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Basic Neuroscience

An efficient method to limit microglia-dependent effects in astroglial cultures

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ABSTRACT

Microglia, the CNS resident macrophages, and astrocytes, the most abundant glial cell population, are both implicated in brain pathologies and can exhibit a pro-inflammatory phenotype. Microglial cells are known to rapidly and strongly react to brain insults. They will promote astrocyte activation and may lead to a vicious, self-perpetuating cycle of chronic inflammation. To obtain a better understanding of the individual role of both cell types, primary cells are frequently used in *in vitro* studies, but the purity of specific cell cultures remains rarely investigated. The aim of this study is to determine the effect of specific removal of microglial cells on the inflammatory properties of different glial cultures. Here, the removal of microglial contamination from mixed glial cultures to obtain astrocyte-enriched cultures was achieved using a magnetic cell sorting approach. Compared to mixed cultures, we clearly showed that these enriched cultures are only weakly activated by pro-inflammatory agents (lipopolysaccharide, interferon-γ or beta-amyloid peptide). This finding was confirmed using twice-sorted astrocyte-enriched cultures and microglia-free cultures composed of neurosphere-derived astrocytes. Thus, we present evidence that the magnitude of the pro-inflammatory response is linked to the percentage of microglia in cultures. Due to their high reactivity to various insults or pro-inflammatory stimuli, microglia-derived effects could be credited to astrocytes in mixed glial cultures. Therefore, we highlight the importance of monitoring the presence of microglia in glial cultures since they can affect the interpretation of the results, especially when inflammatory processes are studied.

1. Introduction

In the central nervous system (CNS), glial cells have supportive roles for neurons but also act as the resident immune system cells, engaging several inflammatory processes to defend the brain from pathogens and helping it to recover from stress and injury (Farfara et al., 2008). However, in brain pathologies, chronic glial activation is a common event, which could promote a progressive dysfunction and loss of neurons in the CNS. Astrocytes and microglia are both glial cells whose physiological roles and responses upon activation are quite distinct. Astrocytes, the most abundant glial cell population, are essential for brain homeostasis and neuronal function. Although astrocytes play a significant role in maintaining normal CNS functions, after injury these glial cells may exert pathophysiological actions leading to neuronal damage (Sofroniew and Vinters, 2010). Microglial cells are the resident macrophages in the CNS (Beutner et al., 2010; Hanisch and Kettenmann, 2007; Dobrenis, 1998). These cells represent the first line of defense against pathogens or brain tissue injury. In their activated state, they have beneficial functions essential for neuron survival (Ekdahl et al., 2009). On the other hand, microglial overactivation can give rise to progressive neurotoxic consequences (Lull and Block, 2010; Block et al., 2007). Microglial activation can also promote astrocyte activation, which in turn can further activate microglia, thus possibly leading to a vicious cycle of overactivation that may contribute to a chronic inflammatory state, with continuously increasing production of pro-inflammatory mediators (Liu et al., 2011; Vallejo et al., 2010; Saijo et al., 2009; Henze et al., 2005; Zaheer et al., 2002).

In vitro experiments using near-pure glial cultures are nowadays widely used in order to avoid heterogeneous cell–cell interactions and to characterize cell-specific signaling pathways. Thus, diverse methods have been developed for cell separation and purification (for review, see Dainiak et al., 2007). Actually, the most widely used technique for harvesting a single glial population is dependent on a mechanical shake-off method (Floden and Combs, 2007), but this is wrought with issues as it often still leaves a mixture of different glial cells. Newer techniques are based on physical criteria like size, shape, and density. They include filtration and centrifugation approaches (Fuss et al., 2009; Lara and Chalmers, 2005; Graham,

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2001), and also affinity approaches based upon biochemical cell surface and biophysical characteristics (Cho et al., 2010). Other approaches are the use of anti-mitotic agents to inhibit microglial growth in primary astrocyte cultures (Hamby et al., 2006a; Solenov et al., 2004) or the differential adherence of various glial cells on cell culture plates (Jana et al., 2007). In this study, we used a more recent technique for primary microglia isolation (Marek et al., 2008). Based on an antigen–antibody-mediated magnetic cell sorting system using CD11b MicroBeads, this technique allowed to rapidly obtain pure astrocyte cultures after microglia depletion.

Our work further stresses the growing consensus that great care must be taken when using glial cultures to assess astrocyte responses to inflammatory stimuli, as even small levels of microglia will have a major influence on the observed effects. Therefore, without a rigorous quality check, microglia-derived effects could be attributed to astrocytes. Due to their high pro-inflammatory capacities, microglia rapidly detect and strongly respond to a large number of stimuli. The presence of microglia must not be underestimated and great caution should be taken when preparing primary glial cultures (Saura, 2007). Thus, we investigated whether classical pro-inflammatory stimuli, such as LPS, IFNγ or beta-Amyloid (Aβ) peptide, were able to activate different types of glial cell cultures: mixed glial cultures (called MGC1), astrocyte-enriched cultures (called AEC-M1 and AEC-M2), neurosphere-derived astrocyte cultures, and primary microglia cultures. We provide clear evidence that the magnitude of the pro-inflammatory response was dependent on the proportion of contaminating microglial cells in our cultures.

