temperature in the dark. After rinsing again, the substrate solution (1:1 A[H_2O_2] + B [tetramethylbenzidine], R&D systems, Cat. DY999) was added and incubated for 20 min, followed by 50µl of the stop solution (R&D systems, Cat. DY994). Plates were read at 450 nm on an Epoch microplate reader using Gen5 software (BioTek, USA). Lastly, the optical densities of the samples were compared to the known standards.

ROS detection assay – The production of reactive oxygen species, including peroxides, superoxides, hydroxyl radicals, and singlet oxygen by treated cells was determined using a cellular ROS kit (cat: ab113851; Abcam, Cambridge UK) according to the manufacturer's instructions. Briefly, 7-9×10^3 microglia 14 DIV were plated with a primary embryonic hippocampal culture containing 2×10^4 cells per well at 21 DIV in a 96-well plate. 48 h after microglia plating, the cells were treated with poly I:C and UA for 24 h as previously described, but now in 1X HBSS medium without phenol red (gives a high autofluorescence for the ROS assay). Next, the cells were treated with 20uM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) for 30 min at 37°C and fluorescence was immediately measured in an Epoch microplate reader at 485 nm. H2DCFDA is deacetylated by cellular estarases to 2',7'-dichlorodihydrofluorescein (H2DCF) and then oxidised by cellular ROS to 2',7'-di chlorofluorescein (DCF). Treatment with tert-butyl hydroperoxide (TBHP) for 4 h served as a positive control for ROS production.

Statistical analysis – Data analysis was performed using Graphpad Prism 9 (Graphpad Software, Inc, United States). Data were presented as mean ± SEM. Differences between experimental groups were determined by one-way ANOVA for analyses with a single variable or by two-way ANOVA for analyses with two variables, followed by Fisher's LSD post hoc test for multiple comparisons. Differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

Urolithin A attenuates microglial activation triggered by poly I:C stimulation – Under pathological conditions, microglial cells proliferate and adopt activated and phagocytic phenotypes to respond to the pathogenic insult (1, 2, 15, 16). To evaluate the microglial activation induced by poly

I:C and the possible beneficial effects of UA, cell cultures were treated with poly I:C and UA simultaneously or alone. For this purpose, immunostaining was performed for Iba-1 and CD68, which are known to be upregulated in activated microglia (32). The results showed that immune stimulation induced by poly I:C increased the expression levels of the microglial activation proteins, Iba-1 ($p < 0.01$) and CD68 ($p < 0.05$) (Fig. 1A-C). Both markers in microglial cells were significantly reduced ($p < 0.05$ and $p < 0.01$, respectively) when cultures were treated simultaneously with poly I:C and 30µM UA. However, lower concentrations of UA did not significantly reduce the increased Iba-1 and CD68 levels in microglial cells triggered by poly I:C.

It has been shown that microglial cells, once exposed to danger signals such as pathogens, neurodegenerative deposits, proinflammatory cytokines, injury, and cellular stress, proliferate (33). To further investigate the possible beneficial role of UA in preventing microglial proliferation

