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ABSTRACT

Microglia cells are the immune effector in the Central Nervous System (CNS). However, studies have showed that they contribute more to glioma progression than to its elimination. Rutin and its aglycone quercetin are flavonoids present in many fruits as well as plants and have been demonstrated to bear anti-inflammatory, antioxidant and antitumor properties also to human glioblastoma cell lines. Previous studies also demonstrated that rutin, isolated from the Brazilian plant *Dimorphandra mollis* Bent., presents immunomodulatory effect on astrocytes and microglia. In this study, we investigate the antitumor and immunomodulatory properties of rutin and its aglycone quercetin on the viability of glioma cells alone and under direct and indirect interaction with microglia. Flavonoid treatment of rat C6 glioma cells induced inhibition of proliferation and migration, and also induced microglia chemotaxis that was associated to the up regulation of tumor necrosis factor (TNF) and the down regulation of Interleukin 10 (IL-10) at protein and mRNA expression levels, regulation of mRNA expression for chemokines CCL2, CCL5 and CX3CL1, and Heparin Binding Growth Factor (HDGF), Insulin-like growth factor (IGF) and Glial cell-derived neurotrophic factor (GDNF) growth factors. Treatment of human U251 and TG1 glioblastoma cells with both flavonoids also modulated negatively the expression of mRNA for IL-6 and IL-10 and positively the expression of mRNA for TNF characterizing changes to the immune regulatory profile. Treatment of microglia and C6 cells either in co-cultures or during indirect interaction, via conditioned media from glioma cells treated with flavonoids or via conditioned media from microglia treated with flavonoids reduced proliferation and migration of glioma cells. It also directed microglia towards an inflammatory profile with increased expression of mRNA for IL-1β, IL-6, IL-18 and decreased expression of mRNA for nitric oxide synthase 2 (NOS2) and prostaglandin-endoperoxide synthase 2 (PTGS2), arginase and transforming growth factor beta (TGF-β), as well as Insulin-like growth factor (IGF). Treatment of U251 cells with flavonoids also reduced tumorigenesis when the cells were xenotransplanted in rat brains, and directed microglia and also astrocytes in the microenvironment of tumor cell implantation as well as in the brain parenchyma to a not favorable molecular inflammatory profile to the glioma growth, as observed in cultures. Together these results demonstrate that the flavonoid rutin and its aglycone quercetin present antiglioma effects related to the property of modulating the microglial inflammatory profile and may be considered for molecular and preclinical studies as adjuvant molecules for treatment of gliomas.

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1. Introduction

Glioblastoma (GBM) is the most common type of glioma. The prognosis for GBM is most often poor. Rapid tumor progression and resistance to chemotherapy and radiotherapy are common in GBM (Hardee and Zagzag, 2012). It is characterized by hypercellular anaplastic glioma cells with marked mitotic activity, as well as the presence of necrosis and microvascular proliferation (Kleihues et al., 2002).

Microglia plays a leading role in the CNS immune response. However, in malignant gliomas, there is M2-polarization of microglia acquiring immunosuppressive and tumor-supportive properties. It occurs under the influence of tumor cytokines, such as Interleukin 10 (IL-10), transforming growth factor beta (TGF-β), and prostaglandin-endoperoxide synthase 2 (PTGS2), exhibiting reduced phagocytic activity (Gabrusiewicz et al., 2011; Borisov and Sakaeva, 2015). *In vitro* co-migration assay studies and the two-dimensional cell co-culture assay provide evidence for duality of glia-associated microglia having been used to show that microglial BV-2 cells migrate to C6 glioma cells and inhibit tumor growth during the initial stage of tumorigenesis (Gu et al., 2017).

Cytokines have been implicated in several stages of glioma progression, participating in tumor onset, growth, angiogenesis and aggressiveness. Interestingly, cytokines also have the ability to inhibit glioma growth upon specific regulation or interplay with other molecules (Christofides et al., 2015).

Rutin (quercetin-3-O-rutinoside) is found in some aliments, such as citrus fruits, onion, wine, grape and buckwheat (Oomah and Mazza, 1996, Thomson et al., 1999). It is also found in greater amounts in the pericarp of the fruits of trees such as the Brazilian *Dimorphandra Mollis* Bent, an important source of this flavonoid (Chaves and Usberti, 2003). The pharmacological properties of rutin, particularly the gastroprotective, hepatoprotective and anti-diabetic effects, have been demonstrated in several studies (Harbone, 1986). Furthermore, rutin has demonstrated pharmacological effects such as anti-inflammatory, antiglycation activities and reduction of anxiety (Hosseinzadeh and Nassiri-Asl, 2014, Hernandez-Leon et al., 2017). Recently in an *in vitro* study we demonstrated that rutin is not toxic to microglial cells and induces activation characterized by a CD150/CD206-positive M2 phenotype, which is associated to down regulation of tumor necrosis factor (TNF), IL-1β, IL-6, Inducible nitric oxide synthase (iNOS) and nitric oxide (NO). In contrast, it was also showed that rutin induces up regulation of IL-10 and arginase in microglial cells reinforcing its anti-inflammatory potential (Bispo da Silva et al., 2017). Rutin also exhibited antiglioma effect, reducing the viability of highly proliferative human glioblastoma multiform cells (GL-15), related to decreased levels of ERK1/ 2 phosphorylation (P-ERK1/2) and accumulation of cells in the G2 phase of the cell cycle. The antiglioma effect of rutin was also associated to the reduction in the secretion of pro-angiogenic factors TGFβ and vascular endothelial growth factor (VEGF) (Santos et al., 2011), inhibition of invasion and down regulation of matrix metalloproteinases (MMPs) activities and expression (Santos et al., 2015).

Quercetin is the aglycone form of a number of other flavonoid glycosides, such as rutin, and is widely distributed in the plant kingdom, with well-known antioxidant effect, including to neural cells (Formica and Regelson, 1995; Bao et al., 2017). In addition, quercetin has revealed an important role in the regulation of key elements in cell signal transduction pathways related to apoptotic cell death and in cell cycle progression (Zamin et al., 2009; Nna et al., 2017). Moreover, studies also demonstrated that quercetin can reduce growth of GL-15 human glioblastoma cells (Santos et al., 2015) and also inhibit viability and induce autophagy of U87 and U251 human glioblastoma cells in a dose-dependent manner (Bi et al., 2016).

Considering the interactions between microglia and glioma cells, favoring glioma growth and invasion, along with the sensibility of both cell types to the flavonoid rutin, and that nothing has been described about the rutin and quercetin effects of the cytokine profile involved in the microglial response to glioma cells and related to antitumor properties, we have underlined the present study to well characterize its antitumorigenic and immunomodulatory effects. In light of that, the present study investigated, using *in vitro* and *in vivo* models of direct and indirect interactions of cells, the potential of the flavonoid rutin and its aglycone quercetin to modulate microglia response and the impact on glioma cell viability, underlying inflammatory mechanisms related to antiglioma properties of both flavonoids (Fig. 1).

2. Material and methods

2.1. Cell lines and culture

Rat C6 glioma cells and human U251 glioblastoma cells were cultured until confluence in polystyrene plates (TPP, Trasadingen, Switzerland), as described previously by Santos et al. (2015) in Dulbecco's modified Eagle's medium (DMEM; Cultilab, Campinas, Brazil) supplemented with 100 UI/mL penicillin G, 100 mg/mL streptomycin, 7 mmol/L glucose, 2 mmol/l L-glutamine, 0.011 g/L pyruvic acid, and 10% fetal calf serum (FCS). TG1 cells derived from the human malignant tumor were cultured and maintained according to the protocol described by Patru et al. (2010). Cells were cultured in complete DMEM/F12 medium, supplemented with 1 mM L-glutamine, 25 mM glucose, 10 mM HEPES and in the presence of growth factors N2, G5 and B27 (Invitrogen). Cultures were maintained in a humidified atmosphere composed of 95% air and 5% CO_2 at 37 °C.

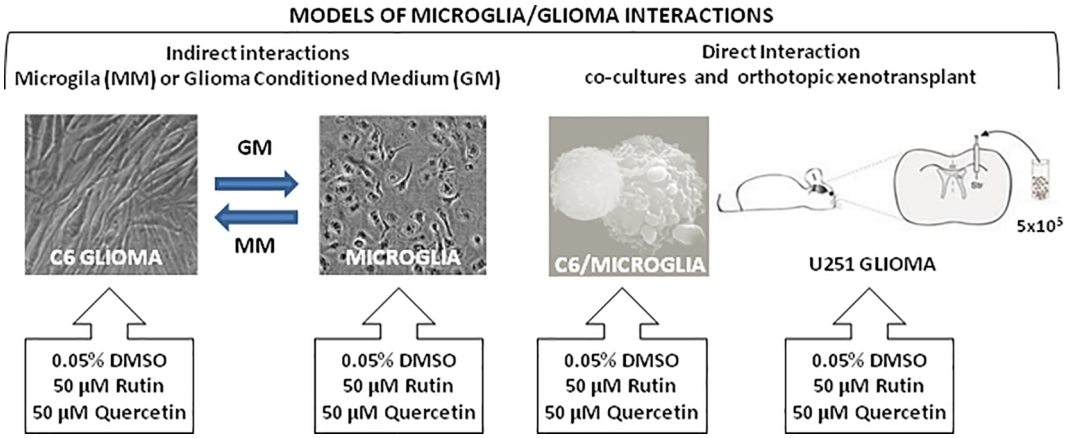

Fig. 1. Experimental design showing models adopted to investigate effects of flavonoid rutin and its aglycone quercetin during microglia/glioma direct and indirect interactions.

2.2. Microglia cultures

Microglial cells were obtained from the cortex of Wistar newborn rats (0–2 days old) from the Animal Facility of the Federal University of Bahia (Salvador, Brazil) and performed according to the Brazilian guidelines for production, maintenance and use of animals for teaching activities and scientific research and the local Ethical Committee for Animal Experimentation, protocol number (0272012, ICS - UFBA). Microglial isolation was performed according to the protocol established at Guaza's Laboratory at the Cajal Institute in Madrid (Mecha et al., 2011). In a nutshell, after decapitation, the fore brains of newborn Wistar rats were dissociated mechanically and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 10% Serum equine (HS), 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured on poly-D-lysine (25 μg/mL)-coated flasks. Upon reaching confluence (7–10 days), adherent microglial cells were harvested by shaking at 165 rpm at 37 °C for 3 h. Isolated microglia were seeded into 24- or 6-well plates at a density of $3 \times 10^4/cm^2$ and experiments were performed after 24 h. In all cases, the microglia cells were at 37 °C in 5% CO_2. In some conditions, microglial cells were cultured in the presence of glioma cells.