2. Materials and methods

2.1. Mixed glial cultures

Mixed glial cell cultures were prepared from newborn C57BL/6 mouse brains (Harlan, The Netherlands) (Fig. 1). Briefly, after carefully removing meninges and large blood vessels, the brains were pooled and then minced in cold phosphate-buffered saline (PBS) solution. The dissociation was completed by a 10 min incubation in 2 mM-cold EDTA. After washes and centrifugations, the cells were plated and grown in complete medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed after 3 days of culture. After 8–10 days, mixed glial cultures (passage 0 or P0) had reached confluence, and microglia were detached by a 30 min shaking on a rotary shaker at 200 rpm at 37 °C. Detached cells, mainly microglia (Iba1-positive cells >95%) were then plated in the same medium (astrocyte-conditioned medium) in a humidified atmosphere containing 5% CO_2 and were ready-to-use 24 h later. Oligodendrocytes were subsequently detached from the mixed glial cultures (P0) by 2 days of washing and shaking steps on a rotary shaker at 200 rpm at 37 °C in a humidified atmosphere containing 5% CO_2. Afterwards, the culture medium was removed and mixed glial cultures were washed with PBS and incubated with 0.05% trypsin/0.53 mM EDTA at 37 °C for 5 min. Then, the trypsin was inactivated by the addition of complete medium and the cellular suspension was centrifuged at $300 \times g$ at 4 °C for 10 min. The cell pellet was resuspended in complete medium and then plated. After 6–7 days, mixed glial cultures (P1), called MGC1, had reached confluence. To detach residual microglia and oligodendrocytes, these cultures were shaken again for 2 days with 3 washings per day on a rotary shaker at 200 rpm at 37 °C in a humidified atmosphere containing 5% CO_2. Finally, fresh complete medium was added and these MGC1 were ready-to-use. Cell culture products were purchased from Invitrogen (Scotland).

2.2. Magnetic cell sorting for astrocyte-enriched cultures

As indicated in Fig. 1, mixed glial cultures (P1) or MGC1, obtained after the trypsinization step (described above), were further worked-up by magnetic cell sorting (MACS). After trypsinization and centrifugation at $300 \times g$ at 4 °C for 10 min, the cell pellet was resuspended in a separation buffer containing PBS, pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The cell suspension was centrifuged at $300 \times g$ at 4 °C for 10 min. Following manufacturer's instruction, 1×10^7 cells were resuspended in 90 μl of buffer and 10 μl of CD11b MicroBeads (MicroBeads conjugated to monoclonal rat anti-mouse CD11b antibody, Miltenyi Biotec, The Netherlands). The suspension was incubated at 4 °C for 20 min with gentle mixing after 10 min. During the incubation step, the CD11b-positive cells, microglia, were magnetically labeled with CD11b MicroBeads. Then, cells were washed with the separation buffer and centrifuged at $300 \times g$ at 4 °C for 10 min. The cellular pellet was resuspended in 500 μl of separation buffer for 1×10^8 cells. A LS column (Miltenyi Biotec) was fixed into the MidiMACS magnetic separation unit (Miltenyi Biotec) and washed with 3 ml of separation buffer. The cell suspension was applied on the column and three washings of 3 ml of separation buffer were performed with the same collecting tube. The unlabeled-cell fraction, mainly astrocytes, was passed through the column. The LS column was then detached from the magnetic separator and flushed with 5 ml of separation buffer. This step was performed to collect CD11b-positive cells. Both cellular fractions were centrifuged at $300 \times g$ at 4 °C for 10 min. The astrocyte-enriched fraction (called AEC-M1), corresponding to the CD11b-negative fraction, was resuspended in complete medium before plating. After 6–7 days, AEC-M1 were confluent and ready-to-use. The CD11b-positive fraction, microglia, were plated in astrocyte-conditioned medium and were ready-to-use 24 h later.

In order to vastly reduce microglial contamination, a second magnetic cell sorting (as previously described) was practiced from AEC-M1 to obtain even purer astrocyte cultures (AEC-M2). After 6–7 days, AEC-M2 were confluent and ready-to-use.

2.3. Neurosphere-derived astrocyte cultures

Primary cultures of neurospheres were obtained from embryonic murine neural stem cells derived from the subventricular zone (SVZ) of embryonic day 14 (E14) C57BL/6 mouse embryos as described previously (Grandbarbe et al., 2003). The SVZ of embryonic brain was extracted, triturated mechanically with a fired-narrowed Pasteur pipette. Dissociated cells were centrifuged at $300 \times g$ for 5 min, washed once and then plated in noncoated 6-well plates until neurospheres appeared. Neurospheres were cultured in Neurobasal-A medium supplemented with 1% B27 without vitamin A, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 ng/ml recombinant mouse Epidermal Growth Factor (rmEGF). These neurospheres were passaged by chemical, non-enzymatic dissociation every 5–6 days and reseeded as single cells at a density of 1×10^6 cells/ml. Neurospheres were differentiated into astrocytes by plating them on poly-L-ornithine coated flasks in DMEM containing 10% FBS and 1% antibiotics (Crocker et al., 2008). The culture medium was renewed after 3 days of differentiation and subsequently once a week. After 6 weeks of differentiation, the cells were considered to be completely differentiated into mature astrocytes.