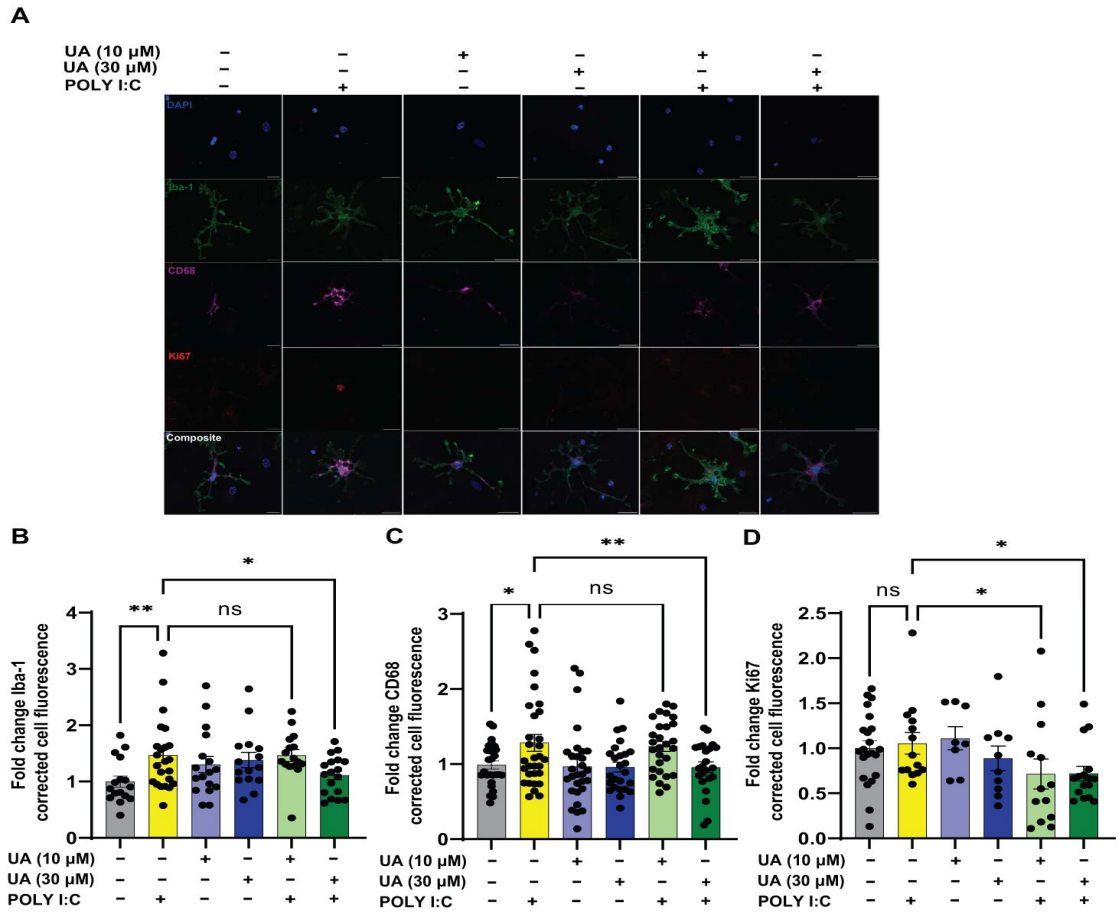


Fig. 1 Urolithin A attenuates activation and proliferation of poly I:C-stimulated microglia in co-cultures containing microglia, neurons, and astrocytes.
Cell cultures were treated with 50µg/ml poly I:C and urolithin A (10µM & 30µM) for 24 hours followed by immunocytochemistry for Iba-1, CD68 and Ki67. Single cell images of microglia were acquired using ApoTome microscope (63X objective) (A) Representative immunofluorescence images showing microglial activation markers Iba-1 (green) and CD68 (magenta), and proliferation marker Ki67 (red). Scale bar: 20µm. (B-C) Relative change in corrected total cell fluorescence intensity of Iba-1 and CD68 indicating the degree of microglia activation in the different experimental groups. Number of experiments, N = 3, sample size, n = 18 - 31 cells per group (D) Relative change in fluorescence intensity of Ki67 indicating cell proliferation in different experimental groups. N = 2, n = 10 - 14 cells per group. Data are presented as mean ± SEM. one-way ANOVA. *$p < 0.05$, **$p < 0.01$.

induced by poly I:C, treated cultures were stained for the nuclear proliferation marker Ki67. The results showed that poly I:C led to a slight increase in microglial cell proliferation, as measured by the fluorescence intensity of Ki67, but was not statistically significant. Interestingly, simultaneous treatment with poly I:C and different concentrations of UA resulted in a significant decrease in microglial proliferation compared to treatment with poly I:C alone ($p < 0.05$, Fig. 1D).

Urolithin A rescues poly I:C-induced morphological changes in microglial cells – In a pathological state, microglia undergo a characteristic morphological change from a branched ramified state to an intermediate activated phenotype and eventually to an amoeboid-like phenotype (1, 2, 15, 16). To determine the potential role of UA in preventing this activation-induced change in microglial morphology induced by poly I:C, individual microglial cells were outlined by MorphData using the fluorescence of Iba-1 or eGFP for wild-type C57BL/6J and CX3CR1$^{eGFP/+}$ cells, respectively, in different experimental groups, and their morphological features were carefully analyzed as binary images (Fig. 2A). The results showed that simultaneous treatment of cultures with poly I:C and UA resulted in a significant concentration-dependent effect ($p < 0.05$ and $p < 0.01$ for 10µM and 30 µM UA, respectively) that suppressed the poly I:C-induced increase in the circularity of cells ($p < 0.01$), the characteristic shape of activated and amoeboid cells (Fig. 2B). Moreover, 30 µM UA was able to reduce the poly I:C-induced increase in microglial cell area ($p < 0.05$, Fig. 2C). Furthermore, UA showed a trend to increase the convex hull area, which was significantly reduced in the poly I:C-administered group (Fig. 2D). Other morphological parameters of microglial cells, such as roughness, fractal dimension, lacunarity, and cell density, showed no significant changes induced by treatment with poly I:C and further UA (Fig. 2E-H).

In the next step, Sholl analysis was performed to determine the branching complexity of microglial cells in the different experimental groups. Strikingly, the results showed that administration of poly I:C resulted in a significant decrease in the branching complexity of microglial cells, which may reveal that microglial cells adopt a more intermediate or amoeboid phenotype after stimulation with poly I:C, confirming their reactivity. However, simultaneous treatment of cultures with poly I:C and UA prevented the poly I:C-induced decrease in microglial cell branching complexity, which was more pronounced at higher UA concentrations (Fig. 2I, $p < 0.05$ for 10µM, $p < 0.001$ for 30µM UA). In addition, the total number of branched junctions of microglial cells was significantly decreased after poly I:C administration, which was significantly restored in the presence of a higher concentration of UA (30µM, $p < 0.001$, Fig. 2J).

To further investigate the morphological shift of microglial cells during immune stimulation by poly I:C and the possible positive role of UA, a principal component analysis (PCA) was performed. This analysis was carried out with three principal components to visualize and combine the morphological characteristics of microglial cells in different experimental groups. The results showed that principal component 1 (PC1) had the largest variation in the data with 43.38%, PC2 had the second largest variation with 24.64%, and PC3 had the third largest variation with 10.45% (Fig. 3A). To analyze the different clusters exhibiting different morphological phenotypes, K-Means clustering was performed with the PCA data. The optimal number of clusters was selected using the elbow method. PCA clustering resulted in three different clusters, 0, 1, and 2, which were identified as amoeboid-shaped, intermediate, and ramified cells, respectively (Fig. 3B). Compared with the control group, poly I:C administration resulted in a 11.09% decrease in the ramified cluster of microglial cells and an 11.54% increase in the intermediate cluster (Fig. 3C). However, in the first cluster, which contained the amoeboid-like microglial cells, only minor changes were observed between the control group and the poly I:C-treated cultures. Simultaneous treatment of cultures with poly I:C and UA reversed this poly I:C-induced morphometric shift in microglial cells in a concentration-dependent manner. Remarkably, treatment with 30 µM UA in combination with poly I:C increased the ramified cluster of microglial cells by 25.07%, reduced the intermediate cluster by 10.80%, and reduced the amoeboid-shape cluster of microglial cells by 14.31%. The lower concentration of UA (10 µM) did not significantly shift the microglial morphology. These results suggest that UA can effectively rescue the poly I:C-induced morphological shift of microglial cells