2.3. Cultures with direct and indirect interactions of microglia and glioma cells

For studies using direct interactions, co-cultures of microglia and C6 glioma cells were produced with a proportion of 2:1 (microglia:C6 cells) on 24- or 6-well plates at the density of 5×10^3 cells/cm^2. After 24 h, the culture medium was changed to fresh medium containing the respective treatment (Kostianovsky et al., 2008, Mecha et al., 2011).

For studies using indirect interactions, two experimental approaches were performed: treatment of C6 cells at the density of 5×10^3cells/cm^2 with conditioned medium derived from microglia cultures (MCM) or treatment of microglia at the density of 5×10^3cells/cm^2 with conditioned medium derived from C6 glioma cells (GCM). In both conditions, cells were treated with 50 μM rutin or quercetin for 24 h. After this time of exposition, the culture media (MCM or GCM) were collected, centrifuged at 1000 RPM for 10 min to deposit eventual cellular wastes and immediately used for treatment of C6 or microglia cultures.

2.4. Drugs and treatments

Rutin (R5143) and quercetin (Q4951) were purchased from Sigma-Aldrich. For treatments, they were dissolved in dimethylsulfoxide (DMSO, Sigma, USA) at a concentration of 100 mM and stored in the dark at −4 °C. Twenty-four hours after cell plating, the culture media were changed for fresh medium without serum. The flavonoids were added directly into the fresh media at a final concentration of 50 μM or equivalent volume of DMSO (0.05%) and analyzed after 24 or 48 h. Control cultures treated with DMSO (0.05%) showed no significant effect on the parameters analyzed compared to cells that did not receive the diluent. In some cases, cultures were treated for 24 h with 1 μg/mL LPS (Sigma Aldrich, USA) to induce an M1 pro-inflammatory microglial profile and were considered as positive control.

2.5. Migration assay

In order to evaluate whether flavonoids affect the migration of C6 glioma cells and during direct (co-cultures) or indirect interaction with microglia (via secretome from microglia cultures exposed to the flavonoids), a wound was created with a 200-μL pipette tip in the monolayer of C6 cells cultures or in C6/microglia co-cultures. Cultures were rinsed three times with DMEM to remove floating cells and cultured with serum-free fresh medium containing rutin or quercetin (50 μM), with MCM (indirect interaction) derived from microglia cultures treated with rutin or quercetin (50 μM) or in control conditions (0.05% DMSO). After 24 h and/or 48 h, the cultures were analyzed with phase-contrast microscopy using an optic phase microscope (Nikon TS-100; Nikon, Melville, New York, USA) and imaged using a digital camera (Nikon E-4300; Nikon). Three independent experiments were performed for each of the experimental variables. Quantification of migrated cells was analyzed using Image J (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA).

2.6. Immunocytochemistry

Morphological changes of C6 glioma cells were analyzed by immunocytochemistry for the cytoskeleton protein α-tubulin. Morphology and activation of microglia were analyzed by immunocytochemistry for the proteins Iba-1, a structural marker of microglia, and CD68, a marker of activate M1 pro-inflammatory profile. For the experiments, cells were seeded on glass coverslips previously sensitized with a solution of 0.1 mg/mL poly-L-ornithine hydrobromide (Sigma Aldrich P3655) in 24-well polystyrene plates. After experiments, cultures were rinsed three times with PBS and fixed in cold methanol at −20 °C for 10 min. Nonspecific antibody binding was blocked by pre-incubating the plates with 3% bovine serum albumin (BSA) in PBS. Cells were incubated with rabbit polyclonal primary antibody for α-tubulin (1:300 Sigma-Aldrich), rabbit polyclonal primary antibody for Iba-1 (1:300 Wako), mouse monoclonal antibody for (1:200, Caltag) or rat monoclonal antibody for CD68 (1:100, Bio-Rad) diluted in PBS with 1% BSA for 12 h at 4 °C under slow agitation. After that, the cells were washed three times in PBS and were incubated with secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400; Molecular Probes, A11008), Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400; Molecular Probes, A11001) or Alexa Fluor 555-conjugated goat anti-rat IgG (1:400; Molecular Probes, A21434). The control of antibody specificity was made with the incubation of control cultures only with secondary antibodies. Nuclear chromatin was stained with the fluorescent dye 4′6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Oregon, USA) at a concentration of 5 μg/mL for 10 min, at room temperature, in a dark chamber. Thereafter, cells were analyzed using an epifluorescence microscope (DCF7000T, Leica). Ten randomized fields were analyzed per each experimental condition. All assays were performed at least three times.

2.7. Bromodeoxyuridine cell proliferation assay

Proliferation was assessed using the bromodeoxyuridine (BrdU) incorporation assay in C6 cultures in control condition (0.05% DMSO) or exposed to rutin or quercetin (50 μM) for 24 h, in microglia cultures ($3 \times 10^4/cm^2$) exposed for 24 h to conditioned medium derived from C6 cultures treated with 50 μM rutin or with 50 μM quercetin (GRCM) or in control condition (GCCM). Before treatments, BrdU (10 μM, clone BU34, St. Louis, MO) was added to cultures. After the time of the experiment, the cells were fixed and the DNA was denatured by treatment with denaturing solution (2 N HCl) for 20 min at room temperature. Anti-BrdU monoclonal antibody produced in mice diluted in PBS (1: 200, Sigma Aldrich, Inc.) was added into the wells and incubated for 1 h. The unbound antibody was washed out and the cells were incubated with Alexa Fluor 488 specific anti-mouse antibody diluted in PBS (1:500, Invitrogen Corporation) for 1 h under slow stirring at room temperature. After incubation, the cell nuclei were stained with 4 ', 6-diamidino-2-phenylindole fluorescent DNA (DAPI) intercalating agent (Molecular Probes, Eugene, Oregon, USA) at a concentration of 5 μg/mL for 10 min at room temperature. All reagents were used according to the manufacturer's instructions. The experiments were performed in triplicate. The cultures were then observed and photographed under a fluorescence microscope (DCF7000T, Leica). Quantification was analyzed using Image J (Wayne Rasband, National Institutes of Health, USA).

2.8. Chemotaxis assays

For microglia chemotaxis assays, C6 cultures were treated with rutin or quercetin (50 μM) or maintained in control conditions (0.05% DMSO) for 24 h. Following, microglia (1×10^5 cells/well) were added to upper microchambers (8-μm diameter pores) separated by polycarbonate membrane (Millipore, 24-well, Millicell), precoated with fibronectin (20 μg/mL, Sigma-Aldrich) (Lee and Chung, 2009). 24 h after microglial were added to Millicell chambers, cells that did not migrate were removed from the upper side of the chamber with a swab and the polycarbonate membrane of each insert was removed. The microglia that migrated to the underside of the membrane were fixed with 4% PFA for 10 min and stained with 0.2% crystal violet (C0775, Sigma-Aldrich). The number of cells that migrated to the underside of the filter was counted in 10 random fields by two raters. The assay was performed in triplicate for each of the experimental conditions. The number of cells emigrated to the bottom of the filter was quantified, considering 10 random fields by two raters.

2.9. ELISA assay for cytokines

Cytokines IL-6, IL10 and TNF were measured in culture medium using ELISA (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, the samples of supernatants were placed in 96-well plates coated with monoclonal capture antibodies and incubated for 2 h. In order to measure TNF, the samples were first subjected to acid treatment to activate the latent form of this growth factor. After washing with PBS, a horseradish peroxidase-conjugated antibody was added to bind to the cytokines. After incubation and washing, a chromogenic substrate was added and the absorbance of each well was measured at 450 nm. The concentrations of IL-6, IL10 and TNF were determined by interpolating from standard curves obtained with known concentrations of standard protein.

2.10. Tumorigenesis assay

For the tumorigenesis assay, male Wistar rats aging about 3 months and weighing between 300 and 350 gs were divided into three groups of 6 animals: animals that received implants of U251 human glioblastoma cells in control conditions, exposed to 0.05% DMSO for 24 h; animals that received implants of U251 human glioblastoma cells treated with 50 μM rutin for 24 h; and animals that received implants of U251 human glioblastoma cells treated with 50 μM quercetin for 24 h. The animals were provided by the Animal Facilities of the School of Veterinary Medicine and were kept in cages with water and food *ad libitum*. This experiment was approved by the ethics committee for use of laboratory animals from the Institute of Health Sciences from the Federal University of Bahia (registration number is 027/2012).

For the surgery, the animals were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (25 mg/Kg), immobilized using stereotactic apparatus (Standard rat/mouse Stoelting TM). With the aid of the atlas for rats, the region of the *caudate putamem* was determined and, with a dental drill, a small hole was opened in the cranial box for the injection of the tumor cells. This procedure was carefully performed to avoid perforation of the meninges or cerebral cortex. For orthotopic xenotransplants, U-251 cells under control conditions or treated with flavonoids rutin or quercetin (50 μM) for 24 h were removed from the culture plates with 0.2% trypsin/0.02% EDTA solution in PBS. After centrifugation, the cells were resuspended in DMEM culture medium without supplements, the viable cells were quantified by trypan blue staining and the medium volume was adjusted to 100,000 viable cells/μL. At the time of injection, the entire cell suspension was aspirated under constant stirring in a Hamilton syringe previously attached to the stereotaxic apparatus and 500,000 cells in a 5-μL volume were injected. At the end of the surgery, the animals were placed in individualized cages and monitored daily.

Thirty days after tumor cell injection, rats were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for perfusion-fixation. Brains were dissected, post-fixed in cold 4% PFA for 24 h and stored at −4 °C before processing.