2.4. Cell treatments

All treatments were applied on confluent cultures. Lipopolysaccharide (LPS 055:B5 from *Escherichia coli*, Sigma, Belgium) was added at 1 μg/ml on mixed and enriched glial cultures but at
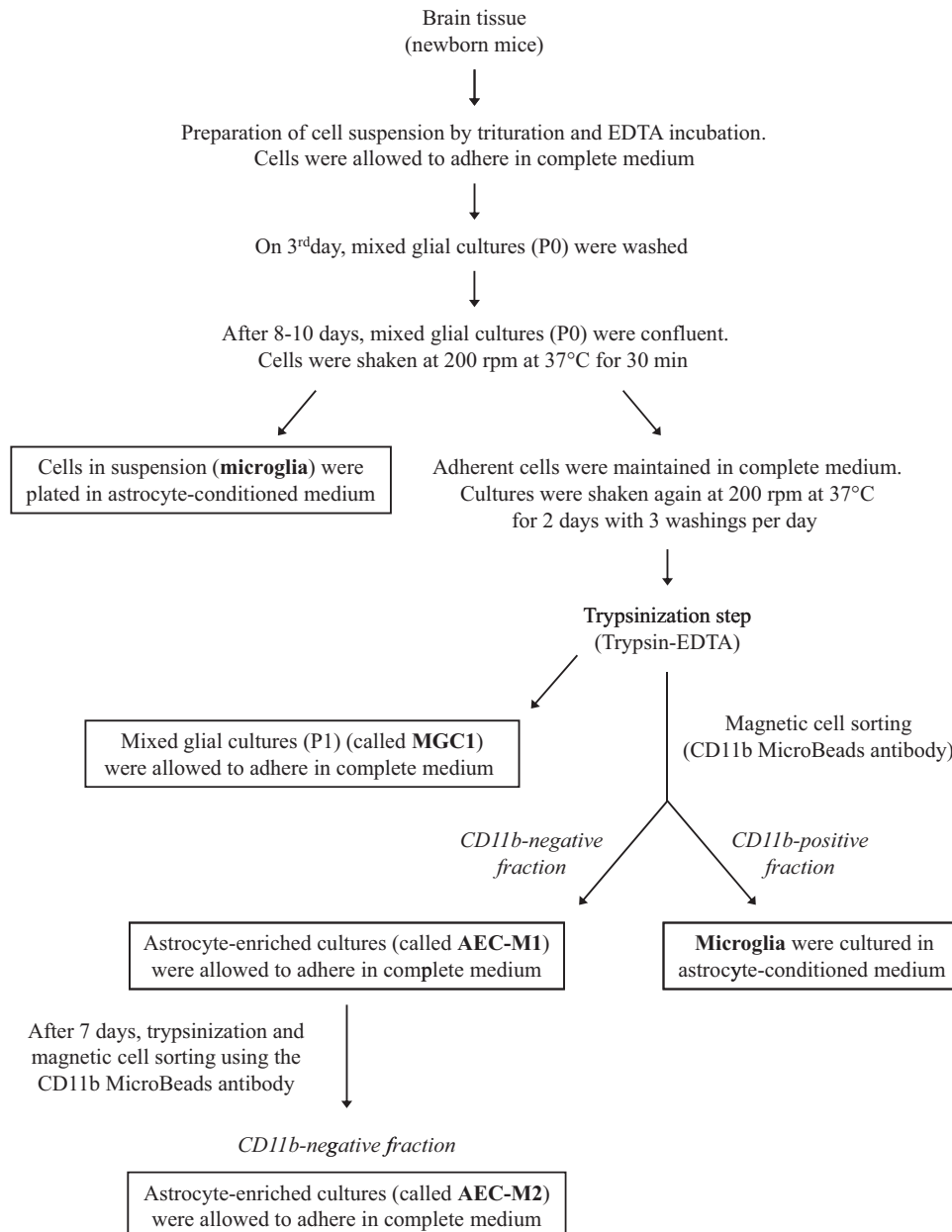
Brain tissue
(newborn mice)

↓

Preparation of cell suspension by trituration and EDTA incubation.
Cells were allowed to adhere in complete medium

↓

On 3rd day, mixed glial cultures (P0) were washed

↓

After 8-10 days, mixed glial cultures (P0) were confluent.
Cells were shaken at 200 rpm at 37°C for 30 min

Cells in suspension (**microglia**) were
plated in astrocyte-conditioned medium

Adherent cells were maintained in complete medium.
Cultures were shaken again at 200 rpm at 37°C
for 2 days with 3 washings per day

↓

Trypsinization step
(Trypsin-EDTA)

Magnetic cell sorting
(CD11b MicroBeads antibody)

Mixed glial cultures (P1) (called **MGC1**)
were allowed to adhere in complete medium

*CD11b-negative
fraction*

*CD11b-positive
fraction*

Astrocyte-enriched cultures (called **AEC-M1**)
were allowed to adhere in complete medium

Microglia were cultured in
astrocyte-conditioned medium

After 7 days, trypsinization and
magnetic cell sorting using the
CD11b MicroBeads antibody

↓

CD11b-negative fraction

Astrocyte-enriched cultures (called **AEC-M2**)
were allowed to adhere in complete medium

Fig. 1. Schematic drawing representing the cell isolation process.

only 1 ng/ml on primary microglia, since a higher concentration is deleterious for these cells. IFNγ (Hycult Biotechnology, The Netherlands) was used at 50 U/ml. The Aβ(1–42) peptide (Bachem, Germany) was initially dissolved to 5 mM in DMSO, separated into aliquots in sterile tubes and stored at −20 °C. The stock aliquot was diluted to 0.5 mM in DMEM containing 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). This Aβ preparation was added directly to cultures at a final concentration of 5 μM.

2.5. Cellular viability assay

Mitochondrial function was assessed as an index of cellular viability using the MTT method. This assay measures the ability of mitochondrial dehydrogenases to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to colored insoluble formazan. Cells were seeded on 48-well plate at an initial density of 2×10^5 cells/well. After treatment, cells were incubated for 3 h at 37 °C with MTT (0.35 mg/ml) diluted in PBS. Then, the medium

was removed, and cells were lysed by addition of 100% DMSO. Absorbance was measured at 540 nm using a microplate reader (TECAN, Austria). Viability was estimated from the absorbance of treated *versus* untreated cells.

