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Lupeol inhibits LPS-induced neuroinflammation in cerebellar cultures and induces neuroprotection associated to the modulation of astrocyte response and expression of neurotrophic and inflammatory factors

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ABSTRACT

In the central nervous system (CNS), neuroinflammation, especially that modulated by the cell response of astrocytes and microglia, is associated with damage to neurons in neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and, Multiple Sclerosis. Lupeol is a dietary triterpene that has demonstrated biological activities as antioxidant. This study investigated the anti-inflammatory and neuroprotective effects of lupeol in an *in vitro* model of neuroinflammation in primary cerebellar cultures. Cultures were obtained from 6-day-old Wistar rats, subjected to inflammatory damage with lipopolysaccharide (LPS, 1 μg/mL) and treated with lupeol (0.1 μM). We observed, after a 48-hour treatment, through Fluorjade-B staining and immunocytochemistry (ICQ) for βIII-tubulin, that lupeol induced neuroprotection in cultures submitted to inflammatory damage. On the other hand, through ICQ for GFAP, it was possible to observe that lupeol modulated the astrocyte morphology for Bergmann glia-like phenotype and, especially for velate astrocyte-like phenotype, both phenotypes associated with the neuroprotective profile. Moreover, RT-qPCR analysis showed that lupeol induced the down-regulation of the mRNA expression for proinflammatory markers TNF, iNOS and NLRP3, as well as the production of nitric oxide (method of Greiss), which were up-regulated by LPS, and also induced up-regulation of the mRNA expression for arginase and IL-6 mRNA. In addition, lupeol induced up-regulation of mRNA expression for neurotrophins GDNF and NGF and also for the sonic hedgehog–Gli pathway. Together, these results lead to the conclusion that lupeol inhibits neuroinflammation in cerebellar cultures and induces neuroprotection associated with the modulation of astrocyte response and expression of neurotrophic and inflammatory factors.

1. Introduction

Inflammation is a process of trying to reach homeostasis, common to any occasion of damage and exposure of the body to pathogens or chemicals [1]. However, in cases of prolonged inflammation, diseases are likely to be triggered, including in the central nervous system (CNS), as for example, in the case of the neurodegenerative diseases (NDDs) Multiple Sclerosis (MS) and Alzheimer's disease (AD) [2]. These conditions, as well as other NDDs, have been the subject of research in neuroscience also to better characterize the inflammatory profile and, consequently, find more effective strategies for treatments [1,3]. An important feature on the cytopathology of these diseases is the involvement of glial cell response in promoting and/or reversing the neuroinflammatory process [2]. Astrocytes and microglia are considered as key players in inflammatory processes triggered in the CNS, and their phenotypic polarization is directly involved in the progression of neuroinflammation [4]. When the inflammatory response in the CNS extends over a long period, it can result in the death of neurons, characterizing what is known as neurodegeneration. During damages to the CNS, reactive profiles of microglia and astrocytes, M1 microglia and A1 astrocytes, respectively, are responsible for producing cytokines, interleukins and proinflammatory chemokines, including TNF-α, IL-6, IL-1β and CXCL-3, which mediate microglial reactivity and astrocyte proliferation, thereby prolonging the inflammatory response [5–8]. However, these cells can acquire an anti-inflammatory type-2 profile, known as M2 microglia and A2 astrocytes, characterized as anti-

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inflammatory and neuroprotective. M2 and A2 cells are also responsible for reducing the production of nitric oxide (NO), modulated for the enzyme inducible oxide nitric synthase (iNOS), in inflammatory conditions [9,10], also associated to the increase in the activity of arginase 1 [11,12]. Moreover, A2 astrocytes, in general, stimulate neuronal proliferation by producing neurotrophins, mainly glia-derived neurotrophic factor (GDNF) [13] and mesencephalic astrocyte-derived neurotrophic factor (MANF) [14].

Lupeol is a triterpene mainly present in fruits of plants such as *Olea europaea* (olive) and *Mangifera indica* (mango) [15]. Its well-known biological effects are anti-inflammatory, anti-arthritic and the anti-tumor activities [16,17]. However, little is known about mechanisms associated with the immunomodulatory and neuroprotective effects of lupeol in the CNS. Badshah et al. [18] observed that lupeol can act as an anti-inflammatory, through the modulation of p-38 pathways, MAPK and JAK in models of neuroinflammation induced by *Escherichia coli* lipopolysaccharide (LPS). Lupeol was also able to modulate the inflammatory response in the CNS in an *in-vivo* model of AD, associated with the regulation of the expression of proinflammatory cytokines and interleukins (TNF-α, IL-6 and IL-1β), and the recovery of short-term memory [19] as well as the down-modulation of BACE-1 activity (*in vitro*, a protease involved in a cleavage of protein TAU and that is deregulated in individuals with AD [20].

In the CNS, the cerebellum is one of the main regions affected in NDDs. Kutzelnigg et al. [21] and Gilmore et al. [22] demonstrated that lesions due to demyelination in neurons are more frequent in the cerebellar cortex than in other regions of the brain. Motor dysfunctions in individuals with MS and tremor in patients with Parkinson's disease (PD) are symptoms closely related to the damage of cerebellar granular neurons [23]. Another important feature that makes the cerebellum an important region in the study of neuroinflammation is its relationship with the telencephalon and the rostral region of the mesencephalon; individuals with PD present a primary inflammatory process in these regions that lead to defects in the molecular communication by neurotransmitters, resulting in paralysis and neuromuscular dysfunction [24]. These characteristics make the cerebellum an important region for the evaluation of processes related to NDDS diseases that present neuroinflammation as one of the main events related to the pathogenesis. In light of this, the present study investigated the anti-neuroinflammatory and neuroprotective effects of lupeol as well as the role of astrocytic response in a cerebellar *in vitro* model of neuroinflammation.

2. Materials and methods

2.1. Primary culture of dissociated cells of the cerebellum

In order to mimic physiological conditions of the CNS in the complexity of the interaction between neurons and glial cells, primary cultures of cerebellar cells from Wistar rats (P6 days) were adopted as a neuroinflammation model of study. The animals were obtained from the Animal Facility of the Federal University of Bahia (Salvador, Brazil) and handled according to Brazilian guidelines for breeding, maintenance and use of animals for teaching activities and scientific research and the local Ethical Committee for Animal Experimentation, protocol number (027/2012, ICS — UFBA). Primary cultures of cerebellar neurons and glia were performed in accordance to the method described previously by Pereira et al. [25]. After euthanasia and decapitation of neonatal Wistars P6–8 rats, the skullcap was opened to expose the entire brain and allow its removal of the cerebellum. The tissue was then cleaved with the aid of a glass pipette by resuspension and the cell suspension was passed through a sterile nylon filter (70-μm pore) and cells collected in Falcon tube, containing DMEM/Ham F-12 (Gibco, 12500-062), BSA (Sigma-Aldrich, A9418) at 10%. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded and the cells suspended with 10 mL of DMEM/Ham F 12 with 10% BSA. After counting the number of cells, they were transferred to polystyrene 96- and 24-well plates (Kasvi), and petri plates of 60 mm, previously coated with 30 μM poly-L-ornithine hydrobromide (Sigma Aldrich P3655) and 20 μM laminin (Sigma Aldrich L2020) at a density of 1.5×10^4 cells/cm^2.