2.11. Immunohistochemistry

In order to perform the immunohistochemical reactions, after perfusion and fixation of the brains, they were immersed in a solution of 30% sucrose for 3 days. Afterwards, 25-μm slices of the whole brain were cut with the aid of a cryostat (SLEE MAIZ Cryostate MCT) at a temperature of − 20 °C and collected on superfrost glass slides (Z692255, Sigma). The tissues were washed 3 times with PBS and then continued with the incubation of 0.3% PBS–Triton X-100 for 20 mi, blocking the non-specific binding with a 5% NGS solution for 1 h. After blocking, the sections were exposed directly to the primary antibodies: polyclonal antibodies (IgG) specific for GFAP protein produced in rabbit BS (1: 200, Z0334, DAKO Cytomation), monoclonal antibodies (IgG) specific for CD68 protein produced in rats (1:100, Invitrogen) or polyclonal antibodies (IgG) specific for Iba-1 protein produced in rabbit (1:100, WAKO) diluted in PBS and incubated overnight in a humid chamber at 4° C. After this procedure, the sections were washed three times in PBS for five min and then exposed to secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400; Molecular Probes, A11008), Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400; Molecular Probes, A11001) or Alexa Fluor 555-conjugated goat anti-rat IgG (1:400; Molecular Probes, A21434) diluted in PBS for 2 h. The slices were then washed three times in the PBS solution and nucleus were stained with the fluorescent DNA intercalating agent DAPI (Molecular Probes, Eugene, Oregon, USA) at a concentration of 5 μg/mL, for 10 min, at room temperature. The sections were then washed with PBS and the slides mounted with Fluoromount (F4680, Sigma-Aldrich). The slices were analyzed through fluorescence microscopy (DCF7000T, Leica) and 10 images were photographed per treatment.

2.12. RNA isolation and cDNA synthesis

Total RNA was isolated from cultures of U251 and TG1 human glioma cells, C6 rat glioma cells, rat microglia and C6/microglia co-culture and from rat brain area with U251 cell implantation and its contra-lateral area with Trizol® reagent according to the manufacturer's specifications. For experiments in cultures, three 60-mm plates for each experimental point containing 1×10^4 cells/cm^2 were treated for 24 h with 1 μg/ml LPS (positive control), flavonoids rutin or quercetin (50 μM) or maintained in control condition. For experiments with brain tissues, 1 mm^3 cuts from the implantation area and from the respective contra-lateral area of three animals from each group were used. The samples were stored at −80 °C with 1 mL Trizol® reagent and RNA was extracted according to the manufactureŕs instructions. The concentration and purity of RNA were determined by spectrophotometric analysis using a nanospectrum Kasvi (K23-0002). DNA contaminants were removed by treating the RNA samples with DNase using the Ambion DNA-free kit (cat # AM1906, Life Technologies™). For cDNA synthesis, Super Script® VILO™ Master Mix was used (catalogue # MAN0004286, Invitrogen™, Life Technologies) in a 10-μL reaction with concentration of 2.5 μg of total RNA.

2.13. 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 (catalogue # 4440038 Invitrogen, Life Technologies™). The assays corresponding to the genes from rats (Rn) quantified in this study were: IL-6 (Rn01410330_m1), IL-1β (Rn00580432_m1), IL-18 (Rn01422083_m1), arginase (ARG)

(Rn00691090_m1), TGFβ (Rn00572010_m1), PTGS2 (Rn01483828_m1), NOS2 (Rn00561646_m1), Hepatoma-Derived Growth Factor (HDGF) (Rn00678055_g1), Insulin-like Growth Factor-1 (IGF1) (Rn00710306_m1), CCL5 (Rn00579590_m1), CCL2 (Rn00580555_m1) and CX3CL1 (Rn00593186_m1). The assays corresponding to the genes from humans (Hs) quantified in this study were: IL-2 (Hs00174411_m1), IL-4 (Hs00174122_m1), IL-6 (Hs00985639_m1), IL-8 (Hs00174103_m1), IL-10 (Hs00961622_m1), NOS2 (Hs01675529_m1), PTGS2 (Hs00153132_m1) and TNF (Hs01113624_g1). 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. The β-actin (ACTB) (Rn00667869_m1 and Hs9999902_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Rn01527840_m1 and Hs02800695_m1) targets were used as reference genes (endogenous controls) for normalization of gene expression data. Data were analyzed using the ΔΔCt method. The results from at least three independent experiments performed in different plates of each cDNA using different cultures were analyzed and averaged to obtain a single average value for each mRNA.

2.14. Statistical analysis

The results are expressed as the mean ± SEM. One-way analysis of variance, followed by Student–Newman–Keuls test was used to determine the significant differences among groups differing in only one parameter. Student's *t*-test was used to compare two groups. Values of $P < 0.05$ were considered significant. All analyses were performed with three independent experiments carried out with triplicate wells.

3. Results

3.1. Flavonoids rutin and quercetin inhibit glioma cell proliferation and migration and modulate inflammatory and growth factors.

Previous studies demonstrated that the flavonoids rutin and its aglycone quercetin induced, at concentrations between 30 and 100 μM, a reduction in the viability and proliferation of GL-15, U251 and TG1 human glioblastoma cells (Santos et al., 2011; Santos et al., 2015). Exposure of GL-15 cells to 50 μM rutin also inhibited cell migration and production of proangiogenic growth factors TGF and VEFG (Freitas et al., 2011). Moreover, previous studies also demonstrated that flavonoid rutin has the capacity to modulate astrocyte and microglia immune response (Silva et al., 2008; Bispo da Silva et al., 2017). Hence, in the present study, we adopted the concentration of 50 μM to analyze microglia response and the impact on glioma cell viability of both the flavonoids rutin and quercetin. For this, we firstly characterized the direct effect of both flavonoids (at 50 μM) on the proliferation and migration of C6 glioma cells. The BrdU incorporation assay was performed 24 h after treatments and showed that compared to control cultures (0.05% DMSO), considered as 100%, the proportion of BrdU positive (BrdU+) C6 cells (that proliferated) was reduced to 24.81% and to 19.75% in cultures exposed to rutin or to quercetin, respectively (Fig. 2 A and C). Immunocytochemistry (ICQ) for the cytoskeleton protein α-tubulin also showed a significant reduction in the proportion of the remaining adherent C6 cells after the flavonoid treatment and cells presented a thinner bipolar phenotype compared to control cultures (Fig. 2B and D). Moreover, in migration assays under control conditions, 24 h after the start of the experiment, it was possible to visualize C6 cells entering the wounded area and almost complete closure of the lesion after 48 h (Fig. 2D–E). On the other hand, treatment of C6 cultures with rutin or quercetin inhibited the migratory property of still viable C6 cells since 24 h after treatments. The proportion of cells that migrated to the wounded area in cultures exposed to rutin was reduced to 24 h and 48 h after the treatment, respectively. In cultures exposed to quercetin, the proportion of cells that migrated to the wounded area was reduced 24 h and 48 h after the treatment, respectively.

In order to investigate whether inflammatory factors are modulated by rutin and quercetin, the levels of cytokine TNF, IL-6 and IL-10 were measured in the medium of C6 cultures 24 h after treatments (Fig. 2G). A predominant regulatory profile was revealed under control conditions with high levels of IL-10 (1.495 pg/mL) compared to TNF levels (310 pg/mL) and IL-6 levels (120 pg/mL). However, 24 h after the treatment with rutin or quercetin, the levels of IL-10 were shown to have decreased significantly. On the other hand, the levels of TNF increased 24 h after the treatment with rutin or quercetin. Moreover, the levels of IL-6 were significantly decreased 24 h after the treatment with rutin.

In order to better characterize the molecular inflammatory profile of glioma cultures that may impact upon microglia response, qRT-qPCR was performed to determine the expression of the mRNA for TNF, IL-1β, IL-6, TGF-β, IL-10, ARG, CCL2, CCL5, CX3CL1, HDGF, IGF and GDNF genes by RT-qPCR 24 h after the treatments (Fig. 3 A and B). When compared to the control cultures (0.05% DMSO), the levels of mRNA expression for TNF and IL1-β and for chemokines CCL2, CCL5 and CX3CL1 increased significantly in C6 cells. Levels of mRNA expression for TGF-β, IL-10, ARG, HDGF, IGF and for GDNF decreased significantly in the treatment with both flavonoids. On the other hand, mRNA expression for Interleukin IL-6 was only significantly increased after treatment with the flavonoid quercetin. LPS (1 μg/mL) treatment, adopted as pro-inflammatory control, induced a significant increase in expression levels of cytokines and chemokines. The capacity of flavonoids to modulate the molecular inflammatory profile of U251 and TG1 human glioblastoma cells was also investigated by means of RT-qPCR for the key inflammatory markers TNF, IL-6 and IL-10 (Fig. 3C and D). In both cell lines, rutin and quercetin induced a decrease in mRNA expression for regulatory cytokine IL-10 and an increase in mRNA expression for the inflammatory cytokine TNF. Expression of mRNA for IL-6 was also reduced by the treatment with rutin in both U251 and TG1 cells, but presented increased expression by TG1 cells after quercetin treatment.

3.2. Treatment of C6 glioma cells with flavonoids rutin and quercetin induces microglia chemotaxis and activation

Studies show that the interaction between microglia and glioblastoma triggers a regulatory immune response responsible for the aggression and invasion of glioblastoma. Moreover, we have recently characterized that the flavonoid rutin (50 μM) can induce the activation and modulate inflammatory profile of isolated microglia *in vitro* (Bispo da Silva et al., 2017). Aiming to investigate whether the treatment with the flavonoids rutin or quercetin interferes with the microglial inflammatory response, chemotaxis assays were initially performed in a Transwell system (Fig. 4A and B). For so, after 24 h of treatment of C6 cultures with the flavonoids, microglia were added to the upper chamber of the Transwell system and the migration to glioma cultures was evaluated 24 h after that. A significant increase was observed in the proportion of microglia that reached the lower chamber of cultures treated with flavonoids, when compared to control cultures. The flavonoid rutin presented greater chemotactic effect. As expected, cultures supplemented with FBS (positive control) induced significant chemotaxis for microglia.