2.6. Flow cytometry

The MACS-based sorting of microglial cells from mixed glial cultures was assessed by flow cytometry. Cells were harvested by trypsinization, centrifuged and resuspended in 3% BSA in PBS. For intracellular markers, cells were previously fixed and permeabilized in PBS-paraformaldehyde 4% and 0.01% of Triton X100 at 4 °C for 30 min. After a washing step, cells were incubated for 45 min with primary antibodies: rabbit anti-Iba1 (1:100, Biocare Medical, USA) or Cy3-labeled mouse anti-GFAP (1:800, Sigma, Belgium). Then, cells were stained with Cy2-conjugated anti-rabbit (1:1000, Jackson ImmunoResearch, England). The cells were washed in PBS after each incubation step. As negative controls, the same staining

procedure was repeated in parallel with the corresponding iso-type control antibodies (Jackson ImmunoResearch). The samples were analyzed on a flow cytometer (FACS Canto, Becton Dickinson) and data analysis was performed using WinMDI software (Scripps Research Institute, USA).

2.7. Immunofluorescence

Cells were cultured on poly-L-ornithine coated coverslips for one week and then fixed with paraformaldehyde (4% in PBS) for 20 min at room temperature, and permeabilized for 5 min in PBS containing 0.3% Triton X100. Following 3 washings in PBS, the blocking step was realized with 3% BSA in PBS at room temperature for 30 min. The cells were then incubated overnight at 4 °C with Cy3-labeled mouse anti-GFAP (1:800, Sigma) and rabbit anti-Iba1 (1:300, Biocare Medical). After washing steps with PBS, cells were incubated with Cy2-conjugated anti-rabbit (1:1000, Jackson ImmunoResearch) at room temperature for one hour. The cells were washed 3 times with PBS, and stained with DAPI (1:5000) for 5 min. Cells were then washed, mounted with Fluoromount G (SouthernBiotech, USA), and observed under a LSM 510 META inverted confocal microscope (Carl Zeiss Micro Imaging, Göttingen, Germany) at a 40-fold magnification.

2.8. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from glial cultures using the Invisorb^{TM} Spin Cell RNA Mini Kit (Invitek, Germany) according to the manufacturer's protocol. Total RNA was recovered in elution buffer and quantified from the absorbance at 260 nm. The purity was verified by spectrophotometry (absorbance ratio at 260 nm *versus* 280 nm). The RNA electrophoresis Experion system (Bio-Rad, CA) was used to confirm the absence of RNA degradation and genomic contamination. Complementary DNA (cDNA) was synthesized from RNA samples using 0.5 μg of total RNA and the ImProm-II Reverse Transcription System with 0.5 μg/reaction oligo(dT) as primer. The RNA and oligo(dT) were denatured at 70 °C for 5 min and immediately chilled on ice for at least 5 min. The reverse transcription reaction was performed in a total volume of 40 μl by mixing 2 μl ImProm-II^{TM} reverse transcriptase, 25 mM MgCl$_2$, 0.5 mM of each dNTP, and 1 μl of Recombinant RNasin® Ribonuclease inhibitor in ImProm-II 5X Reaction Buffer. Samples were incubated for 1 h at 42 °C and 15 min at 70 °C to inactivate the reverse transcriptase. A 1 μl aliquot of cDNA was used for each amplification by real-time PCR and added to the incubation mixture containing 8 μl of 2X iQ^{TM} SYBR Green Supermix, 0.8 μl of each 12.5 μM optimized forward and reverse primers in a final volume of 20 μl. Primer sequences (Table 1) were designed using the Beacon Designer Software (Bio-Rad). PCR analysis were performed on a Bio-Rad iCycler (iQ5 Real-Time PCR Detection System) using a 2-stage program provided by the manufacturer: 3 min at 95 °C followed by 40 cycles, consisting of denaturation at 95 °C for 10 s and annealing/extension at a temperature between 52.5 °C and 60.5 °C for 30 s. The change of reporter fluorescence from each reaction tube was monitored. Analysis of gene expression was performed using the comparative threshold cycle (C_t) method. The threshold cycle of each gene was determined as PCR cycles at which an increase in reporter fluorescence above a baseline signal was observed (Table 2). The target gene was normalized to the endogenous reference gene, Rpl27 (a housekeeping gene coding for a ribosomal protein). The mRNA expression fold change was calculated using the expression 2^{-ddCt}, where $ddCt = (C_{t,target} - C_{t,Rpl27})_{treated sample} - (C_{t,target} - C_{t,Rpl27})_{control sample}$.

Table 1
Sequences of the different real-time PCR primers.

Target gene (accession number)	Sequence (5′–3′)	Product size
Nos2 (NM_010927)	f: AGCCCTCACCTACTTCCTG r: CAATCTCTGCCTATCCGTCTC	100 bp
Ptgs2 (NM_011198)	f: GCCTGGTCTGATGATGTATGC r: GAGTATGAGTCTGCTGGTTTGG	124 bp
Tnf (NM_013693)	f: GGTTCTGTCCCTTTCACTCAC r: TGCCTCTTCTGCCAGTTCC	107 bp
Il1β (NM_008361)	f: GCTTCAGGCAGGCAGTATC r: AGGATGGGCTCTTCTTCAAAG	133 bp
Il6 (NM_031168)	f: ACCGCTATGAAGTTCCTCTC r: CTCTGTGAAGTCTCCTCTCC	117 bp
Ccl2 (NM_011333)	f: ACTCACCTGCTGCTACTCATTC r: GCTTCTTTGGGACACCTGCTG	97 bp
Ccl3 (NM_011337)	f: TTTGAAACCAGCAGCCTTTG r: CAGGTCAGTGATGTATTCTTGG	114 bp
Gfap (NM_010277)	f: GGTTGAATCGCTGGAGGAG r: CTGTGAGGTCTGGCTTTGG	131 bp
Itgam (NM_008401)	f: TGGACGCTGATGGCAATACC r: GGCAAGGGACACACTGACAC	94 bp
Aif1 (NM_019467)	f: TTCCCAAGACCCACCTAG r: TCCTCATACATCAGAATCATTC	142 bp
Rpl27 (NM_011289)	f: ACATTGACGATGGCACCTC r: GCTTGGCGATCTTCTTCTTG	111 bp

All reagents for reverse transcription and real-time PCR were obtained from Promega (CA) and Bio-Rad, respectively.