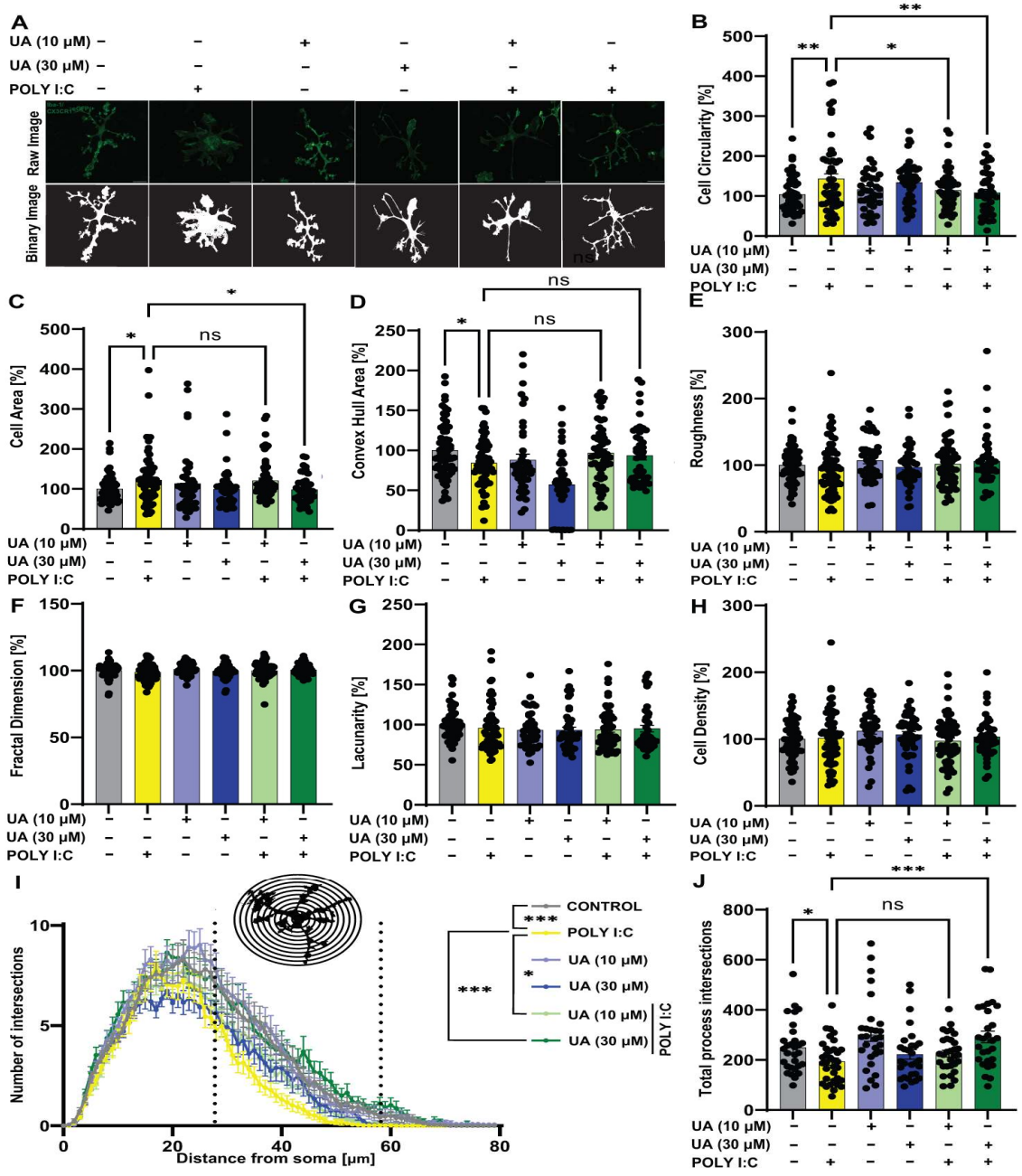


Fig. 2 Urolithin A rescues poly I:C-induced morphological changes in microglial cells
Microglial cells were outlined with MorphData using Iba-1 marker or eGFP, and binary images were generated and analysed with FracLac plugin. (A) Representative immunofluorescence images showing microglia marker Iba-1/CX3CR1$^{eGFP/+}$ (green) and corresponding binary images (black and white) for morphological analysis. Scale: 20µm. (B-H) Morphometric parameters (roughness, circularity, cell area, convex hull area, fractal dimension, lacunarity, density) showing changes in microglial morphology. N = 5, n = 40 - 66 cells per group. Data are presented as mean ± SEM, one-way ANOVA. (I) Sholl analysis showing the complexity of microglial branching in terms of the number of process intersections and distance from cell soma in 1µm increasing radius of concentric circles. N = 3, n = 28 - 34 cells per group. Data are presented as mean ± SEM, two-way ANOVA (treatment and distance). (J) Total number of branching intersections of microglia cells from the cell soma. One-way ANOVA. Data are presented as mean ± SEM, one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001