Aiming to better characterize the response of microglia to the treatment of C6 glioma cells with flavonoids, we performed indirect interaction tests when cultures of microglia were exposed to glioma-conditioned medium (GM) generated 24 h after the treatment with the flavonoids. The morphology of the microglia was characterized by immunocytochemistry for Iba-1+ protein, which allows the visualization of morphology. Proliferation was investigated by BrdU incorporation and immunocytochemistry. In cultures exposed to C6-conditioned medium in control conditions (GMDMSO), the majority of

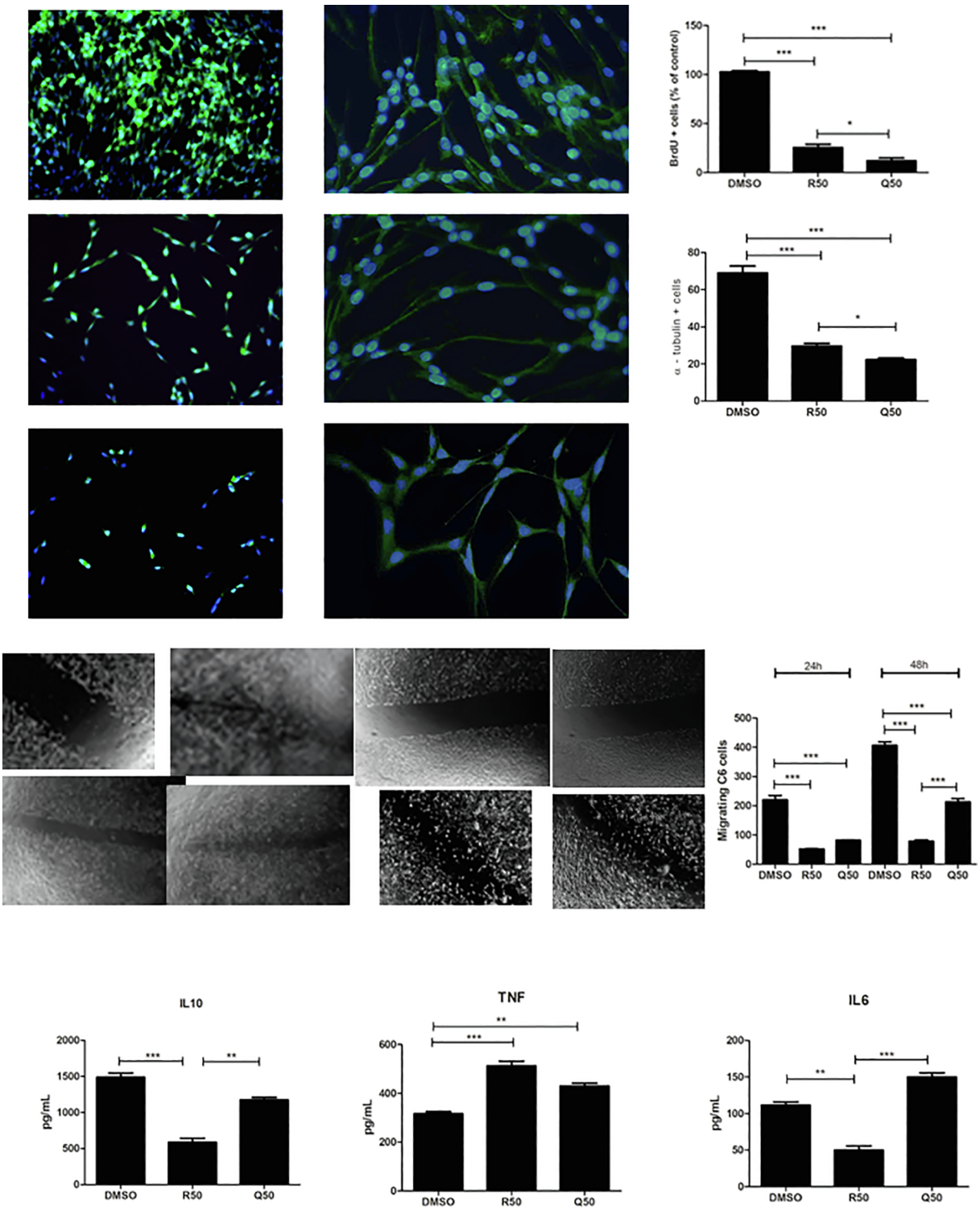

Fig. 2. Effects of the flavonoids rutin and quercetin on the proliferation, migration and inflammatory profile of C6 glioma cells. Cultures were maintained in control conditions (0.05% DMSO) or treated with 50 μM rutin (R50) or 50 μM quercetin (Q50) for 24 h. (A) Analysis of C6 cell proliferation by BrdU incorporation and immunocytochemistry; proliferating BrdU+ cells are stained in green; the nuclear chromatin was stained with DAPI (blue); LPS (1 μg/mL) was adopted as positive control; 20× magnification, scale bars: 50 μm. (B) Analysis of C6 glioma cell morphology by immunocytochemistry for cytoskeleton protein α-tubulin (green); the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm. (C) Bar graph showing the quantification of BrdU positive (BrdU+) adherent C6 cells; results from three independent experiments expressed as mean ± SEM, control cultures considered as 100%; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (D) Bar graph showing the quantification of α-tubulin positive (α-Tub+) adherent C6 cells; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (E) Migration of C6 cells in cultures in the wound assay analyzed with phase-contrast microscopy. Representative photomicrographs of the wounded area at time zero (control) and in different conditions 24 h and 48 h after conditions; 10× magnification, scale bars: 50 μm. (F) Bar graph showing the quantification of cells that migrated to the wounded area 24 h and 48 h after treatments; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (G) Levels of cytokines IL-10, TNF and IL-6 released in the culture medium (pg/mL) measured by sandwich ELISA 24 h after treatment of C6 cultures; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (** $p < 0.01$) (*** $p < 0.001$), one-way ANOVA.

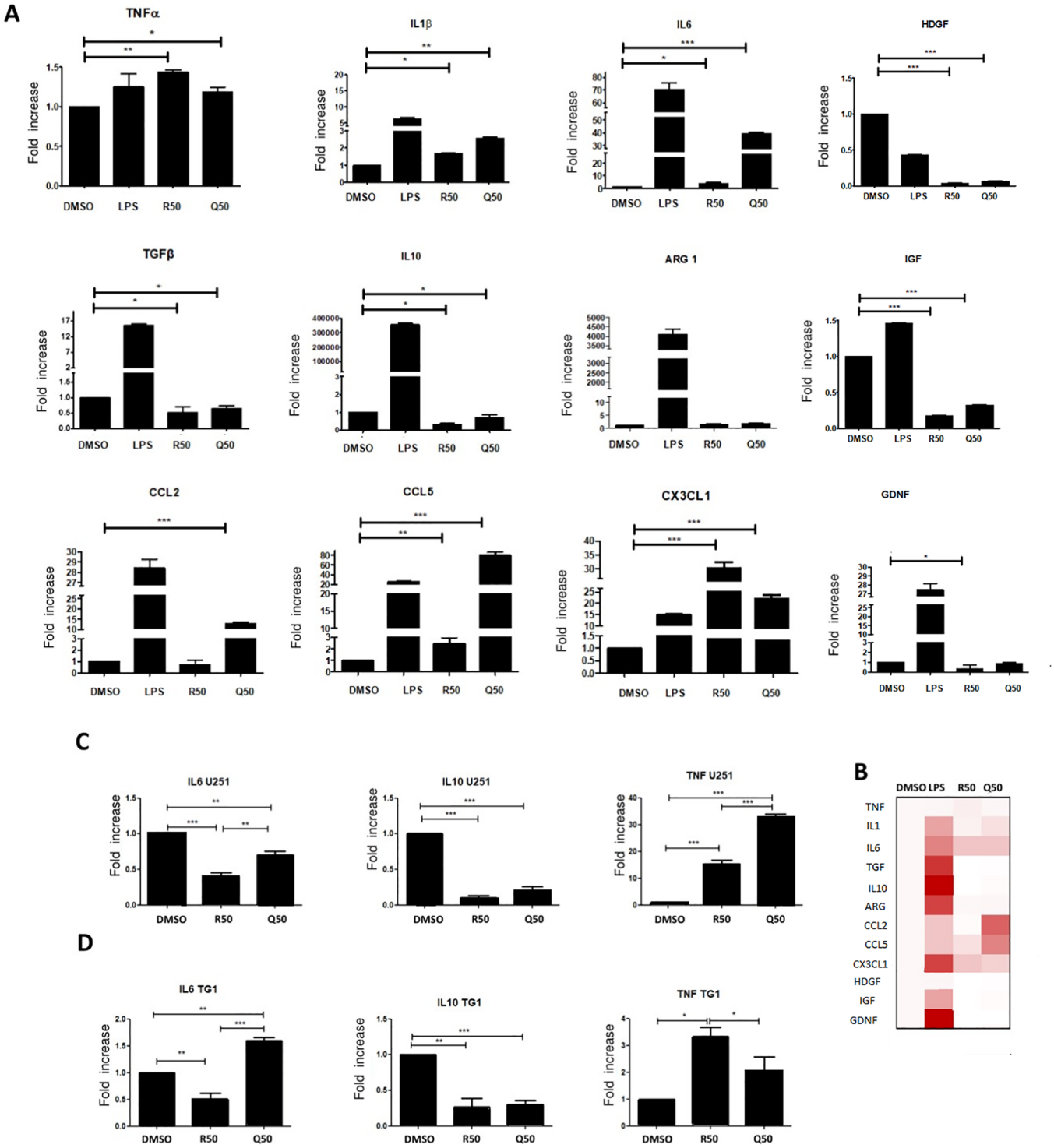

Fig. 3. Effects of flavonoids rutin and quercetin on molecular inflammatory profile of murine and human glioma cells. Cultures were maintained in control conditions (0.05% DMSO) or treated with 50 μM rutin (R50) or 50 μM quercetin (Q50) for 24 h. (A) Real-time qPCR analysis 24 h after treatment of C6 cells with flavonoids or with LPS (1 μg/mL) adopted as positive control, showing the expression of M1 (TNF, IL-1β and IL-6) and M2 (TGFβ, IL-10 and Arg-1) markers, chemokines (CCL2, CCL5 and CXCL3) and growth factors (HDGF, IGF and GDNF). (B) Hot-spot graph shows genes that were up or down regulated according to red color intensity related to control cultures; (C) Real-time qPCR analysis 24 h after treatment of U251 human glioblastoma cells showing the expression of M1 (TNF and IL-6) and M2 (IL-10) markers. (D) Real-time qPCR analysis 24 h after treatment of TG-1 human glioblastoma cells showing the expression of M1 (TNF and IL-6) and M2 (IL-10) markers; Results expressed as mean ± SEM of fold increase in expression of genes related to the expression in control cultures in three independent experiments; (* $P < 0.05$) (** $p < 0.01$) (*** $p < 0.001$), one-way ANOVA.