2.2. Drugs and treatments

The triterpene lupeol (L5632) was purchased from Sigma-Aldrich and dissolved at a concentration of 100 mM in a solution of dimethylsulfoxide (DMSO, Sigma Aldrich, 472301) and ethanol (Sigma Aldrich E7023) (v/v), forming a stock solution kept in the dark at a temperature of −4 °C. The final dilutions were taken at the time of treatment and cells were analyzed after 48 h. Control cultures were treated with DMSO/ethanol diluted in culture medium in a volume equivalent to that used in groups treated with the triterpene, not exceeding a final concentration of 0.01% (v/v). An initial dose-response MTT test was performed to determine non-toxic concentrations of lupeol, as described below. For the neuroprotection and neuroinflammation assays, lupeol was adopted at the final concentration of 0.1 μM. Lipopolysaccharide (LPS) derived from *Escherichia coli* (Sigma Chemical Co., L2630) (St Louis, MO) was dissolved in PBS to form a 1-mg/mL stock solution and was further adopted in experiments at the final concentrations of 1 μg/mL.

Two protocols were adopted to investigate the lupeol effects during neuroinflammation: 1) cultures were treated with 1 μg/mL LPS for 24 h, then the medium was removed and replaced for either fresh medium or medium containing 0.1 μM lupeol, or vehicle; and 2) cultures were treated with 0.1 μM lupeol for 24 h, then the medium was removed and replaced for either fresh medium or medium containing 1 μg/mL LPS, or vehicle. The experimental analyses were performed 48 h after the treatments, as shown in Fig. 1.

2.3. Cell viability assay

The cytotoxicity of lupeol to cerebellar cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Confluent cells cultured in 96-well plates (TPP) were exposed to lupeol for 48 h. Two hours before the end of the exposure time, the culture medium was replaced by a solution of MTT diluted in DMEM (5 mg/mL) and then the plates were incubated for 2 h in a humidified atmosphere with 5% CO^2, at 37 °C. Thereafter, cells were lysed with 20% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) acetic acid and 2.5% (v/v) 1 mol/L HCl, and the plates were kept overnight at 37 °C to allow the formazan crystals to dissolve. Cytotoxicity was investigated by measuring the conversion of yellow MTT into purple MTT formazan by mitochondrial dehydrogenases of living cells. The optical density of each sample was measured at 540 nm using a spectrophotometer (Varioskan™ Flash Multimode Reader, Thermo Plate). Three independent experiments were conducted and eight replicate wells were used for each experimental condition.

2.4. Nitric oxide production

Nitric oxide (NO) production was assessed as sodium nitrite ($NaNO_2^-$) accumulation in the culture medium using a colorimetric test based on Griess reagent [26]. Samples (50 μL) were collected 48 h after treatments. Equal volumes of culture medium and Griess reagent composed of 1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2% phosphoric acid (NEED, Sigma-Aldrich 1465-25-4) were mixed. The mixture was incubated for 10 min at room temperature, and then the absorbance at 550 nm was measured using a microplate reader (Varioskan™ Flash Multimode Reader, Thermo Plate). The concentrations of nitrite in the samples were determined based on a sodium nitrite standard curve (1.26–100 mmol/L $NaNO_2$). Three independent experiments were performed.

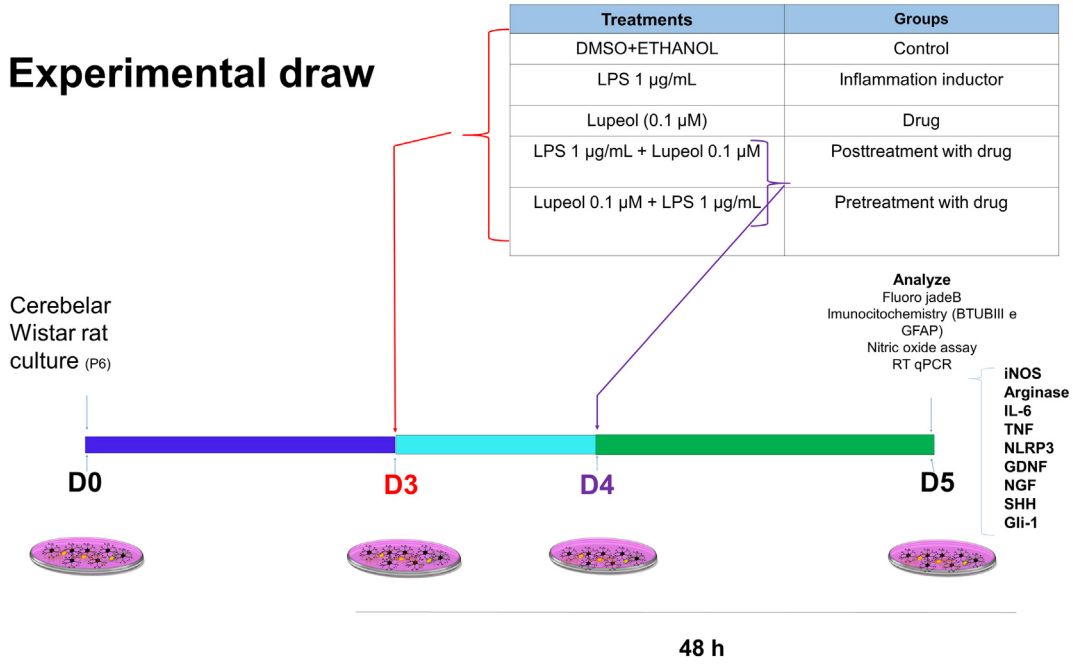

Fig. 1. Experimental design. Primary cerebellar cultures of neuron and glial cells were obtained from cerebellum from Wistar rats (P6–8). After 3 days, cultures were treated with vehicle DMSO/ethanol v/v (control condition), treated with LPS (1 µg/mL) for 24 h and with fresh medium for additional 24 h, treated with lupeol (0.1 µM) for 24 h and with fresh medium for additional 24 h, pretreated with LPS (1 µg/mL) for 24 h and then treated with lupeol (0.1 µM) for additional 24 h, or pretreated with lupeol (0.1 µM) for 24 h and then treated with LPS (1 µg/mL) for additional 24 h. Analyses were performed 48 h after the beginning of treatments.

2.5. Fluoro-Jade B staining

In order to investigate neuronal loss staining with the fluorescent reagent Fluor Jade B (Merck-Millipore, AG310), a specific marker of neuronal degeneration in the process was used. After the treatments, the medium of the cultures was removed and the cells were fixed with ethanol (4 °C) for 10 min. The cultures were washed for three times with PBS and permeabilized with Triton X-100 (Sigma-Aldrich T8787) at a 0.3% solution in PBS for 10 min. After this period, the cultures were washed three times with distilled water and incubated with Fluor Jade B solution (0.001%) for 30 min at room temperature under slow agitation and protected from light. Then, the cultures were washed three times with PBS and incubated with a fluorescent DNA intercalating agent 4′,6-diamidino-2-phenilindol dihydrochloride (DAPI, Molecular Probes, Eugene, OR) for 5 min and washed again with PBS. After this process, the plates were observed in a fluorescence microscope (Leica DMIL model 801). The result of the quantification of cell death was evaluated by the ratio of the fluorescence intensity (Fluoro Jade B/DAPI fluorescence intensity). Three independent experiments were performed.