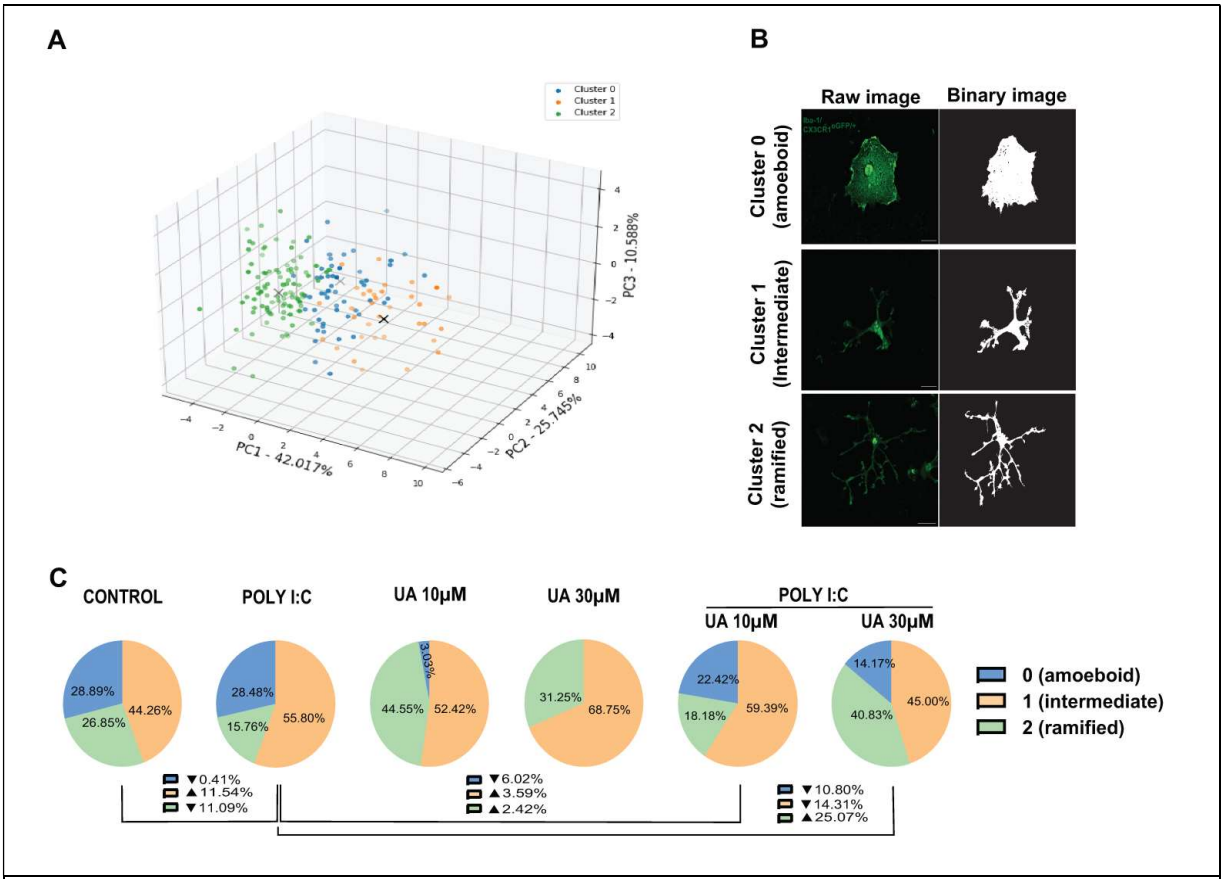


Fig. 3 Urolithin A reverses the poly I:C-induced shift in microglial morphology from ramified to intermediate forms

(A) Principal component analysis by dimensionality reduction and K-Means clustering of microglial cells into three distinct clusters based on the degree of dissimilarity of their morphological parameters. Shown are PC1, PC2, and PC3. (B) Representative immunofluorescence images showing Iba-1-positive or CX3CR1-eGFP-labeled microglia (green) and corresponding binary images (black and white). Scale bar: 20µm. (C) Quantification of the PCA data showing changes in percentages (%) of clusters between experimental groups.

from ramified towards the intermediate phenotype.

Urolithin A reduces levels of secreted proinflammatory mediators induced by poly I:C in culture supernatant – Proinflammatory cytokines and chemokines are molecules secreted by peripheral immune cells, microglia, astrocytes, and neurons in the CNS (34). The production of proinflammatory mediators in the brain is one of the most important mechanisms that develop in many pathological conditions (35, 36). Previously, it has been shown that immune stimulation by viral or bacterial insults can trigger the production of proinflammatory mediators in the CNS or in the cell cultures (7, 33). On the other hand, several studies have shown that UA is able to limit inflammatory signals under various pathological conditions (2, 24). Therefore, the anti-inflammatory properties of UA were analyzed here with respect to a possible reduction of proinflammatory mediators secreted in the culture supernatant after poly I:C administration. As shown in Figs. 4 A-C, 24 hours after poly I:C administration, the levels of proinflammatory chemokine CCL2 ($p < 0.001$) and cytokines; TNF-α ($p < 0.001$), and