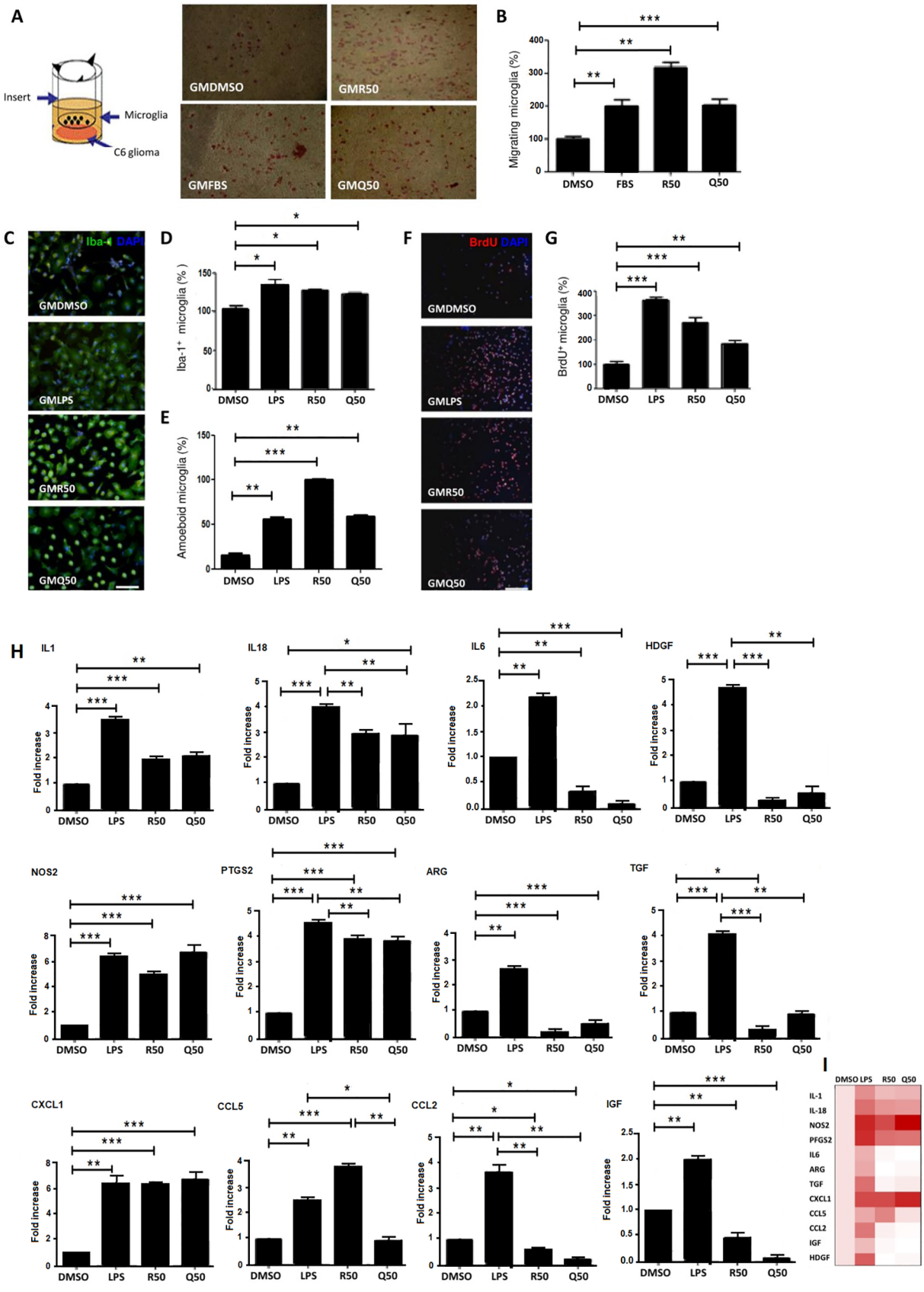

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Fig. 4. Effects of the conditioned medium derived from C6 glioma cells (GM) after the treatment with the flavonoids rutin and quercetin on microglia response. Microglia were exposed to the medium from C6 cultures treated for 24 h with 50 μM rutin (GMR50), 50 μM quercetin (GMQ50), or 0.05% DMSO (GMDMSO). (A) Chemotaxia assay was performed 24 h after treatment of C6 cells. Treatment of C6 cultures with fetal bovine serum (5% FBS) was adopted as positive control. Twenty four (24) h after treatments, microglia were added to the Transwell System and analyzed after further 24 h. Microglia that migrated across the membrane in the Transwell System were stained with violet crystal and counted; 10× magnification. (B) Bar graph showing the quantification of microglia that migrated; results from three independent experiments expressed as mean ± SEM, control cultures considered as 100%; (** $P < 0.01$) (*** $p < 0.001$), one-way ANOVA. e control; (* $P < 0.05$) (** $p < 0.01$) (*** $p < 0.001$), one way ANOVA. (C) Analysis of microglia morphology by immunocytochemistry for protein Iba-1 (green); the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm. The medium from C6 cultures treated with LPS (1 μg/mL) was adopted as positive control. (D) Bar graph showing the quantification of Iba-1 positive (Iba-1+) microglia; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (E) Bar graph showing the quantification of amoeboid microglia; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (F) Analysis of microglia proliferation by BrdU incorporation and immunocytochemistry; proliferating BrdU+ cells are stained in green; the nuclear chromatin was stained with DAPI (blue); 20× magnification, scale bars: 50 μm. The medium from C6 cultures treated with LPS (1 μg/mL) was adopted as positive control. (G) Bar graph showing the quantification of BrdU positive (BrdU+) microglia; results from three independent experiments expressed as mean ± SEM, control cultures considered as 100%; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (H) Real-time qPCR analysis showing the relative expression of M1 (IL-1β, IL-6, IL-18, NOS2 and PTGS2) and M2 (TGFβ and Arg-1) markers, chemokines (CCL2, CCL5 and CX3CL1) and growth factors (IGF, HDGF). (I) Hot-spot graph shows genes that were up or down regulated according to red color intensity related to control cultures. The medium from C6 cultures treated with LPS (1 μg/mL) was adopted as positive control; (* $P < 0.05$) (**$p < 0.01$) (***$p < 0.001$), one-way ANOVA.

microglia (Iba-1+) presented polygonal phenotype and some cells with amoeboid phenotype (Fig. 4C–E). However, cultures exposed to MCG derived from glioma cultures treated with rutin (GMR50) or quercetin (GMQ50) showed a significant increase in the proportion of microglia with contracted cytoplasm and amoeboid phenotype, suggesting proliferation. The quantification of the proportion of microglia (Iba-1+) with ameboid phenotype was 97.74% and 58.28%, respectively, in cultures exposed to GMR50 and GMQ50, respectively, when compared to control cultures. In cultures exposed to LPS, a classical inductor of M1-activated phenotype, a significant increase in the density of cells was observed with predominance of cells with amoeboid to round cytoplasm.

The induction of microglia proliferation after treatment with glioma-derived conditioned medium was confirmed by BrdU incorporation and immunocytochemistry. The proportion of $BrdU^+$ microglia (in proliferation) was about 278.18% and 198.8% in cultures exposed to GMR50 and GMQ50, respectively (Fig. 4E–F). As expected in cultures treated with LPS, adopted as a pro-inflammatory positive control, the proportion of proliferating microglia was almost tripled (344.84%).

The inflammatory profile of microglia exposed to conditioned medium of C6 glioma cells previously treated with flavonoids was also determined by RT-qPCR 24 h after culture. It was observed that relative mRNA expression of IL-1β, IL-18, CX3CL1, CCL5, CCL2, PTGS2 and NOS2 increased in microglia cultures treated with conditioned medium of glioma cells treated with rutin (GMR50) or quercetin (MGQ50). On the other hand, under the same conditions, the levels of arginase, HDGF, TGF-β, IL-6 and IGF relative mRNA expression were decreased.

Together, these results indicate that exposure of C6 glioma cells to the flavonoids rutin or quercetin indirectly induces through the secretion of soluble factors the activation and proliferation of microglia.

3.3. Flavonoids rutin or quercetin induce microglia activation and indirectly inhibit glioma cell migration

Aiming to investigate whether microglia response directly modulated by flavonoids rutin or quercetin interferes with glioma viability, we firstly characterized the morphology and inflammatory profile of isolated microglia exposed to the flavonoids and then investigated the effects of microglia-conditioned medium in the property of C6 glioma cells to migrate (Fig. 5). Microscopy and Iba-1 immunostaining showed characteristic morphology of microglia in control cultures that was changed after flavonoid treatments. In control conditions, microglia presented a rounder phenotype; after rutin treatment, more than 50% of cells acquired a branched, multipolar phenotype and the others acquired the amoeboid phenotype, both indicating activation (Fig. 5A–C). However, after treatment with quercetin, most cells acquired the amoeboid phenotype.

Characterization of inflammatory profile by ELISA showed that microglia cultures under control conditions present a predominant regulatory profile with high levels of IL-10 (about 189 pg/mL) compared to TNF levels (18 pg/mL) in the culture medium. However, the 24-hour treatment with rutin induced a significant increase in the levels of TNF and IL-10. Treatment of microglia with quercetin induced a significant increase in the levels of TNF and a decrease in levels of IL-10 in the culture medium compared to control cultures, indicating that both flavonoids can modulate the microglia inflammatory profile (Fig. 5D).

Moreover, the effect of conditioned medium from microglia cultures (MM) previously treated with the flavonoids (indirect contact) on the migration of C6 glioma cells submitted to a monolayer lesion assay was investigated. Under this indirect contact, in control conditions (MMDMSO), it was possible to visualize C6 cell reaching the wounded area that was almost completely closed after the 48-hour treatment (Fig. 5E–F). On the other hand, the migration of glioma cells was almost abolished in cultures exposed to the medium of microglia cultures pretreated with 50 μM rutin (MCR50) or with 50 μM quercetin (MCQ50), 24 h after exposures.

3.4. Flavonoids rutin and quercetin modulate microglia inflammatory profile and cell migration during microglia/glioma direct interaction in co-cultures

Aiming to investigate whether the treatment with the flavonoids rutin and quercetin interfere with the microglia activation during direct interaction with glioma cells, ICQ was initially performed for Iba-1 and for CD-68, a marker of activated microglia in M1 phenotype, in microglia/C6 co-cultures (Fig. 6A–C). The inflammatory stimulation with LPS (1 μg/mL) was adopted as positive control. In control cultures exposed to the vehicle DMSO (0.05%), the proportion of microglia (Iba-1+ cells) 24 h after the experiment was about 100%. However, in microglia/glioma co-cultures exposed to rutin or quercetin, a reduction was observed on the cellularity and the proportion of microglia (Iba-1+ cells) was increased to 75.15% and to 68.42%, respectively. In control conditions, microglia co-cultured with C6 cells presented a rounder phenotype. However, after the rutin or quercetin treatment, some cells acquired a more branched phenotype or amoeboid with enlarged cytoplasm phenotype. Moreover, in comparison to the control cultures, the proportion of $Iba\text{-}1^+/CD68^+$ activated microglia in the co-cultures increased by 20.32% after treatment with rutin, but was not significantly affected after treatment with quercetin. As expected, the proportion of $Iba\text{-}1^+/CD68^+$ microglia in the co-cultures increased by 50.62% after treatment with LPS.