2.9. Measurement of pro-inflammatory mediator release

Cells were seeded on 6-well plates at an initial density of 10^5 cells/well. After 6–7 days, cultures were confluent and ready-to-use. The release of nitrite, a stable metabolite of nitric oxide (NO), was measured after 24 and 48 h of LPS, IFNγ and Aβ exposure. The concentration of nitrite was determined in culture supernatants by the Griess assay. The production of prostaglandins E$_2$ (PGE$_2$) and TNFα was measured after 24 h of LPS, IFNγ and Aβ exposure. PGE$_2$ and TNFα release were measured in the medium of the different cultures with a commercially available Enzyme ImmunoAssay kit (Assay Designs, USA), and a sandwich Enzyme Linked ImmunoSorbent Assay (R&D Systems, USA), respectively. The protein content was determined using the Dye Reagent (Bio-Rad) by measuring the absorbance at 595 nm, using bovine serum albumin standards.

2.10. Statistical analysis

For comparison of means between two different treatments, statistical analysis was done by Student's *t*-test (two-sample assuming equal variances). For comparison of multiple treatments, results were analyzed by an ANOVA followed by a Fisher's exact test. Differences between groups were considered as significant when *p*-values were less than 0.05. Results are expressed as mean ± SEM from at least three independent experiments.

3. Results

Following the experimental procedure described in Fig. 1, we have prepared mixed glial cultures (P0) from the brains of newborn mice. From these cultures, we were able to prepare other glial cultures, such as mixed glial cultures (MGC1), but also astrocyte-enriched cultures (AEC-M1), twice-sorted astrocyte-enriched cultures (AEC-M2) and microglial cultures. In this study, we have also generated microglia-free cultures composed of neurosphere-derived astrocytes.

Table 2
Threshold cycle (C_t) value of the target genes in the different cultures.

Target gene (accession number)	MGC1	AEC-M1	Neurosphere-derived astrocytes	Primary microglia
Nos2 (NM.010927)	36.0	36.0	36.0	37.0
Ptgs2 (NM.011198)	28.0	27.5	33.5	30.0
Tnf (NM.013693)	29.0	36.0	34.5	26.5
Il1β (NM.008361)	30.5	35.5	36.5	29.0
Il6 (NM.031168)	31.5	31.5	37.0	35.0
Ccl2 (NM.011333)	25.0	26.5	28.0	25.0
Ccl3 (NM.011337)	27.0	35.0	31.5	22.0
Gfap (NM.010277)	21.0	21.0	20.5	–
Itgam (NM.008401)	29.2	34.4	36.0	22.6
Aif1 (NM.019467)	24.6	30.8	38.2	21.8
Rpl27 (NM.011289)	20.0	20.5	20.5	20.0

3.1. Analysis of the different glial cell culture preparations

Immunocytochemistry analysis revealed that the number of microglial cells (Iba1-positive cells) in MGC1 was much higher compared to the ones contained in AEC-M1 (Fig. 2). Mouse astrocytes obtained by this separation process showed typical astrocyte morphology as well as astrocytic marker expression (GFAP), which was slightly lower than in MGC1. In both cultures, some cells were negative for both Iba1 and GFAP markers. Furthermore, quantitative data have been obtained by FACS analysis (Fig. 3). As shown in Fig. 3A, a MACS separation increased the GFAP-positive cells from about 60% in MGC1 to 70% in AEC-M1. The percentage of microglial cells (Iba1-positive cells) has also been determined in both cultures. Before cell sorting separation, Iba1-positive cells represented 9–10% of the culture (Fig. 3B), whereas Iba1-positive cell number decreased to 1–1.5% of the total cells in enriched cultures (Fig. 3C).

Cell sorting by CD11b MicroBeads was used to obtain astrocyte-enriched cultures, but also microglial cultures (>95% Iba1-positive cells, data not shown).

### 3.2. Effects of pro-inflammatory stimuli: assessment of mRNA levels

In order to evaluate the consequences of the presence of microglial cells in our astrocyte cultures, pro-inflammatory conditions were induced by treating MGC1 and AEC-M1 with LPS, IFNγ and Aβ. Pro-inflammatory gene expressions were assessed by real-time PCR (Fig. 4).

In MGC1, a 6 h LPS exposure strongly induced the expression of Nos2 (Fig. 4A), Ptgs2 (Fig. 4B), Tnf (Fig. 4C), Il1β (Fig. 4D) and Il6 (Fig. 4E) as well as Ccl2 and Ccl3 chemokines (data not shown). In AEC-M1, where the percentage of microglial cells was about 10-fold lower than in MGC1, the expression of these pro-inflammatory genes was much lower compared to mixed culture responses: Nos2 (−90%), Ptgs2 (−85%), Tnf (−95%), Il1β (−97%), Il6 (−95%), Ccl2 (−50%, data not shown) and Ccl3 (−90%, data not shown).