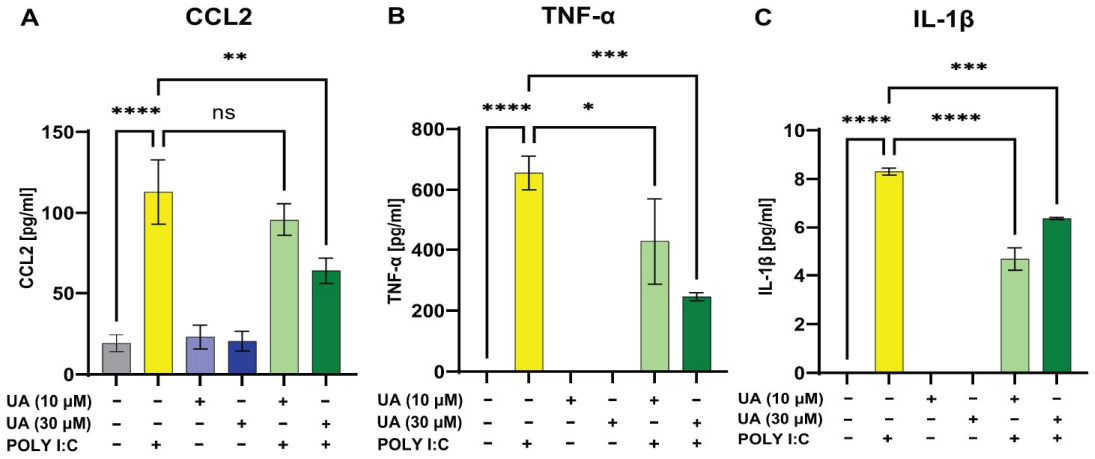


Fig. 4 Urolithin A reduces the release of proinflammatory mediators induced by poly I:C in culture supernatant.
The cell cultures were treated with poly I:C (50µg/ml) and UA (10µM & 30µM) for 24 hours and proinflammatory mediators were analyzed in supernatant by ELISA. Protein levels of proinflammatory (A) chemokine; CCL2 and (B-C) cytokines; tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) in the cell supernatants are shown. N = 4. One-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

IL -1β ($p < 0.001$) were increased in the culture's supernatant. However, simultaneous treatment with UA and poly I:C reduced the levels of these proinflammatory mediators; CCL2 (Fig. 4A, ns for 10µM, $p < 0.01$ for 30µM UA), TNF-α (Fig. 4B, $p < 0.05$ for 10µM, $p < 0.001$ for 30µM UA), and IL-1β (Fig. 4C, $p < 0.0001$ for 10µM, $p < 0.001$ for 30µM UA). The beneficial anti-inflammatory properties of UA were more pronounced after treatment with the higher concentration of UA (30µM).

To test the possible role of UA treatment in the production of anti-inflammatory mediators, the levels of IL-10 were also measured but were below the detection level in all experimental groups.

Urolithin A may induce mitochondrial biogenesis and attenuate poly I:C-induced ROS production – Previously, viral infection was shown to trigger microglial cell activation associated with mitochondrial dysfunction and excessive production of reactive ROS (9, 19). In addition, preliminary published data suggest that UA can improve mitochondrial function under various pathological conditions by stimulating mitophagy, a process in which damaged mitochondria are recycled to allow renewal with healthy mitochondria (25). Under pathological conditions, UA was also able to decrease the production of ROS in cells, inhibiting the mitochondria-related apoptosis pathway (27).

To investigate the role of UA in preventing mitochondrial dysfunction in microglial cells induced by poly I:C in this scenario, the expression level of the inner mitochondrial protein cytochrome C oxidase type 4 (COX4), the regulator of oxidative phosphorylation, was first quantified in the microglial cells in the different experimental groups. The results showed that there were no significant differences between the different experimental groups, although administration of poly I:C alone or together with UA slightly changed the COX4-positive area (Fig. 5B), fluorescence intensity (Fig. 5C), and the number of particles

(Fig. 5D) in the microglial cells, which was not statistically significant.

To test the potentially positive role of UA in preventing the expression of ROS, indicating a reduction in oxidative stress in the cultures after poly I:C stimulation, the next step was to determine the ROS content in the supernatant of the cultures by a colorimetric assay. The results showed that administration of a higher concentration of UA (30µM) alone resulted in a decrease in ROS levels in the supernatant of the cultures ($p < 0.001$). However, the slight increase in the levels of ROS in the supernatant of the cultures induced by poly I:C was not significantly reduced in the presence of UA, although the levels of ROS in the supernatant of the cultures treated with poly I:C and 30µM UA were slightly reduced compared to the cultures treated with poly I:C alone, which may confirm the positive role of UA in suppressing oxidative stress in this scenario as well (Fig. 5E).