In order to evaluate the effect of the flavonoids rutin and quercetin on the expression of genes associated to inflammatory response during direct interaction of microglia with glioma cells, the expression of

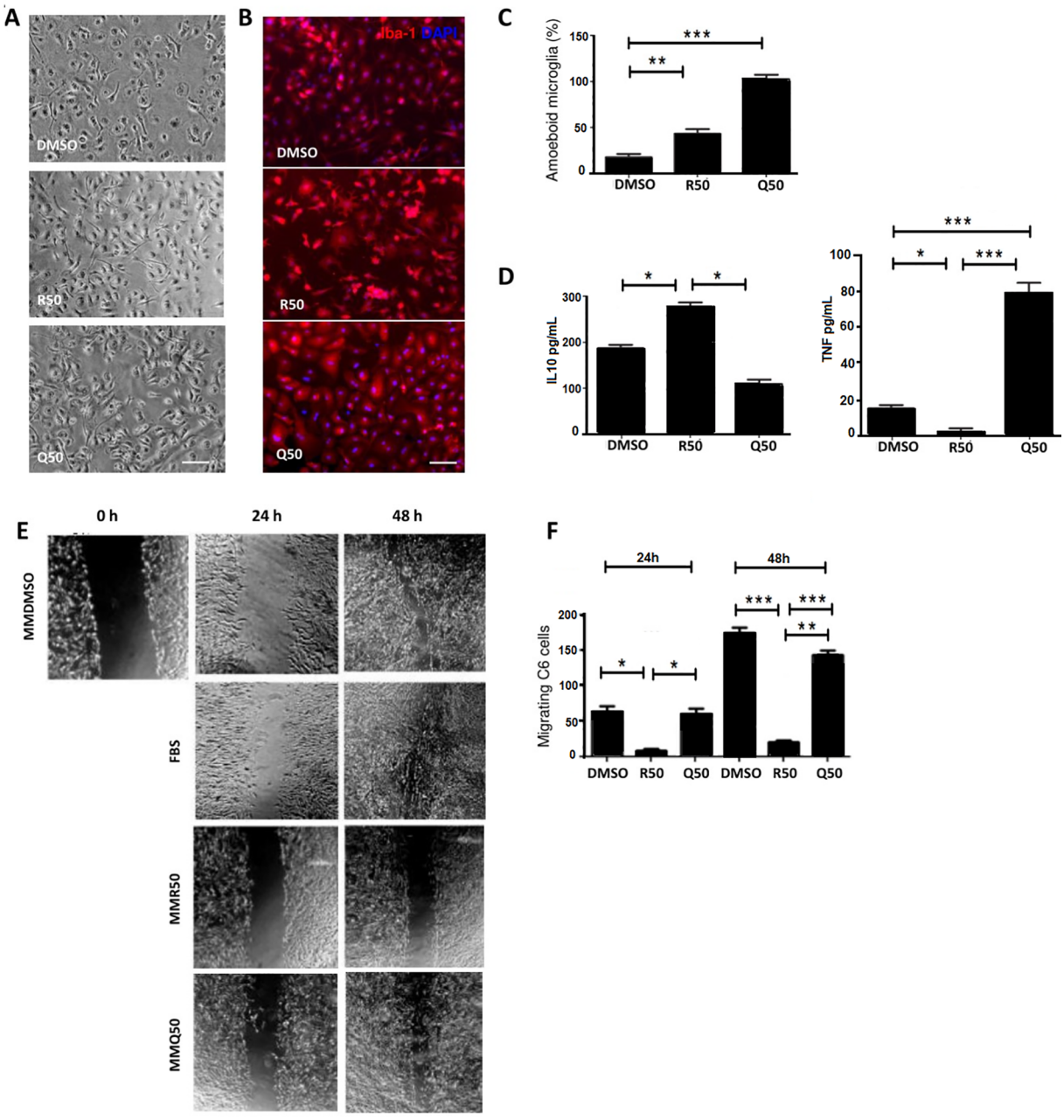

Fig. 5. Effects of flavonoids rutin and quercetin on the inflammatory profile of microglia and on the migration of C6 glioma cells. Microglia were maintained in control conditions (0.05% DMSO) or treated with 50 μM rutin (R50) or 50 μM quercetin (Q50) for 24 h. C6 cultures were treated with the conditioned medium derived from microglia cells (MM) treated for 24 h with 50 μM rutin (MMR50), 50 μM quercetin (MMQ50), or 0.05% DMSO (MMDMSO). Treatment of C6 cultures with fetal bovine serum (5% FBS) was adopted as positive control. (A) Representative photomicrographs of microglia cultures by phase-contrast microscopy; 20× magnification, scale bars: 50 μm. (B) Analysis of microglia morphology by immunocytochemistry for protein Iba-1 (red); the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm; (C) Bar graph showing the proportion of amoeboid microglia; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA. (D) Levels in the culture medium of cytokines IL-10 and TNF (pg/mL) measured by sandwich ELISA; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (** $p < 0.01$) (*** $p < 0.001$), one-way ANOVA. (E) Migration of C6 cells in cultures in the wound assay analyzed with phase-contrast microscopy exposed to conditioned medium of microglia cultures in control condition or exposed to flavonoids as described above. Representative photomicrographs of the wounded area at time zero (control) and in different conditions 24 h and 48 h after treatments; 10× magnification, scale bars: 50 μm. (F) Bar graph showing the quantification of cells that migrated to the wounded area 24 h and 48 h after treatments; results from three independent experiments expressed as mean ± SEM; (* $P < 0.05$) (*** $p < 0.001$), one-way ANOVA.

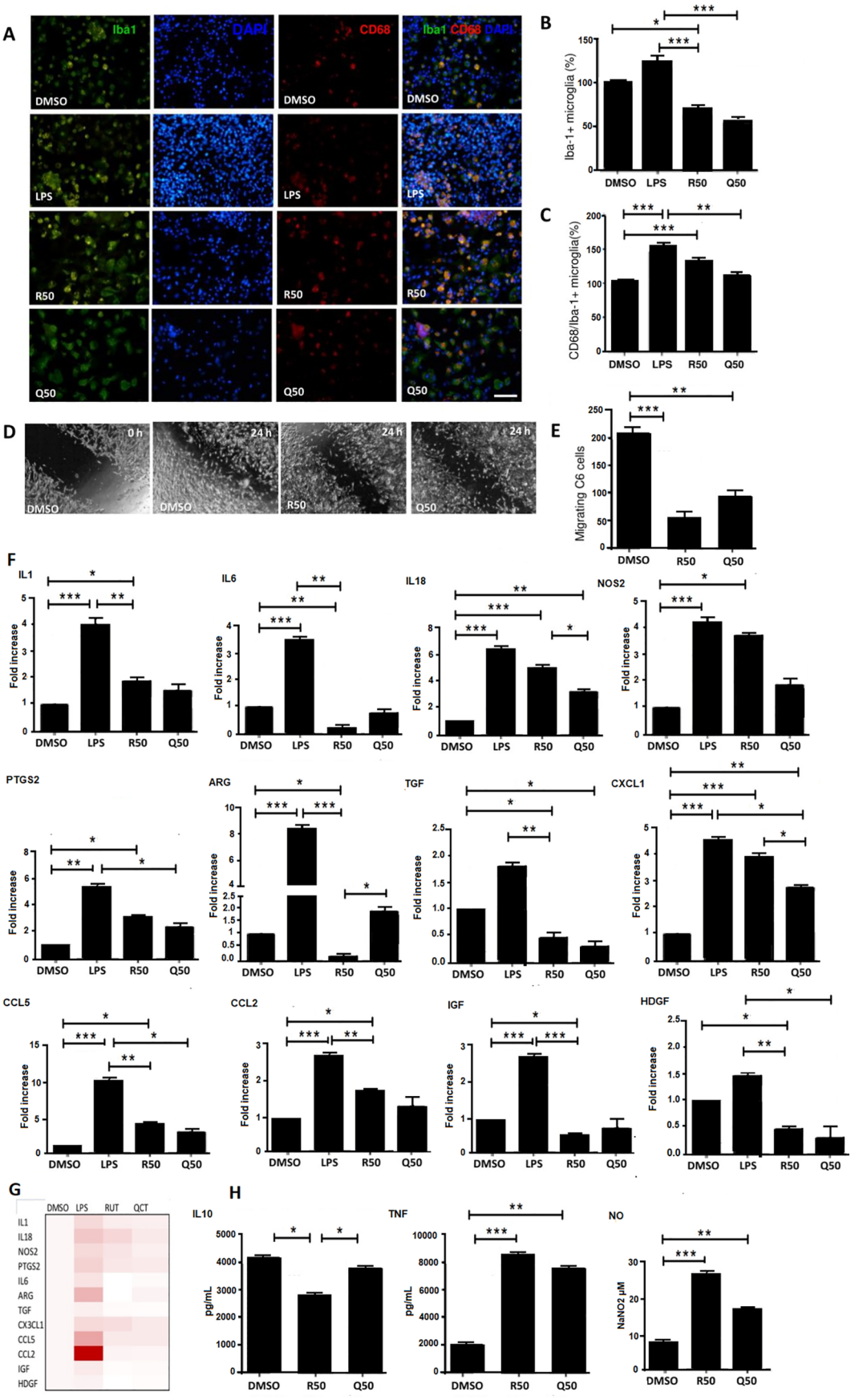

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Fig. 6. Effects of flavonoids rutin and quercetin on microglia morphology, cell migration and inflammatory profile of microglia/C6 co-cultures. Co-cultures were maintained in control conditions (0.05% DMSO) or treated with 50 μM rutin (R50), 50 μM quercetin (Q50) or 1 μg/mL LPS for 48 h after treatments. (A) Analysis of microglia morphology and activation by immunocytochemistry for proteins Iba-1 (green) and CD68 (red); the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm. (B) Bar graph showing the quantification of Iba-1 positive (Iba-1+) microglia in co-cultures; results from three independent experiments expressed as mean ± SEM; (* P < 0,05) (*** p < 0,001), one-way ANOVA. (C) Bar graph showing the quantification of Iba-1/CD68 positive (Iba-1+/CD68+) microglia in co-cultures; results from three independent experiments expressed as mean ± SEM; (* P < 0.05) (*** p < 0.001), one-way ANOVA. (D) Migration of cells in microglia/C6 co-cultures analyzed by phase-contrast microscopy. Representative photomicrographs of the wounded area at time zero (control) and in different conditions 24 h after treatments; 10× magnification, scale bars: 50 μm. (E) Bar graph showing the quantification of cells that migrated to the wounded area 24 h after treatments; results from three independent experiments expressed as mean ± SEM; (* P < 0.05) (*** p < 0.001), one-way ANOVA. (F) Real-time qPCR analysis showing the relative expression of M1 (IL-1β, IL-6, IL-18, NOS2 and PTGS2) and M2 (TGFβ and Arg-1) markers, chemokines (CCL2, CCL5 and CX3CL1) and growth factors (IGF, HDGF) in co-cultures; (* P < 0.05) (** p < 0.01) (*** p < 0.001), one-way ANOVA. (G) Hot-spot graph shows genes that were up or down regulated according to red color intensity related to control cultures. (H) Levels in the culture medium of cytokines IL-10 and TNF (pg/mL) measured by sandwich ELISA, and NO levels, expressed as NaNO2 measured by Greiss reaction; results from three independent experiments expressed as mean ± SEM; (* P < 0.05) (** p < 0.01) (*** p < 0.001), one-way ANOVA.