IFNγ and Aβ were less potent than LPS in both glial cultures, but their effects were still different between MGC1 and AEC-M1 cultures. In MGC1, IFNγ and Aβ induced Nos2 expression (Fig. 4F)

MGC1   **AEC-M1**
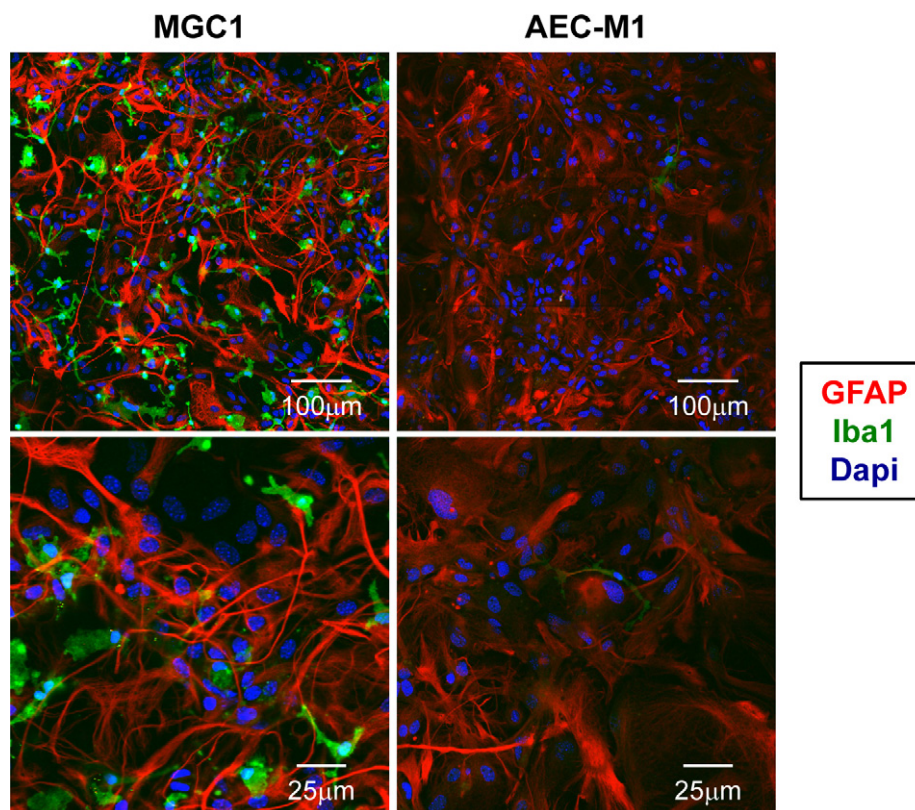
GFAP
Iba1
Dapi

Fig. 2. Astrocyte and microglial cells in cultures. MGC1 and AEC-M1 were labeled with anti-GFAP antibody (red) in combination with the microglial marker Iba1 (green) and counterstained with nuclear stain DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
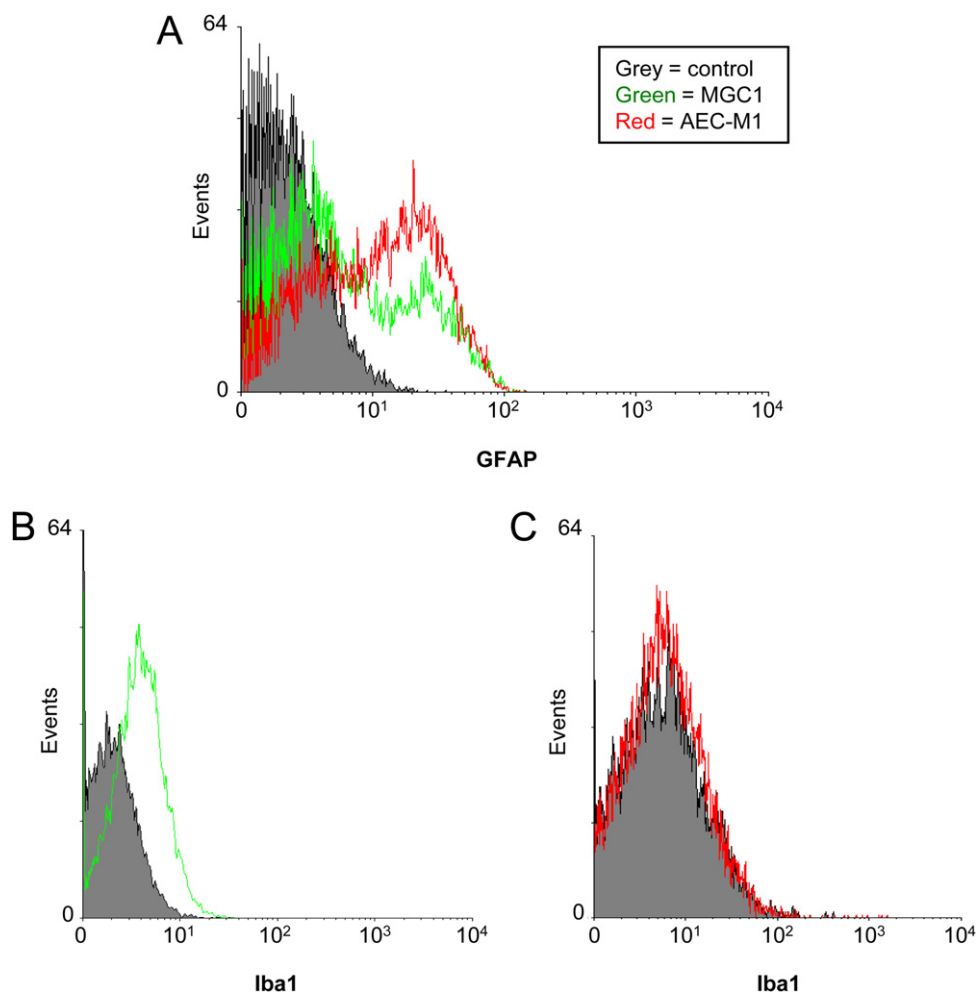
Fig. 3. Quantitative assessment of astrocyte and microglia populations by flow cytometry. The different glial cell populations were immunostained for flow cytometry analysis. GFAP (A) and Iba1-positive cells (B and C) were determined in both MGC1 (green events) and AEC-M1 (red events). For negative control (grey events), the same staining procedure was repeated with the secondary antibodies alone. Data analysis was performed using WinMDI software (Scripps Research Institute, USA). This is a representation of one out of three experiments. All experiments showed similar profiles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by respectively 25-fold and 5-fold compared to the control level. On the contrary, after magnetic separation, an IFNγ exposure induced lower Nos2 expression (4.5-fold), whereas Aβ had no effect on it. In both conditions, Ptgs2 up-regulation (Fig. 4G) was similar. Control expression levels of Tnf and Il1β (respectively Fig. 4H and I) were higher in MGC1 due to increased basal expression levels in microglia compared to astrocytes (Table 2). Thus, in MGC1, IFNγ and Aβ up-regulated Tnf by respectively 4.4-fold and 2-fold (Fig. 4H). Aβ also increased Il1β expression (4-fold) contrary to IFNγ, which decreased Il1β expression (−20%). In AEC-M1, these two pro-inflammatory compounds had different effects on the expression of Tnf and Il1β. An IFNγ exposure up-regulated Tnf expression by 1.6-fold whereas Aβ had no effect. However, an Aβ exposure up-regulated Il1β expression (2-fold induction) whereas IFNγ had no effect. IFNγ and Aβ also induced a higher Il6 overexpression in MGC1 compared to AEC-M1 (Fig. 4J).