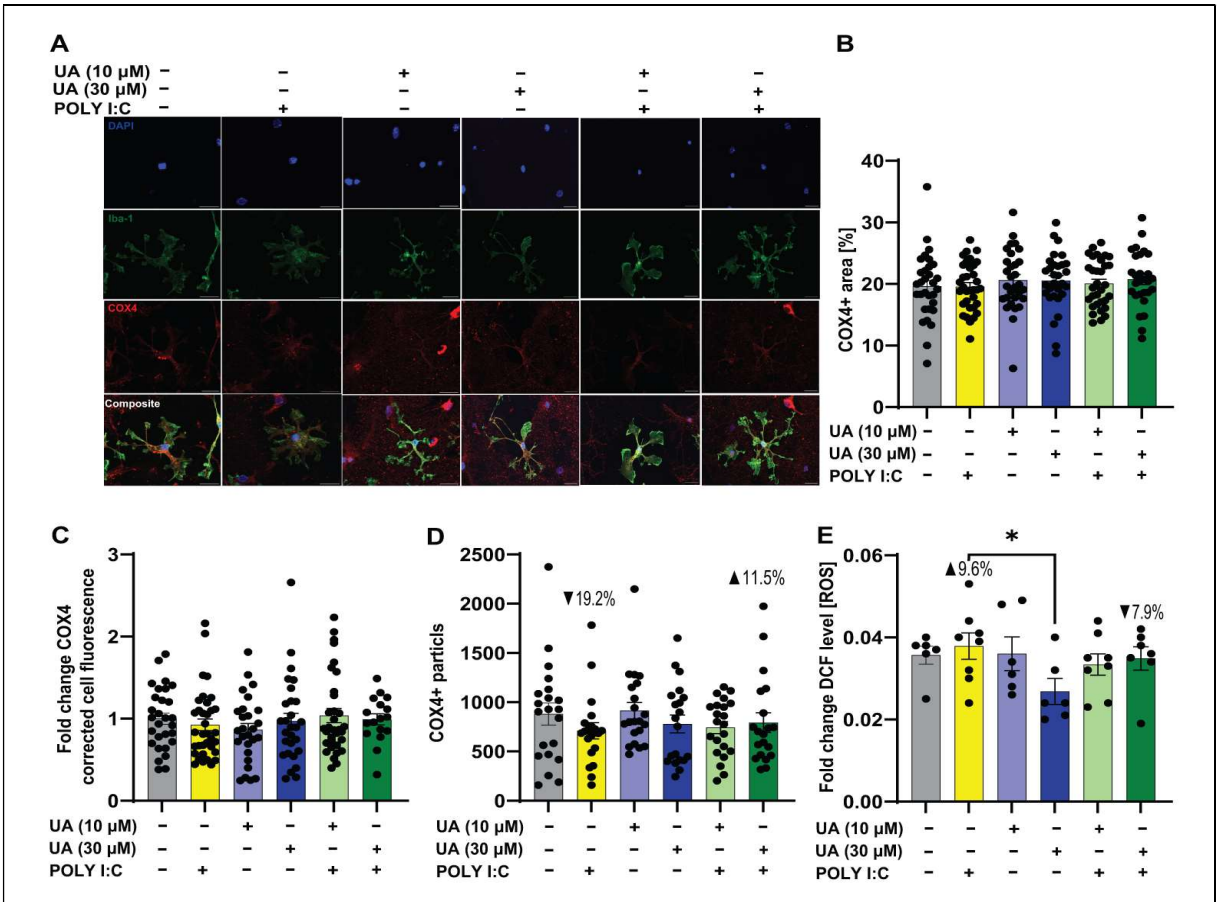


Fig. 5 Urolithin A may increase mitochondrial biogenesis in microglial cells and limit cellular ROS production after poly I:C treatment of cultures.

Cultures were treated with 50µg/ml poly I:C and urolithin A (10µM & 30µM) for 24 hours and immunocytochemically assayed for the internal mitochondrial protein, cytochrome c oxidase (COX4). For ROS measurements, cells were incubated in H2DCFDA for 30 minutes and read with a microplate spectrophotometer. (A) Representative immunofluorescence images showing microglia, Iba-1 (green), and mitochondria, COX4 (red). Scale bar: 20µm. (B) Relative COX4 positive area, (C) Fold change corrected total cell fluorescence of COX4, and (D) Number of COX4+ particles in treated microglial cells are shown. N = 3, n = 18 - 31 cells per group (E) Relative ROS level - the amount of DCF generated upon oxidation of H2DCF by cellular reactive oxygen species. Data are presented as mean ± SEM. One-way ANOVA. **$p < 0.01$

Overall, the results of this study suggest that UA may play a beneficial role in preventing microglial activation and proliferation, limiting the excessive production of proinflammatory mediators (CCL2, TNF-α, and IL-1β) and suppressing cellular ROS levels induced by poly I:C stimulation *in vitro*.

DISCUSSION

Viral infections represent a major threat to human health today. Although most viruses target specific tissues, such as neurotropic viruses that can invade and infect brain cells, some non-neurotropic viruses can exploit weaknesses in the immune system that eventually allows them to also affect the CNS (37). Once in the CNS, viruses can cause severe neuronal damage, sometimes with long-lasting, life-threatening consequences. However, the ability to enter the CNS does not determine whether a virus can cause neurological complications (3, 38). Viral infections are becoming increasingly important in the development or progression of neurodegenerative diseases (39). To date, the cellular mechanisms underlying the neurological consequences of viral infections are not fully understood, but they involve neuroimmune interactions that have so far focused mainly on microglia. As the major immune cells in the brain, reactive microglia play a central role in neuroinflammation by responding directly or indirectly to viruses. Chronic reactivity of microglia, characterized by an alteration in microglial morphology, proliferation, and release of neurotoxic molecules, leads to functions that are distinct from their beneficial roles under physiological conditions and may result in neuronal damage that contributes to the pathogenesis of various neurological diseases (6, 7). Because the CNS is highly susceptible to inflammatory responses and has limited regenerative capacity (40), disruptions in its homeostasis can have profound consequences for important functions and severely affect patients' quality of life. Therefore, it is of great importance to find an effective way to prevent harmful neurological consequences of viral infections of the brain. Therefore in this study, to investigate the possible beneficial effects of a metabolite of intestinal bacteria, urolithin A (UA), which has shown anti-inflammatory effects in various pathological conditions (24-27), on the prevention of neuroinflammation induced by viral infections, the co-culture system containing all three major types of brain cells, including neurons, astrocytes, and microglia, was used. The viral mimetic used was a synthetic analog of viral double-stranded (ds) RNA, poly I:C, which is known to induce microglial activation and inflammatory response (41). The results indicated that UA could significantly prevent microglia activation and rescue the phenotypic shift from ramified cells to activated intermediate cells after poly I:C stimulation. In addition, UA significantly reduced the concentrations of proinflammatory mediators (CCL2, TNF-α, and IL-1β) and a trend toward a reduction in ROS concentrations released in the supernatant of poly I:C-administered cultures.