IL-1β, IL-6, TGF-β, IL-18, PTGS2, NOS2, IGF, HDGF, ARG, CX3CL1, CCL2 and CCL5 were evaluated by means of RT-qPCR. It was observed that relative mRNA expression for IL-1β, IL-18, CX3CL1, CCL5, CCL2, PTGS2 and NOS2 increased in co-cultures treated with both rutin and quercetin. On the other hand, the flavonoid treatment induced a reduction on mRNA levels for arginase, HDGF, TGF-β, IL-6 and IGF. In general, treatment with LPS, adopted as positive pro-inflammatory control, induced an increase in the expression levels of all genes studied.

3.5. Flavonoids rutin and quercetin reduce tumorigenesis of glioma cells and induce glial activation in vivo

The antitumorigenic effect of the flavonoids rutin and quercetin and *in vivo* glial activation were evaluated considering the importance of cellular interactions and a more reliable simulation of the reactions that occur in the tumor microenvironment, in addition to allowing a broader understanding of the phenomena related to the pathogenesis of these tumors. The antitumorigenic effect of the flavonoids was investigated by the analysis of tumor formation after 30 days of orthotopic xenotransplantation of U251 human glioblastoma cells under control conditions, or pretreated for 24 h with rutin or quercetin, in the brain of Wistar rats (Fig. 7).

In the brain of control animals, which had received xenotransplants of U251 cells previously treated with the vehicle DMSO (0.05%), macroscopic lesions with darker central areas, suggestive of necrosis, were observed in the xenotransplantation region (Fig. 7A). However, in the brain of animals that received U251 cells pretreated with rutin or quercetin, a predominance of whitish and homogeneous areas in the xenotransplantation region was observed.

In order to observe the behavior of glial cells in the presence of U251 human glioblastoma cells, we investigated the morphology and activation of astrocytes and microglia through immunohistochemistry (IHQ) for the GFAP and Iba-1 proteins, respectively, and the microglia activation profile through IHQ for CD68, intracellular glycoprotein associated with cytoplasmic granules and with macrophage membranes, what characterizes classically activated subtypes of M1 macrophages. This analysis was also performed in the contralateral hemispheres of neoformed gliomas (Fig. 7B and C). In the hemispheres that received the glioblastoma cells under control conditions, a number of GFAP+ but unreactive astrocytes was observed, which are evenly distributed in the contralateral hemispheres. In the brain of animals injected with cells previously treated with rutin or quercetin, the proportion of astrocytes in the areas of tumor cell implants was reduced when compared to that of animals that received untreated cells, and presented a more reactive phenotype with GFAP+ well-defined cellular processes. The analysis of microglia morphology and activation demonstrated an increase in the proportion of microglia in the region of tumor cell implants under control conditions with predominance of cells with branched morphology, the same morphology as that observed in the contralateral hemisphere of the implant of tumoral cells. However, in the hemispheres of animals injected with cells previously treated with rutin or quercetin, the proportion of microglia in the areas of tumor cell implants and in the contralateral hemispheres of the tumors is quite similar, but with predominance of microglia with amoeboid phenotype, typical of activated microglia. The analysis of the inflammatory activated profile of microglia by labeling for the CD68 protein showed that microglia were predominantly directed to this phenotype in the areas of implants of tumor cells previously treated with rutin or quercetin, as well as in the contralateral areas. On the other hand, a lower proportion of microglia with this M1 phenotype was observed in the contralateral cerebral hemispheres and tumor cells implant areas in animals injected with the glioblastoma cells under control conditions.

The reduced capacity of the immune response in the glioblastoma microenvironment is mainly due to the microglia/glioma interaction. Microglia and astrocytes are immunoreactive and secrete cytokines and trophic factors that influence tumor growth and migration. Thus, the modulation of the expression of genes related to the inflammatory response was also investigated in the brains of rats with xenotransplants of glioblastoma cells through RT-qPCR, aiming to better characterize the response profile of the tumor environment (Fig. 7D). The results obtained showed a trend towards a proinflammatory profile in the cerebral areas of xenotransplants of U251 human glioblastoma cells pre-treated with rutin or quercetin because there was a significant increase in mRNA expression for IL-1β, IL-18, CX3CL1, PTGS2 and NOS2 and a significant decrease in mRNA expression levels for IL-6, TGFβ, arginase, HDGF and IGF. In this condition, there was also a decrease in mRNA expression for TNF, suggesting control of an exacerbated inflammatory response.

4. Discussion

In the present study we investigated the antitumor and immunomodulatory properties of rutin and its aglycone quercetin on the viability of glioma cells alone and under direct and indirect interaction with microglia. Treatment of rat C6 glioma cells with both flavonoids resulted in the inhibition of proliferation and migration, corroborating with previous studies with other human glioblastoma cells (Santos et al., 2011; Santos et al., 2015). Treatment of C6 cells with rutin or quercetin also induced microglia chemotaxis, which was associated to the up regulation of TNF, CCL2, CCL5 and CX3CL1 and down regulation of IL-10. These chemokines as CX3CL1 and its cognate receptor CX3CR1 are expressed in CNS cell, and are also expressed in glioma cells, negatively regulating glioma invasion (Marchesi, et al., 2010, Sciumè et al., 2010; Liu et al., 2016). In addition to providing trophic support to neurons, microglia-derived trophic factors can also act in an autocrine way by regulating the ability of microglia to maintain the anti-inflammatory state.

A similar profile of regulation by the flavonoids of the inflammatory and growth factors was observed in co-cultures of microglia/glioma cells and in the xenotransplanted pre-treated glioma cells, showing down regulation of HDGF, IGF and GDNF growth factors. The levels of

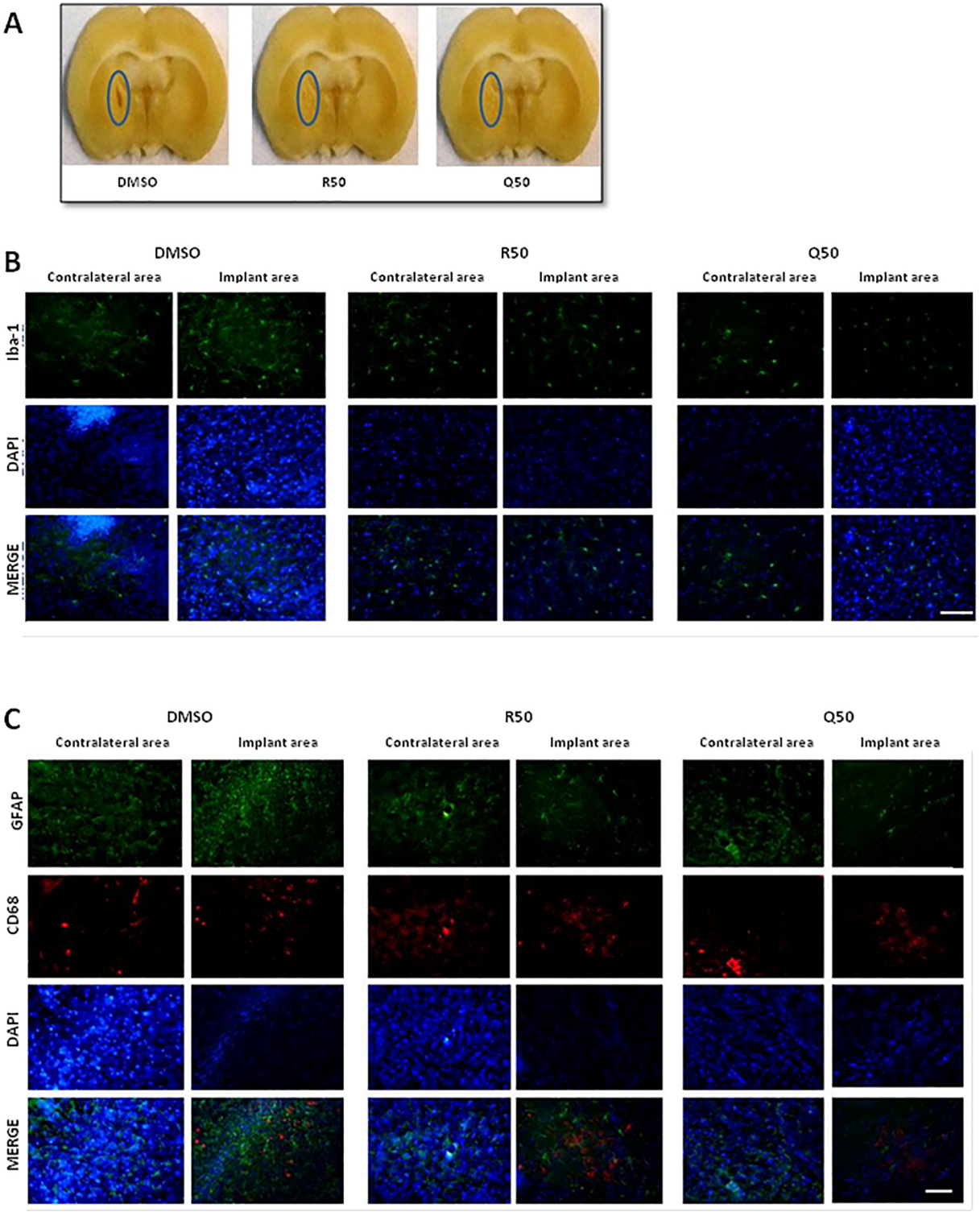

Fig. 7. Effects of flavonoids rutin and quercetin on tumorigenic potential of human U251 cells and glial reactivity after orthotopic xenotransplant in the brain of Wistar rats. U251 cells were maintained in control condition (0.05% DMSO) or treated with 50 μM rutin (R50) or 50 μM quercetin (Q50). Twenty four (24) hours after the treatment, 5×10^5 viable U251 cells were xenotransplanted by stereotaxic injection in the striatum and after 30 days the animals were sacrificed. (A) Macroscopic evaluation of the encephalon of one animal that received xenotransplants of U251 cells in control conditions or treated with flavonoids; the area of the transplant is surrounded, and it is possible to evidence the dark region of tumor growth in animals that were transplanted with cell under control conditions. (B) Analysis of microglia morphology in the transplant and contra-lateral areas of the brains by immunohistochemistry for Iba-1 (green); the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm. (C) Analysis of astrocyte and microglia activation in the transplant and contra-lateral areas of the brains by immunohistochemistry for proteins GFAP (green) and CD68 (red), respectively; the nuclear chromatin was stained with DAPI shown in blue; 20× magnification, scale bars: 50 μm. (D) Real-time qPCR analysis showing the relative expression of M1 (IL-1β, IL-6, IL-18, NOS2 and PTGS2) and M2 (TGFβ, IL-10, IL-4 and Arg-1) markers, chemokines (CCL2, CCL5 and CX3CL1) and growth factors (IGF, HDGF) in co-cultures; (* $P < 0.05$) ($^{**}p < 0.01$) ($^{***}p < 0.001$), one-way ANOVA. (E) Hot-spot graph shows genes that were up or down regulated according to red colour intensity related to control cultures.