2.6. Immunocytochemistry

For immunocytochemistry, the cerebellar cells were plated on coverslips previously sensitized with a solution of 0.1 mg/mL poly-L-ornithine hydrobromide (Sigma Aldrich P3655) and 1 mg mL laminin (Sigma Aldrich L2020) at a density of 1.5×10^4 (150,000) cells/m^2. After the treatments, the cultures were washed three times with PBS at pH 7.4 and fixed with 4% paraformaldehyde (Sigma Aldrich, P6148) in PBS during 20 min, at −20 °C. Excess paraformaldehyde was discarded and the well plates were washed three times with PBS to remove paraformaldehyde excess. For immunocytochemical staining, cells were rehydrated with PBS and permeabilized with a solution of 0.33% Triton X-100 (Sigma Aldrich, T8787) in PBS and blocked with 5% bovine serum albumin solution (BSA, Sigma Aldrich, A9418) in PBS for 1 h. Cultures were incubated with the following primary antibodies: mouse monoclonal antibodies specific for mouse β-III Tubulin (β-IIITUB) (1:500, Abcam — AB78078), structural marker of young neurons, rabbit polyclonal antibodies specific for glial fibrillary acidic protein (GFAP) (1:500, Wako — G9269), structural marker of astrocyte, diluted in PBS/BSA (1%) and kept in a humid chamber at 4 °C, for 12 h. After three washes of 10 min with PBS under slow agitation, the cultures were incubated with a solution containing specific goat secondary antibodies for rabbit IgGs of or mouse IgGs conjugated with Alexa Fluor 488 or Alexa Fluor 594, diluted in 1% PBS/BSA (1:1000) for 2 h, at room temperature. After that, the cultures were incubated with PBS containing 0.3 mM DAPI, for 5 min, protected from light, at room temperature. The coverslips were then assembled on the slides with anti-fade N-propyl gallate solution (0.1 mM). In all experiments, including the cultures where the primary antibodies were not added, unspecific stained was not observed in negative controls. Thereafter, cells were analyzed using a fluorescence microscope (Leica, DFC7000) and with a confocal microscope (LEICA DMIL 801, analyzed by DMI 6000B). All assays were performed at least three times. Ten randomized fields were analyzed per each experimental condition and quantified using the Image J software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA).

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated from cerebellar cultures with Trizol® reagent according to the manufacturer's specifications. Therefore, 1×10^4 cells/cm^2 were seeded in 60-mm plates and after treatments, the cultures were washed three times with PBS and 1 mL of Trizol® reagent was added. The samples were stored at −80 °C until the time of the analysis. The concentration and purity of RNA were determined by spectrophotometric analysis using a nano spectrum Kasvi (K23-0002). DNA contaminants were removed by treating the RNA samples with DNAse using the Ambion DNA-free kit (#AM1906, Life Technologies™). For cDNA synthesis, SuperScript® VILO™MasterMix (#MAN0004286, Invitrogen™, Life Technologies) was used in a 20-µL reaction with a concentration of 2.5 µg of total RNA.

2.8. Quantitative PCR (qPCR)

Quantitative real-time PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems, CA, USA) containing two primers to amplify the sequence of interest, a specific Taqman® MGB probe and TaqMan Universal Master Mix II with UNG (catalog# 4440038 Invitrogen, Life Technologies™). The assays corresponding to the genes quantified in this study were: Arginase 1 (Rn00681090_m1), Nitric Oxide Synthase Induced/iNOS (Hs01075529_m1), Interleukin 6/IL-6 (Rn01410330_m1), NLRP3 (Rn04244620_m1), Tumor Necrosis Factor/TNF (Rn99999017_m1), nerve growth factor/NGF (RN01533872_m1) and glial-derived neurotrophic factor/GDNF (RN00569519_m1), Sonic Hedgehog/SHH (Rn00568129_m1), Gliome-1-related oncogene/Gli-1 (Rn01504237_m1) distributed by Thermo Fisher (TaqMan® Gene Expression assay). Real-time PCR was performed using the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, CA, USA). The thermocycling conditions were performed according to the manufacturer's specifications. HPRT1 (Mm01545399_m1) was used as reference gene (endogenous controls) for normalizing gene expression data.

2.9. Statistical analysis

For each analysis, three independent experiments were performed. The results were analyzed using the Graph Pad Prism 7.10 statistical software (California, USA) and reported as mean ± standard deviation or median ± standard deviation of the parameters assessed. One-way ANOVA analysis was used followed by Student's Newmann-Keuls' post-test. p values ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Lupeol is neuroprotective to neurons submitted to inflammatory damage

Initially, the effect of lupeol at different concentrations (0.1, 1 and 10 μM) on the viability of cerebellar cells was evaluated through the MTT test (Fig. 2). No toxicity was observed at the concentrations tested. Hence, the concentration of 0.1 μM was adopted for all assays upon the inflammatory stimulus.

The effect of lupeol (0.1 μM) alone and during inflammatory stimulus with LPS (1 μg/mL) on the viability of neurons was investigated through the techniques of Fluoro-Jade B staining (Fig. 3), specific for neurons in degeneration, and through immunocytochemistry (ICQ) for β-III tubulin (β-IIITUB) protein, exclusive of the cytoskeleton of neurons (Fig. 4). Analysis with Fluoro-Jade B staining (Fig. 3) shows that cultures in control conditions present little proportion of neurons in degeneration (5.39 ± 0.53%), and it was observed that the LPS treatment induced a significant increase in the proportion of neurons in degeneration, attainting about 12.47 ± 0.78% of cells in the culture. However, treatment with lupeol reduced the proportion of degenerating neurons compared to the control cultures, as well as when it was adopted either as pretreatment or post-treatment to inflammatory stimulus with LPS, resenting proportions of 1.39 ± 0.39%, 8.96 ± 0.60% and 1.74 ± 0.20% of neurons in degeneration, respectively. Moreover, it is interesting to observe that lupeol protection of neuronal damage was higher when it was adopted as pretreatment to inflammatory stimulus with LPS.

β-IIITUB immunostaining confirmed the neuroprotective effect of lupeol in cerebellar cultures (Fig. 4). The cultures in control conditions have a proportion of neurons (βIIITUB+) of about 19.39 ± 0.66%. It was observed that treatment with LPS induced a significant reduction in the proportion of βIIITUB+ cells to about 7.38 ± 0.12% of total cells in cultures. Lupeol treatment increased to about 24.57 ± 0.07% the proportion of βIIITUB+ cells when compared to control cultures. Moreover, pre-treatment of cultures with lupeol before LPS inflammatory stimulus presented a proportion of neurons similar to that of control cultures (19.62 ± 1.24%). Lupeol post-treatment to inflammatory stimulus with LPS also increased the proportion of neurons compared to cultures only exposed to the toxin, but in a lesser extension (12.72 ± 0.27%).