The results of this study revealed that co-administration of UA at higher concentration (30μM) and poly I:C in cultures results in decreased expression of Iba-1 and CD68 in microglial cells. It is suggested that Iba-1 can only label microglia/infiltrating macrophages and that its expression level may be related to the activation of microglia in brain tissue (38). On the other hand, high expression levels of CD68 in microglia, indicating higher phagocytic activity, suggested the activation status of microglial cells. Consistent with these results, the positive role of UA in attenuating microglial activation in primary microglial cultures after stimulation with other pathogenic molecules, such as lipopolysaccharide (LPS), has also been demonstrated (27, 42, 43).

Although poly I:C led to microglial cell activation that was attenuated by UA, it did not significantly increase microglial cell proliferation as measured by Ki67 expression in cells. This was consistent with the observation of *He et al.*, who showed that poly I:C did not induce significant proliferation of primary microglial cells (33). Nevertheless, the low proliferation of microglial cells after stimulation with 50 μg/ml poly I:C may suggest that long-term stimulation or higher concentrations are required to produce stronger effects. Indeed, viral infection is progressive, and the degree of microglial activation depends on the concentration of the immunogenic molecule(1, 41). It is also plausible that the complex interactions between microglia, neurons, and astrocytes in the co-culture used here limited the extent of microglial proliferation compared with monocultures of microglia. However, Ki67 expression was also

lower in microglial cells after simultaneous treatment with poly I:C and UA, again confirming the positive role of UA in this scenario. Because UA can cross the blood-brain barrier (44), this finding may suggest that UA may be able to limit microglial activation and proliferation as a therapeutic agent after viral infections, which remains to be investigated.

In addition to higher expression of activation-related markers in microglial cells triggered by poly I:C, this stimulus resulted in a shift in microglial morphology from small branched cells to larger and rounder cells. Consistent with previous studies, an increase in cell circularity, an increase in the cell area, and a decrease in the convex hull area were observed after poly I:C administration as signs of microglial activation (29, 45). Nevertheless, administration of poly I:C in cultures did not result in significant changes in fractal dimension, lacunarity, and density as observed by *Fernández-Arjona et al.* after injection of the viral enzyme neuraminidase into rat brain (29). This could be due to the different signaling pathways triggered by viral proteins and poly i:C, as well as the characteristics of microglial cells *in vivo* compared to *in vitro* conditions. Remarkably, UA was able to reduce the poly I:C-induced increase in cell circularity and cell area that define the characteristic intermediate and amoeboid shape of activated microglial cells. In addition, there was a trend toward an increase in the convex hull area of microglial cells, indicating an increase in the expansion of cell processes, as cell areas tended to be smaller in poly I:C-treated cultures that were simultaneously treated with UA.

To date, the role of UA in modulating immunostimulant-induced microglial activation, particularly in the morphological changes, has not been recognized. PCA analysis showed that poly I:C stimulation shifted microglia to the intermediate-activated phenotype rather than ameboid cells. This was consistent with the results of *He et al.*, who found that unlike LPS, which induces ameboid morphology, poly I:C mainly induces intermediate activated morphology (33). Here, UA not only rescued poly I:C induction of intermediate and reduced ramified microglia but also significantly reduced the number of amoeboid microglial cells. This result suggests that UA treatment reverted microglia to their ramified state, with the reduced circularity of cells, reduced cell area, and increased branching. This branched phenotype is important for the performance of beneficial functions such as immune surveillance (46). Thus, UA can restore the homeostatic functions of microglia after immune stimulation. *Fernandez-Arjona et al.* previously demonstrated *in vivo* aberrations in microglial morphology after treatment of rats with surface protein of influenza viruses, neuraminidase (29). Whether our results indicate a possible rescue of these morphological changes of microglia *in vivo* by UA treatment remains to be investigated. On the other hand, as described by *Woodburn et al.*, morphology does not always define the functional state of microglial cells, and because of their high plasticity, it is not always easy to characterize their structural changes (41). Therefore, the observed restoration of microglial morphology by UA treatment should not be hastily interpreted as a linear change in their functions and requires further detailed investigation. But overall, in line with previous observations of the anti-inflammatory role of UA in various pathological conditions, the results of this study regarding the reduction of Iba-1 and CD68 and the changes in microglial morphology toward a reduced activation status suggest a potential beneficial role of UA in virus-induced microglial activation.