3.3. Comparison with neurosphere-derived astrocyte cultures

Neurosphere-derived astrocytes are considered as pure astrocytes. In response to an inflammatory stimulus (Fig. 5), neurosphere-derived astrocytes reacted to TNFα (data not shown) and IFNγ exposure. The presence of IFNγ increased gene expression levels of Nos2, Ptgs2, Tnf and Il6 (respectively Fig. 5A–D)

but, in contrast to AEC-M1 cultures, LPS exposure did not increase Nos2, Tnf, and Il6 expression. Moreover, a LPS treatment induced a little increase of COX2 expression (2.2-fold). After IFNγ and Aβ treatments, the activation profile observed in neurosphere-derived astrocytes was the same than the one found in AEC-M1. However, Il1β expression was not detectable in neurosphere-derived astrocytes. In these conditions, the Gfap expression level was not modulated by pro-inflammatory activations (Fig. 5E).

3.4. Comparison with primary microglia cultures

Compared with MGC1 and AEC-M1, primary mouse microglia were highly sensitive to LPS (1 ng/ml), IFNγ (50 U/ml) and Aβ (5 μM) exposures (Table 3). As expected, primary microglia showed

Table 3
Pro-inflammatory phenotype in primary microglia.

| Target genes	LPS	IFNγ	Aβ
Nos2	22,737.9 ± 1932.8	41.9 ± 5.8	11.2 ± 2.2
Ptgs2	3008.3 ± 240.0	37.3 ± 3.6	28.5 ± 3.8
Tnf	1986.1 ± 253.6	5.7 ± 1.6	15.1 ± 2.1
Il1β	3482.8 ± 214.2	0.2 ± 0.04	20.7 ± 1.1
Ccl2	636.7 ± 54.2	12.3 ± 0.3	3.3 ± 0.5
Ccl3	101.4 ± 11.3	0.2 ± 0.03	7.5 ± 0.8
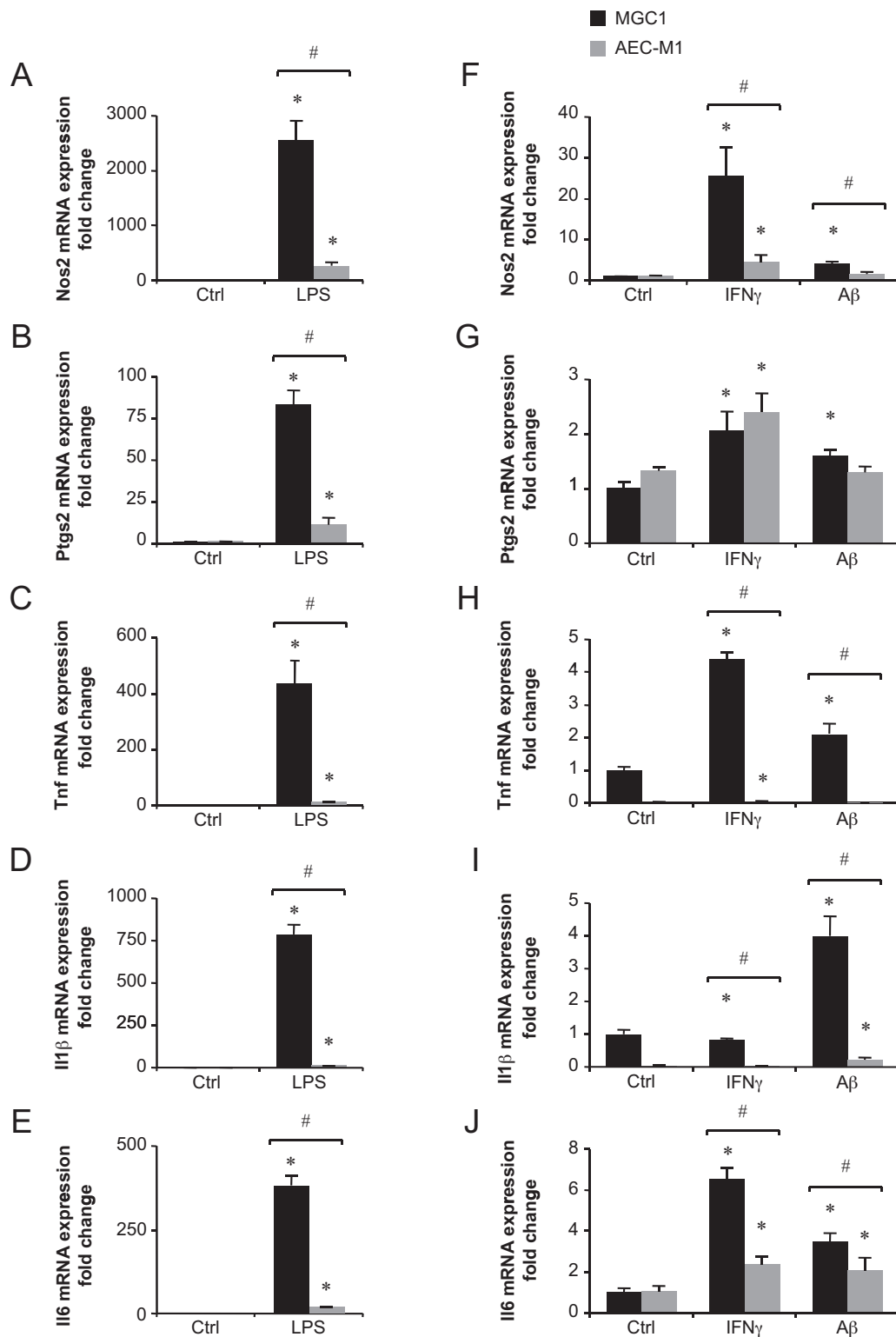
Fig. 4. Evaluation of pro-inflammatory gene expressions. Pro-inflammatory gene Nos2 (A and F), Ptgs2 (B and G), Tnf (C and H), Il1β (D and I) and Il6 (E and J) expressions were determined in MGC1 and AEC-M1 after 6 h of LPS (1 μg/ml), IFNγ (50 U/ml) and Aβ (5 μM) exposure. The transcription levels were analyzed by real-time PCR. Control expression levels were fixed at 1.0 in mixed culture condition. All gene expressions were normalized to Rpl27 expression level. Results are given as mean ± SEM ($n = 5$ independent experiments). *$p < 0.05$, significantly different from control; #$p < 0.05$, significantly different between MGC1 and AEC-M1.