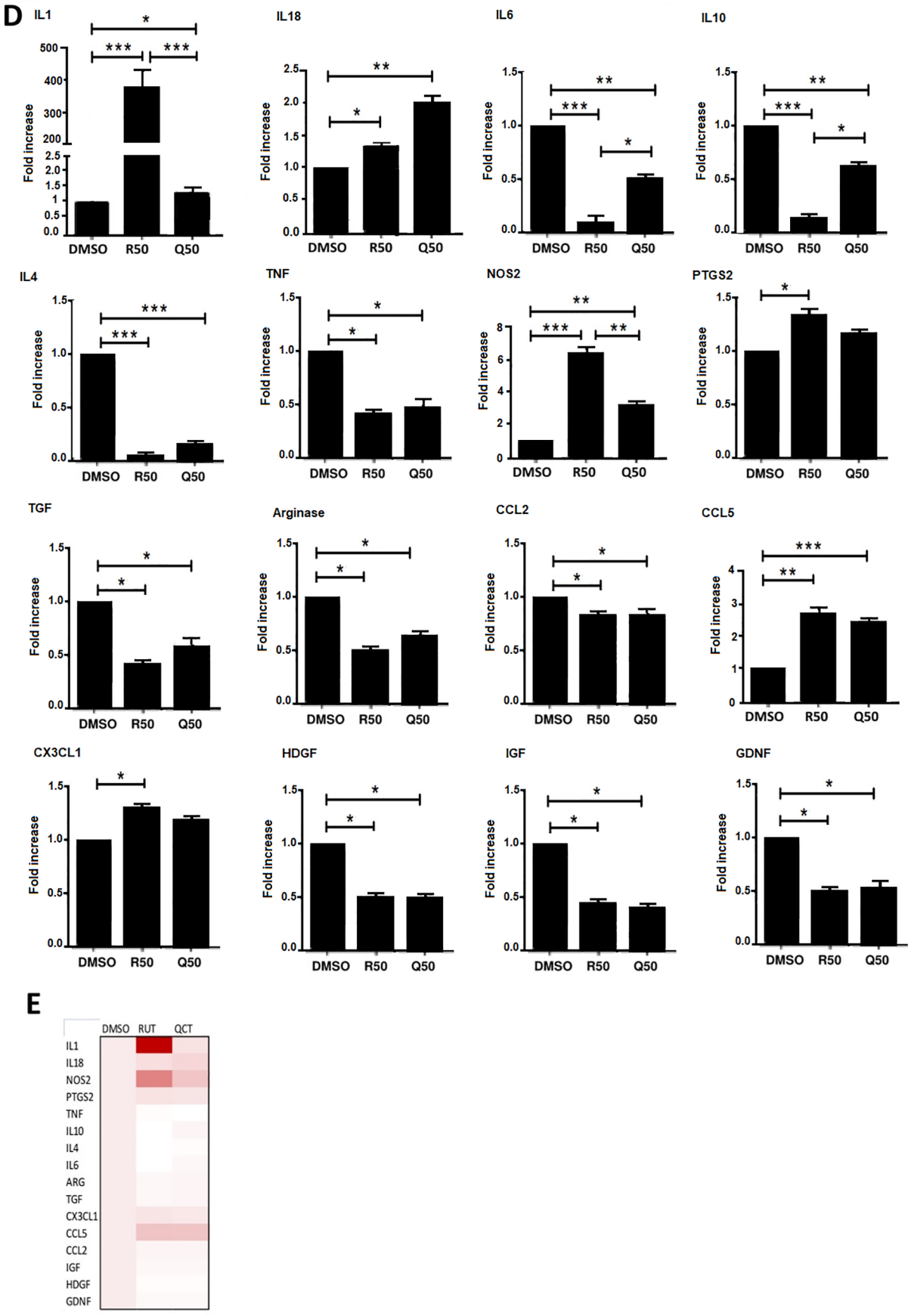

Fig. 7. (*continued*)

HDGF e IGF expression has been associated with the poor prognosis in glioma patients (Yang et al., 2017), and the reduction of HDGF expression significantly decreases proliferation, migration, invasion and cellular tumorigenesis of glioma cells (Song et al., 2014). Thus, the fact that rutin and quercetin modulated expression of these chemokines and growth factors infers that it contributes to a decrease in the growth and migration of gliomas cells besides microglia response that appears to favor tumor progression without the treatment.

Therefore, the treatment of microglia and C6 cells with flavonoids either in co-cultures or in conditioned media, besides reduction on proliferation and migration of glioma cells, also directed microglia towards an inflammatory profile with increased expression of IL-1βand IL-18, and decreased expression of lL-6, IL-10, NOS2, PTGS2, arginase-1, TGF-β, IGF and HDGF. The conditioned medium from U87, GBM-8401 and C6 glioma cells significantly induced iNOS expression and NO production (Shen et al., 2014). Another marker of malignant glioma is the increase expression of COX2 and arginase 1 enzymes. Arginase 1 was highly expressed by tumor-associated microglia and macrophages in human GBM samples (Zhang et al., 2016), and it has been associated with low survival of patients with high level malignant glioma (Ochs et al., 2015).

IL-6 has a broad effect on cells of the immune system and those not of the immune system, with context-dependent pro- and anti-inflammatory properties (Hunter and Jones, 2015). An oncogenic role has been attributed to cytokine IL-6 in gliomas, and is related to the invasiveness and prognosis (Shan et al., 2015; Wang et al., 2016). Similar reduction on expression of mRNA for IL-6 regulatory cytokine was observed after treatment with rutin, but not quercetin. IL-6 mRNA expression was increased in the different models of glioma microglia interactions. Increased expression of IL-6 has been associated to changes on oxidative status of cells (Waxman and Kolliputi, 2009). Hence, increased IL-6 expression after quercetin exposure may be related to the well know antioxidant effect of the flavonoid (Bao et al., 2017). Similar inflammatory profile was also observed in the microenvironment of flavonoid treated glioblastoma cells in xenotransplant, reinforcing the antiglioma and immunomodulatory effects of the compounds. In the microenvironment of glioma, tumor IL-18 appears to be involved in the activation of microglia, possibly through fibronectin and vitronectin, secreting factors that can promote migration (Yeh et al., 2015; Kast, 2015). On the other hand, several genes regulated by TGF-β are responsible for the progression of glioma, including members MMPs and VEGF (Edwards et al., 1996, Pepper, 1997). The suppression of type 2 TGF-β receptor II (TGFBRII) in GBM cells is related to the inhibition of migration and invasion of human U251 glioblastoma cells (Hu et al., 2017). Moreover, using a mathematical and computational model, it was possible to show that microglia can stimulate the invasion of tumor cells by secreting the TGF-β growth factor (Kim et al., 2017). Previous studies demonstrated that the reduction on GL15 human glioma cells migration after exposure to rutin was associated with a decrease in MMP activity and expression (Santos et al., 2015), and a reduction in secretion of VEGF and TGF-β was also observed after flavonoid treatment (Freitas et al., 2011). Considering that there was an increase in the expression IL-18 and a decrease in the expression of TGF-β after treatment with flavonoids rutin and quercetin in microglia/glioma co-cultures and in the microenvironment of xenotransplants of glioma cells, it can be inferred that equilibrium on levels of both regulatory molecules can impact on the reduction of glioma cell migration.

In the present study, we sought to find alterations in the pattern of microglia expression in the different forms of culture interacting directly or indirectly with glioma. It seems possible to admit a more targeted microglial response to an inflammatory profile and less favorable to glioma, since both flavonoids in co-cultures promoted an increase in the pro-inflammatory cytokine gene profile such as IL1β and IL18, in addition to NOS2 and PTGS2, and decreased arginase and TGF-β levels. The immunomodulatory effects were also observed in the *in vivo* tumorigenic assay with an increase in the expression of inflammatory factors (IL1β, IL18, NOS2 and PTGS2) in addition to the downregulation of anti-inflammatory or regulatory factors (IL-10, IL-4, IL-6, TGFβ) in the brain area of implant of glioma cells pre-treated with flavonoids.

5. Conclusion

In light of the above, about the interaction between microglia and glioma, in the control groups, a predominance of a regulatory profile that facilitates tumor growth was demonstrated. Flavonoids were able to induce the microglia to mount an immune response to an inflammatory profile, migrating to the tumor environment, acquiring the ability to reduce proliferation in glioma cells, associated to the inhibition of growth factor expression in cultures.

The discovery of new drugs including those from natural sources in current therapy is of great interest. Rutin and quercetin have been shown to be able to induce microglial proliferation and production of proinflammatory mediators. These results suggest that these flavonoids present an interesting subject of study due to their potential effect as immunoregulating microglia and inhibitors of glioma growth. Considering that rutin had a more prominent effect than quercetin on the induction of change in profile of expression and secretion of inflammatory cytokines and chemokines, and growth factors, associated with tumor progression, it is possible to suggest that rutin exhibits a more potent antitumor action considering the ability to inhibit proliferation and migration of tumor cells, associated with its ability to modulate the inflammatory response in a tumor microenvironment.

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