To determine whether the rescue of poly I:C-induced morphological changes by UA can positively affect microglial functionality, the results showed that UA dramatically reduced the release of neurotoxic molecules in the microenvironment of cells 24 hours after poly I:C stimulation. UA significantly reduced the expression of proinflammatory cytokines (TNF-α and IL-1β) and chemokines (CCL2) after poly I:C treatment. This result confirms that UA can exert anti-inflammatory functions in microglia during immune stimulation, which is consistent with several studies in other pathological conditions (43, 47). *DaSilva et al.* have previously shown that reducing proinflammatory mediators in microglia can prevent neuronal apoptosis in microglia-neuron co-cultures (47). Moreover, UA did not induce detectable changes in IL-10 levels 24 hours after treatment. This suggests that UA may only attenuate inflammation by suppressing proinflammatory mediators and not stimulate the production of anti-inflammatory mediators. However, *Abdelazeem et al.* found that UA

increased IL-10 levels 2 hours after LPS stimulation of bone marrow-derived macrophages but decreased at later time points (48). Therefore, cytokine analysis at different time points or mRNA detection is useful to evaluate the role of UA in the release of IL-10 and other proinflammatory cytokines. Nonetheless, the decrease in proinflammatory molecules may have a significant physiological effect in limiting neuroinflammation and the resulting neuronal damage.

Much evidence has accumulated over the past decade suggesting that patients infected with RNA viruses are under chronic oxidative stress (49). Oxidative stress from RNA virus infections can contribute to several aspects of viral disease pathogenesis, including inflammatory response, cell death, immune dysregulation, and enhanced viral replication (50). Consequently, the accumulation of oxidative damage to mitochondrial DNA in microglia under pathological conditions, including viral infections, leads to increased production of ROS, which subsequently results in neuronal damage and death (19, 23).

Next, the role of UA in attenuating mitochondrial damage and oxidative stress, which have been shown to occur during viral infections, was investigated (19-21). In contrast to other studies showing that UA induces mitophagy by decreasing mitochondrial accumulation and thus the expression of mitochondrial proteins such as COX4, here UA instead resulted in a very slight increase in COX4 expression. Although this change was statistically insignificant, it may indicate an increase in mitochondrial biogenesis. This is similar to the observations of *Esselun et al.*, who found that UA did not trigger mitophagy but did trigger the transcription of genes for mitochondrial biogenesis (51). Another group found that UA triggered cellular autophagy rather than mitophagy in ischemic neuronal injury (52). While these results appear contradictory at first glance, they could represent observations at different stages of the same pathway. Indeed, an increase in mitophagy would degrade accumulated damaged mitochondria in the short term and eventually increase mitochondrial biogenesis after the recycling of materials. However, to determine the role of UA in preventing mitochondrial dysfunction in microglial cells triggered by an immune stimulus, a more robust analysis is required.

In addition, UA showed a small trend to decrease cellular ROS production in poly I:C-treated cultures, although not statistically significant. This antioxidant property suggests that UA may limit oxidative stress in activated microglia (27). In this case, the 7.9% decrease in ROS levels induced by poly I:C following 30 µM UA co-treatment may have a physiologically relevant effect in modulating neuroinflammation and maintaining brain homeostasis during viral infections. Specific quantification of mitochondrial ROS levels will give a better idea of the role of UA in inhibiting ROS production. However, other studies have suggested that the role of UA in improving mitochondrial function is independent of ROS production (25, 51). Therefore, further studies are needed to objectively investigate the antioxidant role of UA in viral infections.

However, this study had the following limitations. First, this study primarily focused on elucidating the protective role of UA against viral mimetic-induced microglial dysfunction because these cells, as resident innate immune cells of the CNS, play an important role in neuroinflammation and subsequent neuronal injury. However, recent data suggest that viruses and other immune stimuli can also activate astrocytes (37, 53). Thus, astrogliosis may also play an important role in neuroinflammation, which should be considered in future studies of therapeutic approaches. On the other hand, it is important to investigate the potentially positive role of UA on poly I:C-induced neuronal death and damage to confirm that the role of UA in reducing microglial activation is sufficient to protect neurons in this scenario.

Here, we used an optimized co-culture system that includes primary microglia, neurons, and astrocytes to account for the complex interactions between these cells *in vivo* during neuroinflammatory processes. However, *in vitro*, models cannot adequately represent the physiological state of the cells. Therefore, future studies in animal models and using real RNA viral infections will be useful to determine the role of UA *in vivo* in virus-induced neuroinflammation and to exploit it clinically in the long term. One area of microglial biology that has been relatively neglected until recently is sex differences, and this is in spite of the fact that sex is a risk factor in several diseases that are characterized by neuroinflammation and, by extension, microglial

activation (54). Therefore, sex differences should also be taken into account in further studies. Finally, this study does not reveal the intracellular signaling mechanisms of UA in modulating microglial structure and function during viral stimulation, which warrants further investigation.

CONCLUSION

In conclusion, these results demonstrate for the first time that UA can prevent microglial activation induced by stimulation with the viral poly I:C mimetic in vitro. UA was able to attenuate microglial activation, inhibit the production of proinflammatory signaling molecules, and rescue the morphological changes of microglia after poly I:C stimulation. In addition, UA appeared to induce mitochondrial biogenesis and suppress oxidative stress, which could prevent neuronal damage and death. Moreover, the results of this study add detailed microglial morphometric findings at the single cell level to other studies showing the positive role of UA in preventing neuroinflammation under pathological conditions.

Because urolithins are natural metabolites of the gut microbiota from ellagitannins and ellagic acid and can be obtained from abundant foods such as pomegranates, strawberries, and walnuts, they have promising therapeutic potential. Therefore, this study may contribute to the future use of UA in probiotics or as a dietary supplement to protect neurons from damage caused by viral infections. However, further studies are needed to evaluate the *in vivo* applicability of UA so that it can be used in a clinical setting.

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