3.2. Lupeol modulates morphology and reactivity of astrocyte submitted to inflammatory damage

Cell morphologies may represent a cell group. In the CNS, astrocytes, and microglia, especially, have different phenotypes that may indicate resting/activated or inflammatory/anti-inflammatory profiles in cultures and tissues, and according to the structural subtype. Hence, the morphological analysis of these cells is a very useful tool to understand and to characterize responses to CNS damages. Through the immunocytochemistry staining for GFAP (Fig. 5A–E), a protein exclusive of the cytoskeleton of astrocytes and the classical marker of astrogliosis (astrocyte reactivity), these cells could be identified in cerebellar control cultures, presenting a homogeneous distribution of GFAP in the cell body, characterizing the different patterns of glial phenotypes in the cerebellum: Bergmann glia (7.20%, Fig. 5A), known for five cytoplasmic processes, one of which is a long extension; velate astrocyte (9.38%, Fig. 5B), with cytoplasm in the form of veil, hyperplasic and peripheral nucleus; reactive astrocytes (8.98%, Fig. 5C),

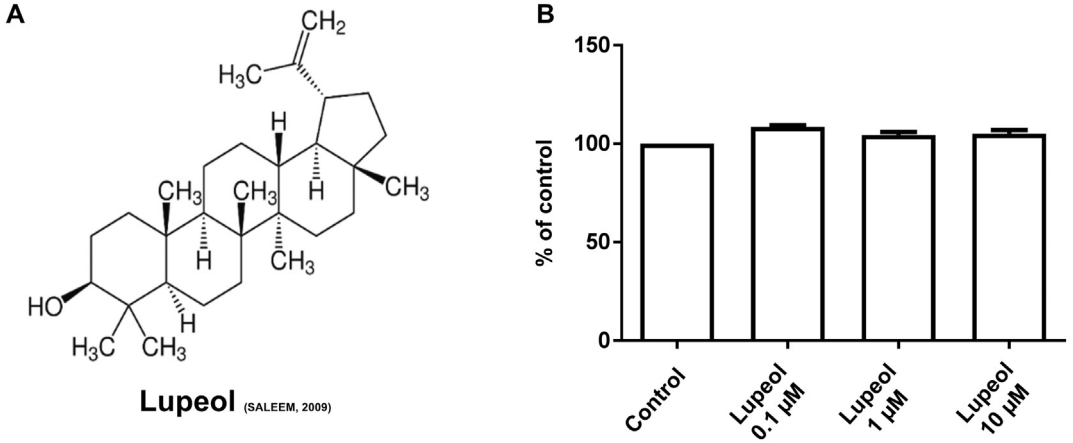

Fig. 2. Analysis of the effect of lupeol on the viability of cerebellar cells through MTT test. Cells were cultured in control conditions (vehicle) or in the presence of 0.1–10 μM lupeol, and the activity of mitochondrial dehydrogenases was measured after 48 h. Results expressed as percentage of media ± SD related to control, considered as 100%, in three independent experiments.

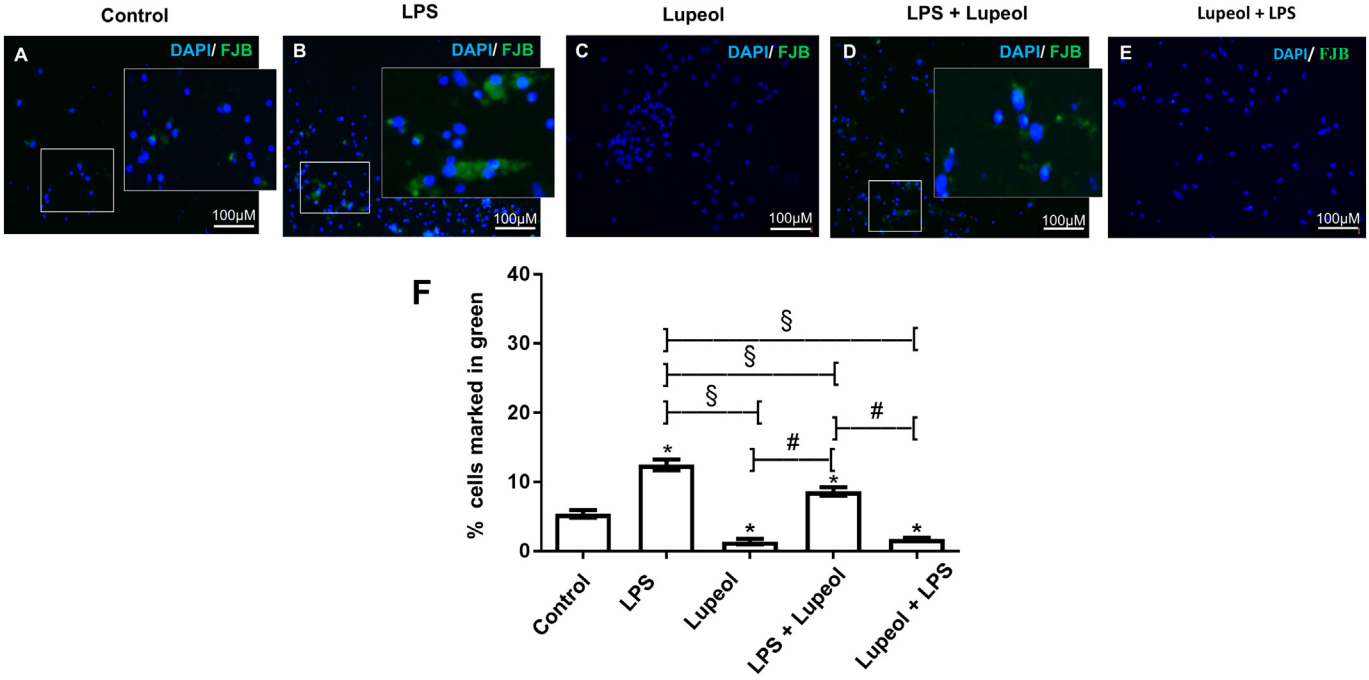

Fig. 3. Analysis of the effect of lupeol during LPS-inflammatory stimulus on the viability of cerebellar neurons through the Fluoro-Jade B (FJB) staining. (A) Photomicrographs of cerebellar cultures in control conditions; (B) treated with LPS (1 μg/mL); (C) treated with lupeol (0.1 μM); (D) pretreated with LPS for 24 h and then treated with lupeol; (E) pretreated with lupeol for 24 h and then treated with LPS. FJB positive cells are stained in green and cell nuclei co-stained with DAPI appear in blue, obj. ×20, scale bars of 100 μm; inserts of images at obj. ×40 are seen in B and D showing neurons in degeneration in green. (F) Quantification of the proportion of FJB positive cells (green) related to the total of cell nuclei counted (DAPI stained nucleus, blue), expressed as the media of percentage in three independent experiments. The results were analyzed by Kruskal-Wallis ANOVA followed by Dunn's post-test; * representing $p \leq 0.05$ compared to control; § representing $p \leq 0.05$ compared to LPS 1 μg/mL; # represent $p \leq 0.05$ compared to lupeol 0.1 μM; obj. ×20, scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whose morphology presents contracted cytoplasm, most condensed nucleus and great cytoplasmic extensions; polygonal astrocytes (30.96%, Fig. 5D), which can present a hyperplasic or triangular cytoplasm; and GFAP positive cells (GFAP+) with other uncharacterized phenotypes (37.45%). In general, in cerebellar cultures exposed to LPS and/or lupeol, the proportion of GFAP+ cells was increased when compared to control (Fig. 5F). Concerning the Bergman glia (BG), which appears early in the cerebellum during development, treatment with LPS induced a reduction in the proportion of astrocytes with this morphology, from 7.20 ± 0.52% in control cultures to 3.36 ± 0.92%; this proportion was increased in cultures treated only with lupeol (9.89 ± 0.45%) or in both conditions of pre- or post-treatment of the inflammatory process (8.09 ± 0.75% and 13.19 ± 0.41%, respectively) (Fig. 5G). In cultures treated only with lupeol, a large increase was observed in the proportion of astrocytes with the velate morphology, from 9.38 ± 0.52% in control cultures to 36.1 ± 0.06% (Fig. 5H). This increase was also observed in cultures stimulated with LPS and treated with lupeol (19.10 ± 0.45%), and mainly in cultures treated with lupeol before LPS stimuli (35.87 ± 0.68%). Moreover, LPS induced most astrocytes to acquire mainly a reactive phenotype (43.18 ± 0.33%, Fig. 5B and I). However, treatment of cultures with lupeol before stimulation with LPS induced a decrease in the proportion of reactive astrocytes (16.73 ± 0.57%) (Fig. 5E and I). On the other hand, the presence of astrocytes with polygonal morphology was higher when Lupeol was adopted alone and as pretreatment to LPS (37.33 ± 1.50 and 45.42 ± 0.81, respectively) (Fig. 5E and J).

3.3. Lupeol alters the inflammatory molecular profile in cerebellar cultures

The effect of lupeol on the inflammatory profile of the cerebellar culture submitted or not to LPS-induced inflammatory stimulus was evaluated by measuring the nitric oxide levels with spectrophotometry analyses and the expression of mRNA for some inflammatory cytokines and interleukins with RT qPCR (Fig. 6). It was observed that LPS induced an increase in NO levels; however, pretreatment of the cultures with lupeol induced a reduction in LPS-induced NO levels to values similar to those of the control. Moreover, compared to control cultures, LPS treatment also induced an increase in the expression of mRNA of iNOS (14.2 ± 2.93-fold), IL-6 (3.28 ± 0.46-fold), TNF 2x (2.66 ± 0.43-fold) and NLRP3 inflammasome (9.99. ± 0.97-fold). Treatment with lupeol did not alter any of the inflammatory parameters analyzed. The inflammatory profile when lupeol was adopted as post-treatment to LPS was similar to that observed in cultures treated only with LPS concerning levels of NO and mRNA for iNOS (13.51 ± 2.44-fold increase). Nonetheless, it was capable to revert the induction of mRNA expression for pro-inflammatory cytokine TNF and to NRLP3 inflammasome, both induced by LPS. Moreover, lupeol when adopted as pretreatment to LPS, was capable of maintaining the inflammatory profile similar to that observed in control conditions. Both treatments with the associations of lupeol and LPS induced upregulation in mRNA for the regulatory molecules arginase and IL-6.

The expression of some neurotrophins and differentiation factors were also investigated using RT-qPCR (Fig. 6G–J). It was observed that LPS only modulated expression of mRNA for NGF that was increased in 4.19 ± 1.80-folds. Treatment with lupeol induced a great increase in the levels of mRNA expression for GDNF (30.24 ± 6.90-fold), NGF (10.10 ± 0.78-fold), SHH (about 20-fold) and its GLI-1 transcription factor (about 30-fold), markers of astrocyte and neuronal differentiation. This effect was also observed, but in a lower proportion, in cultures pretreated with lupeol and stimulated with LPS.

4. Discussion

The cerebellum is a CNS region rich in immature neurons and

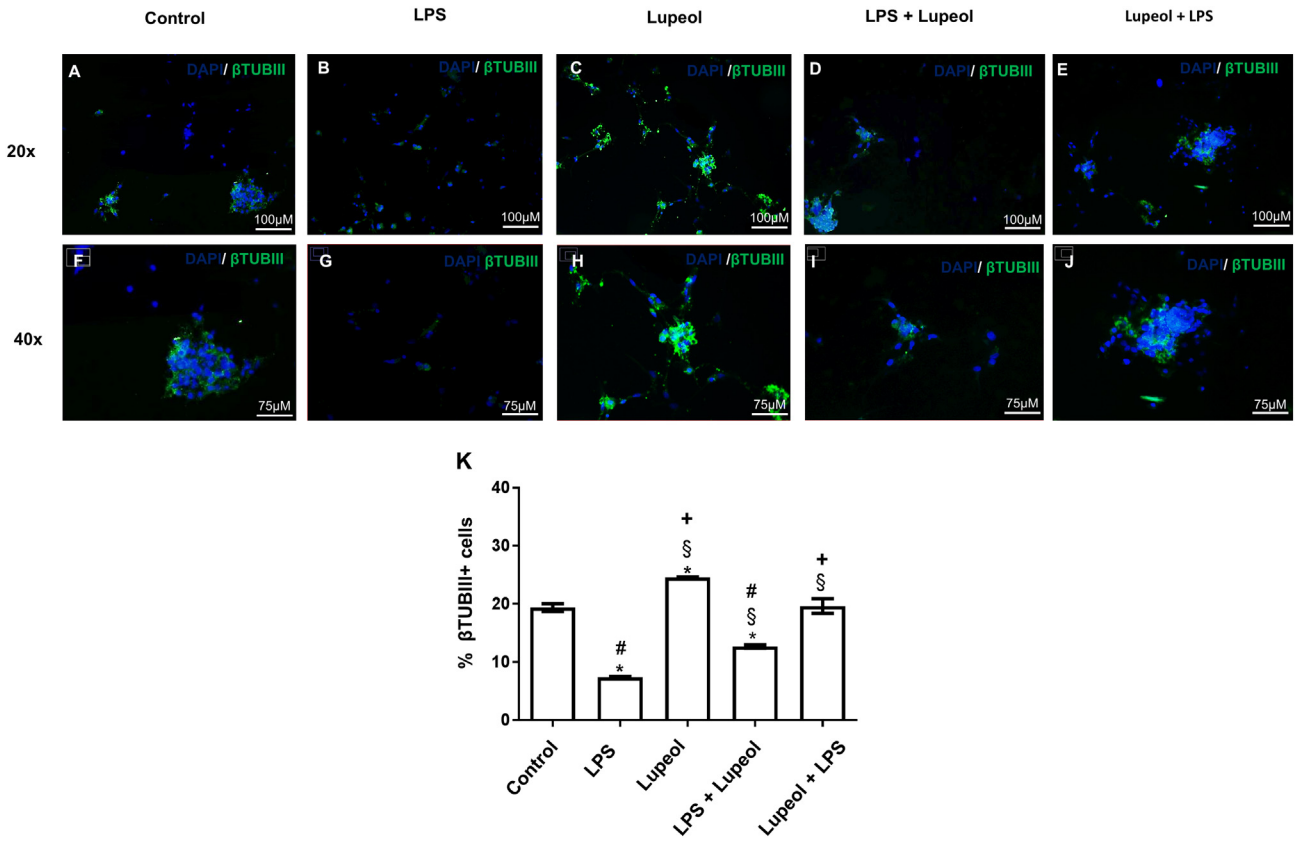

Fig. 4. Analyze of the effect of lupeol during LPS inflammatory stimulus on the viability of cerebellar neurons through immunocytochemistry for β-III tubulin (βIIITUB) protein. (A) Photomicrographs of cerebellar cultures in control conditions; (B) treated with LPS (1 μg/mL); (C) treated with lupeol (0.1 μM); (D) pretreated with LPS for 24 h and then treated with lupeol; (E) pretreated with lupeol for 24 h and then treated with LPS. βIIITUB positive cells are stained in green and cell nuclei co-stained with DAPI appear in blue, obj. ×20 and ×40, scale bars of 100 μm and 75 μm respectively; (F) quantification of the proportion of βIIITUB positive cells (green) related to the total of cell nuclei counted (DAPI stained nucleus, blue), expressed as the media of percentage in three independent experiments. The results were analyzed with Kruskal-Wallis ANOVA followed by Dunn's post-test; * represents $p \leq 0.05$ compared to control; § represents $p \leq 0.05$ compared to LPS 1 μg/mL; # represents $p \leq 0.05$ compared to lupeol 0.1 μM; + represents $p \leq 0.05$ compared to LPS 1 μg/mL + lupeol 0.1 μM. obj. ×20, scale bar = 100 μm; obj. ×40, scale bar = 75 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neuronal precursors [27,28]. The triterpene lupeol has shown promising results in studies conducted to assess its anti-inflammatory and cytoprotective capacity [15]. In order to investigate the neuroprotective activity of lupeol for cerebellar neurons and astrocytes and the association with the modulation of neuro-inflammation, its effects were tested in primary cultures of neonatal rat cerebellum submitted or not to inflammatory stimulus with LPS.

It was observed through Fluoro Jade-B staining specific for neurons in degeneration and also through ICQ for neuronal protein β-III tubulin that lupeol induces neuroprotection during the development of neurons in cultures, as well as when adopted as the pretreatment with LPS inflammatory damage. This protective effect of lupeol can be attributed to the ability to reverse the degenerative processes and apoptosis commonly generated by LPS activity, as it has already been observed by Badshah et al. [18] in the brains of adult mice.

The approach on the astroglial activity in the culture is very important to understand whether these cells are participating in the neuroprotection process generated by lupeol [29–31]. In the cerebellum Bergmann, glia cells assist Purkinje neuron maturation and axonal extension, while the framework of velate astrocytes operates in the maintenance of granule neurons [27,30]. Therefore, it is possible that in situations of damage, these subtypes of astrocytes can act favoring neuroprotection. Using a protocol of standard variations on the morphology of cerebellar astrocytes, it was possible to identify the prevalence of about 30% of polygonal astrocytes in the cerebellum cultures in control situation. On the other hand, few astrocytes with a reactive profile (about 10%) that are characteristic of an inflammatory process in the cerebellum and other regions of the brain [32,33] were observed in control cultures. Moreover, two morphological profiles of resident cerebellar astrocytes were identified in cerebellar cultures in a similar proportion (about 10%): the morphology of Bergmann glia (BG) [34–36] and velate astrocyte (VA), already described by Palay and Chan-Palay [37,38], and more recently by Farmer et al. [30]. In this study, an increase was observed in the proportion of GFAP-positive astrocytes (GFAP+) with variations in the proportion of different morphologies in cultures exposed to inflammatory injury induced by LPS and treated with lupeol and in their association. The reduction in the proportion of BG observed in this study due to the treatment with LPS may have influenced neuronal viability and, apparently, the positive modulation in the proportion of BG in cultures subjected to lupeol can be related to the reduction of neuronal death. Moreover, in cultures treated with lupeol, with or without inflammatory damage, there was a prominent increase in the proportion of VA cultures compared to control conditions, which suggests a positive relationship with the increase of neuronal viability.

As expected, LPS induced an increase in the proportion of reactive astrocytes configuring an inflammatory profile in cerebellar cultures, which corroborates the increased expression of NO, iNOS, IL-6, TNF and activation of inflammasome NLRP3. Lupeol, however, acted reducing the proportion of reactive astrocytes and the expression of markers of neuroinflammation, which may infer anti-neuroinflammatory activity associated with modulation of astroglial response. The induction

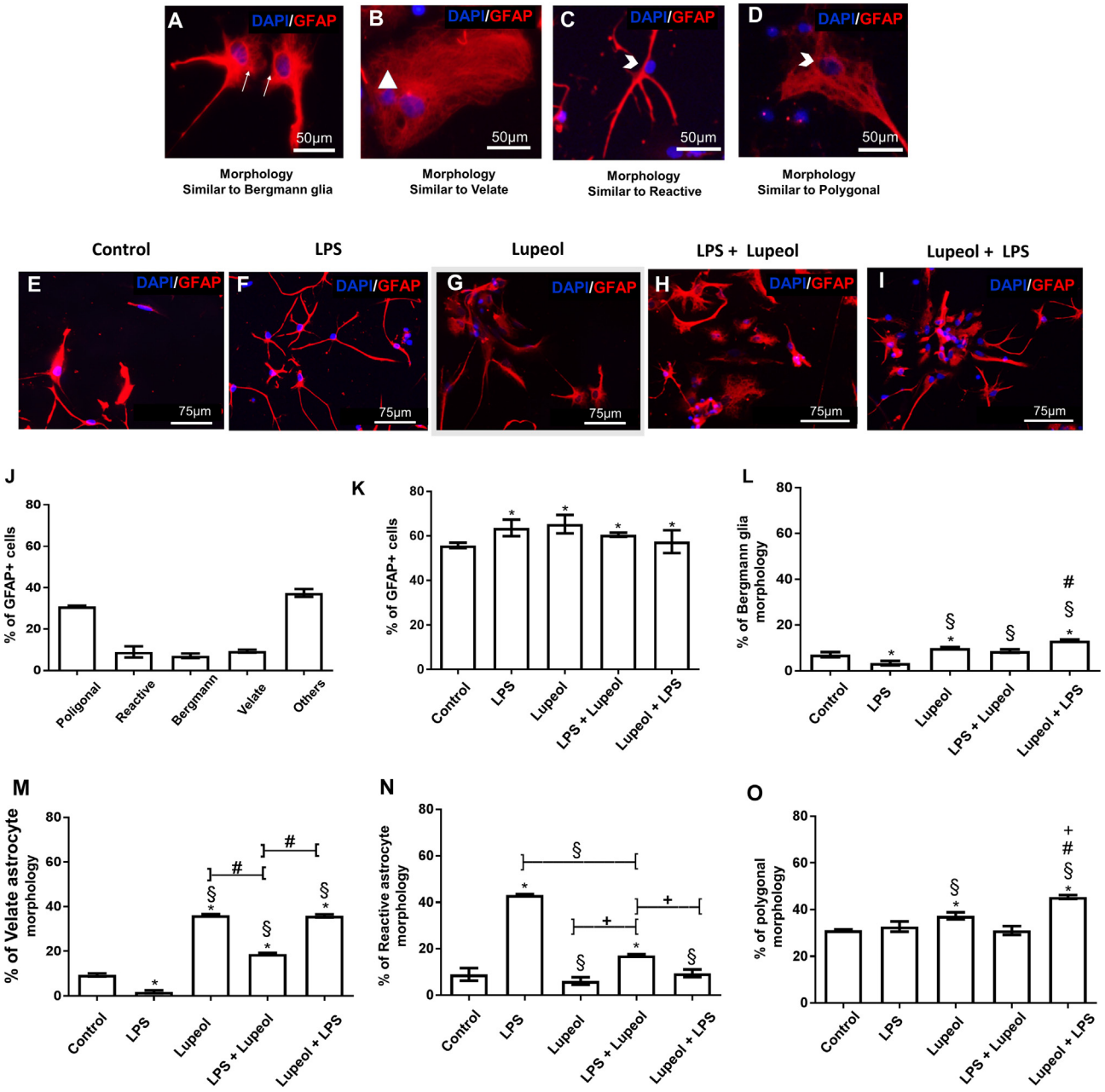

Fig. 5. Characterization of different astrocyte population in cerebellar cultures submitted to inflammatory stimulus by immunocytochemistry for GFAP. (A–D) Photomicrographs of Bergmann glia, velate, reactive and polygonal astrocyte phenotypes in cerebellar cultures in control conditions; GFAP + proteins are stained in red and cell nuclei stained with DAPI are seen in blue; obj. ×60, scale bar = 50 μm. (E–I) Photomicrographs of astrocytes (GFAP +) in different experimental conditions: control cultures (E); cultures treated with LPS (1 μg/mL) (F); cultures treated with lupeol (0.1 μM) (G), cultures pretreated with LPS for 24 h and then treated with lupeol (H) and cultures pretreated with lupeol for 24 h and then treated with LPS (I); obj. ×40, scale bar = 75 μm. (J) Quantification of the proportion of astrocytes (GFAP + cells) presenting the different phenotypes in control conditions; results express the proportion of GFAP + cells related to cell nuclei stained with DAPI. (K) Quantification of the total astrocytes (GFAP + cells) related to the cell nuclei stained with DAPI. (L) Quantification of total astrocytes (GFAP +) with Bergmann glia phenotype, related to cell nuclei stained with DAPI. (M) Quantification of velate astrocytes (GFAP +), related to cell nuclei stained with DAPI. (N) Quantification of reactive astrocytes (GFAP +) related to cell nuclei stained with DAPI. (O) Quantification of polygonal astrocytes (GFAP +) related to cell nuclei stained with DAPI. The results were analyzed by Kruskal-Wallis ANOVA followed by Dunn's post-test; * represents $p \leq 0.05$ compared to control; § represents $p \leq 0.05$ compared to LPS 1 μg/mL; # represents $p \leq 0.05$ compared to lupeol 0.1 μM; + represents $p \leq 0.05$ compared to LPS 1 μg/mL + lupeol 0.1 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of TNF by LPS can activate apoptosis pathways, and mitochondrial deregulation can trigger mechanisms resulting in the production of apoptosis activators, this being one of the mechanisms of neurodegeneration induced by this toxin — which may infer anti-neuroinflammatory activity associated with the modulation of astroglial response [39,40]. While lupeol down-regulated the mRNA levels for this cytokine after the inflammatory stimulus to levels similar to those of control cultures, maybe this modulation is contributing to its neuroprotective activity. Another important factor involved in the neurodegeneration is the molecule NLRP3 component of inflammossome, which is involved in the production of proinflammatory cytokines, including IL-6, and in the apoptosis and pyroptosis pathways [41,42]. In cerebellar cultures, compared to the control cultures, LPS was able to induce an increase in about 10 times in mRNA levels for NLRP3, while

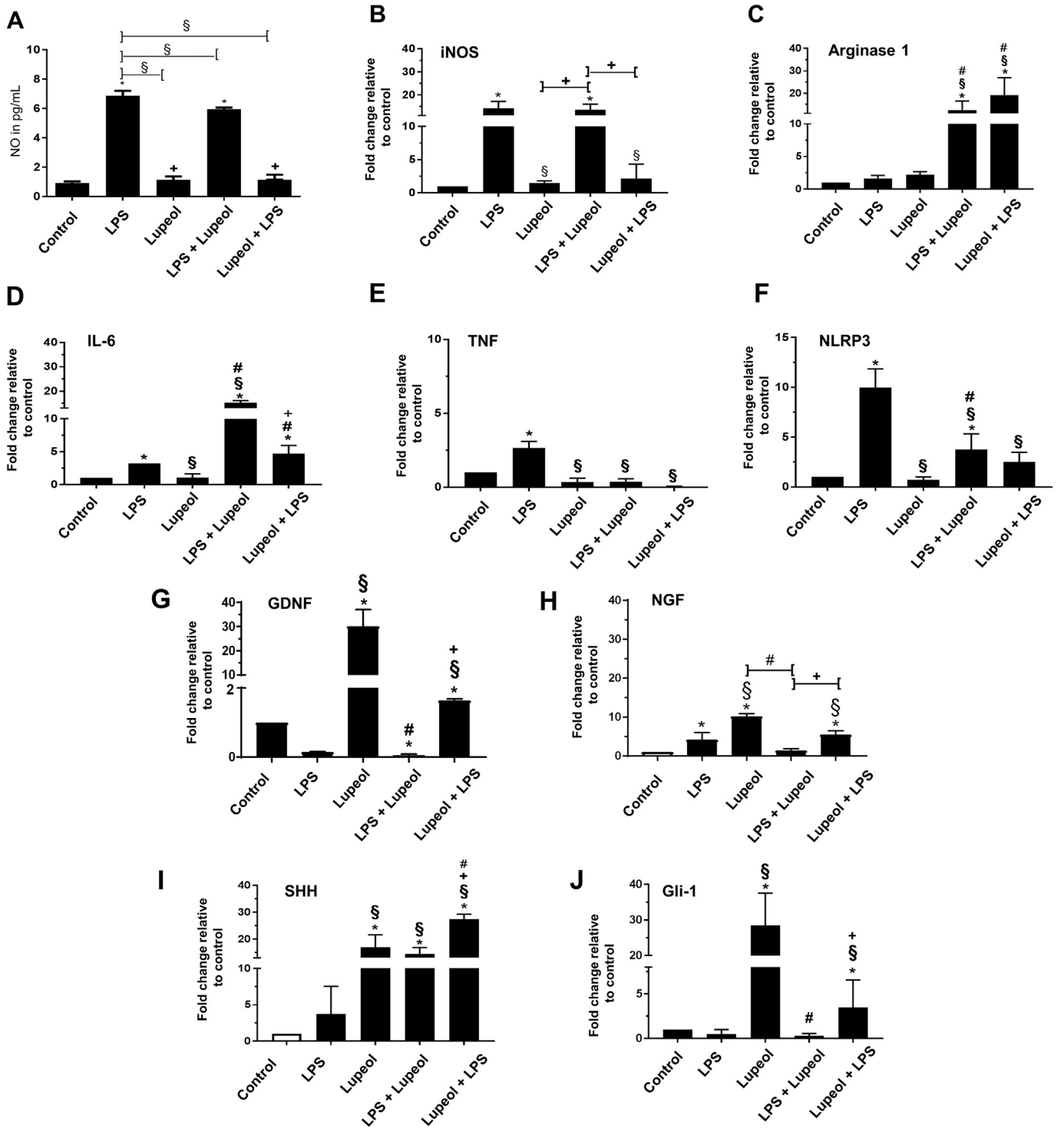

Fig. 6. Analysis of the effect of lupeol on the expression profile of neurotrophic factors and components of the immune response in cerebellar cultures submitted to an inflammatory stimulus. The cultures were treated with LPS (1 μg/mL), lupeol (0.1 μM), pretreated with LPS for 24 h and then treated with lupeol, or pretreated with lupeol for 24 h and then treated with LPS. (A) Levels of nitrite ($NaNO_2$), the stable form of nitric oxide (NO) in the medium of cultures based on the Griess reaction; results expressed media ± SD of three independent experiments. (B–J) Expression of mRNA for iNOS, arginase, TNF and IL-6 cytokines, NRLP3 inflamassome, GDNG and NGF growth factors, Sonic hedgehog (SHH) differentiation factor, and for the transcriptional factor activated by SHH GLI-1; results of 3 independent cultures were considered and analyzed with Kruskal-Wallis ANOVA followed by Dunn's post-test; * represents $p \leq 0.05$ compared to control; § represents $p \leq 0.05$ compared to LPS 1 μg/mL; # represents p ≤ 0.05 compared to lupeol 0.1 μM; + represents $p \leq 0.05$ compared to LPS 1 μg/mL + lupeol 0.1 μM.

lupeol was able to maintain these levels similar to those of control. Moreover, the association of lupeol with LPS induced a negative modulation of mRNA for NLRP3. It has already been shown that the levels of NLRP3 have an important relationship with astrocyte activation, mainly due to its ability to interfere with the production of reactive oxygen species, nitric oxide and the release of proinflammatory cytokines such as TNF [42–44]. There is evidence that lupeol reduced liver injury and the protein expression of TLR4, MyD88, IRAK-1 and TRAF6, and NF-κB nuclear translocation in LPS/D-GaIN-induced fulminant hepatic failure in mice [45]. Therefore, the anti-neuroinflammatory and

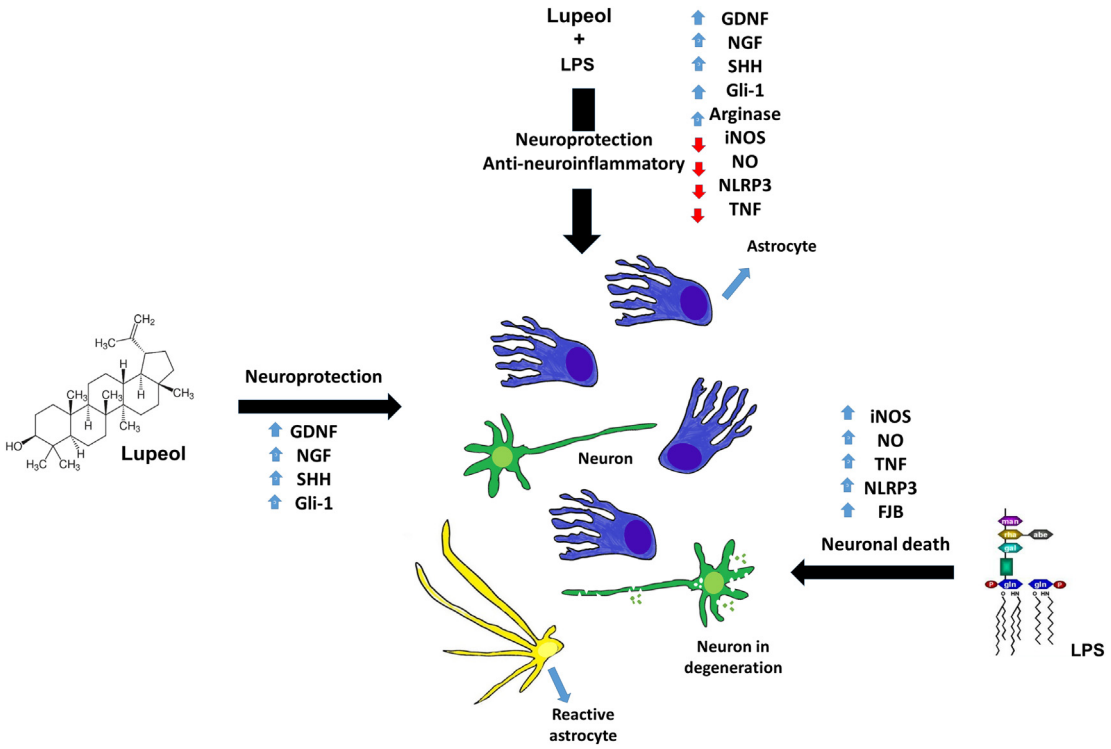

Fig. 7. Diagram demonstrates the first cell population with which it interacts with lupeol and, at last, represents the biological activities observed in this study that resulted in neuroprotection and improved neuronal viability. The black arrows indicate activities regulated by Lupeol or LPS, the blue arrows are the markers that were shown as high and the red arrows those who were negatively expressed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

neuroprotective activity by lupeol in cerebellar cells can be related to modulation of these molecules.

iNOS modulation by microglia and astrocytes in inflammatory processes, resulting in NO high production especially related to M1 and A1 phenotypes is responsible for generating neuronal damage [46–49]. In this study, it was observed that compared to the cerebellum cultures stimulated with inflammatory damages, cultures pretreated with lupeol and exposed to LPS showed lower levels of NO and mRNA expression for iNOS, as well as for TNF and inflammasome NLRP3 mRNA, and conversely, increased levels in mRNA expression for arginase and IL-6 regulatory factors, highlighting the anti-inflammatory potential of the triterpene. Lupeol is likely to be modifying the physiology of astrocytes, related to phenotypic changes and modulation of inflammatory factors [11,50–53]. Some studies show that arginase 1 can act on the change of activated astrocytes and microglia profiles for non-reactive and neuroprotective phenotypes [11]. On the other hand, an increase in IL-6 expression may be related to the control of astrocyte activation during neuroinflammation and production of neurotrophic factors. It has been shown that during neuroinflammatory damage, high levels of IL-6 are related to the prolongation of inflammation and increased production of GDNF by astrocytes [54,55]. On the other hand, it was demonstrated that the inhibition of IL-6 production by astrocytes induces lower GDNF production [56,57].

Neurotrophins are mainly responsible for the modulation of biochemical activities of the neuronal population [58,59]. Studies involving inflammatory induction by LPS in CNS cells have shown that the reduction in the neurotrophin levels as NGF (Neuronal Growth Factor), BDNF (Brain-derived Neurotrophic Factor) and GDNF are related to its neuroinflammatory and degenerative responses [60–63]. Increased mRNA levels for NGF and GDNF were observed in cultures treated with lupeol, suggesting that neuronal protection is related to an increase in these neurotrophins. It can also be associated with the modulation of astrocyte response, given that increased production of NGF and GDNF have been shown to be associated with neuroprotection [51,64].

SHH (Sonic Hedgehog protein), especially produced by astrocytes, plays an important role in neuronal maturation and differentiation during and after development, acting through the regulation of Gli-1 transcription factor [65]. In this study, lupeol induced a large increase in the mRNA expression of these two molecules, induction also observed after submission to inflammatory injury with LPS. Farmer et al. [30] highlighted the importance of the SHH pathway in the regulating population of astrocyte with velate phenotype (VA) in the cerebellum. Chechneva et al. [66] demonstrated that SHH pathway has a very important relationship with neuroprotection mediated by astrocytes, and that was confirmed recently by Farmer et al. [30] and Cheng et al. [67]. A relationship between SHH pathway and neuroprotection mediated by astrocytes has also been shown *in vitro* and *in vivo* studies after neurotoxic damages induced by kainic acid [65], and hemorrhagic damages [57]. PTCH2 protein, a SHH receptor, is highly expressed on the plasma membrane of VA and BG astrocytes [30] and can be activated by various mechanisms, one of them being hormone receptors linked to G-protein-coupled receptors (GPCRs) [68,69]. The triterpene lupeol has a chemical analogy with estrogen and other derivatives of cholesterol [15]. Thus, this drug is likely to be interacting with hormone membrane receptors like PTCH2, activating the SHH/Gli-1 pathway promoting astroglial modulation and neuroprotection.

5. Conclusions

Lupeol showed the ability to preserve the population of neurons in cerebellar cultures, promoting neuroprotection to the inflammatory damage induced with LPS, which is associated with a higher modulation of velar type astrocytic response, something not yet reported in the literature as a probable neuroprotection pathway in neuro-inflammatory models (See Fig. 7). Reduction of pro-inflammatory factors (NO, mRNA for: iNOS, TNF, NLRP3), suggesting a primary mechanism

of reversal of the neuroinflammatory process being performed by lupeol. An increase in immunoregulatory factors such as mRNA for IL-6 and arginase 1, which may be the modulation mechanism of morphologies and astrocytic response and increased neurotrophin mRNA (GDNF and NGF) and modulation of the SHH/Gli-1 signaling pathway, suggests that the modulation of these mechanisms may be related to the modulation of astrocytic morphologies and neuroprotection.

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