a much stronger inflammatory response than any of the other cell populations. The three stimuli induced high expression levels of Nos2, Ptgs2, Tnf as well as the Ccl2 chemokine. However, an IFNγ exposure reduced Il1β and Ccl3 expression in contrast to LPS and Aβ treatments.

3.5. Effects of pro-inflammatory stimuli: assessment of inflammatory protein release

As a result of Nos2, Ptgs2 and Tnf up-regulation, nitrites, PGE₂ and TNFα releases increased significantly after treatments in MGC1
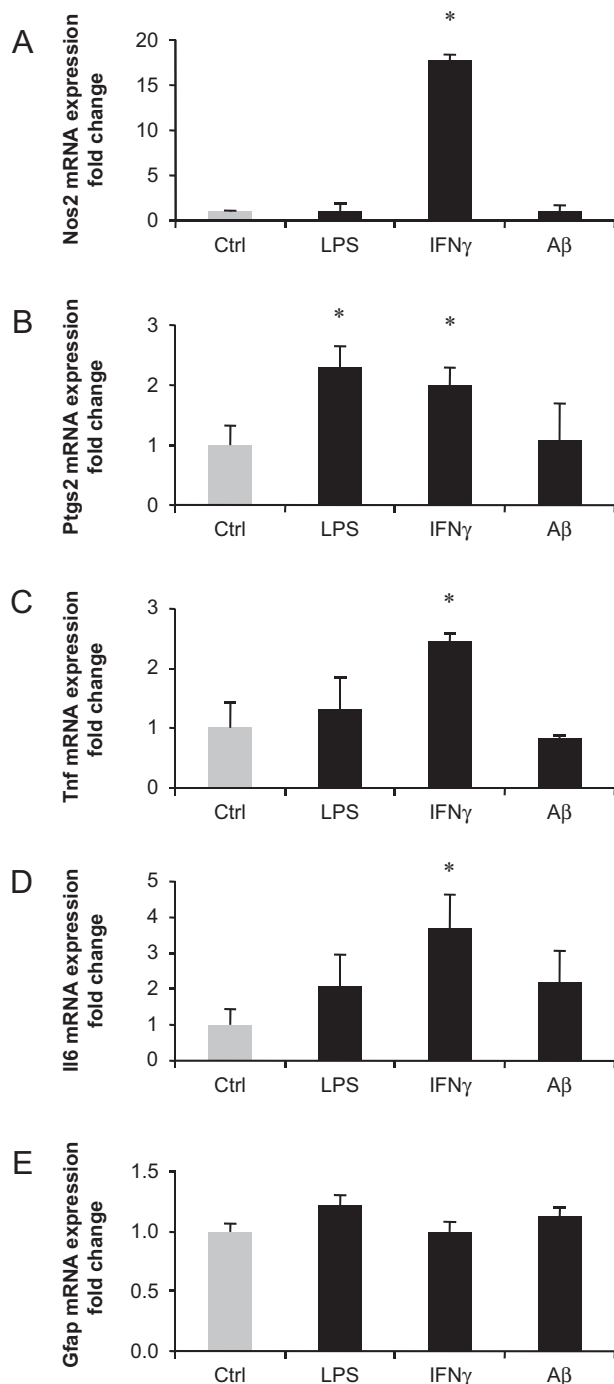
Fig. 5. Effect of pro-inflammatory compounds on neurosphere-derived astrocytes gene expression. Pro-inflammatory gene Nos2 (A), Ptgs2 (B), Tnf (C) and Il6 (D) expressions as well as Gfap expression (E) were determined in neurosphere-derived astrocytes after 6 h of LPS (1 μg/ml), IFNγ (50 U/ml) and Aβ (5 μM) exposure. The transcription levels were analyzed by real-time PCR. Control expression levels were fixed at 1.0. All gene expressions were normalized to Rpl27 expression level. Results are given as mean ± SEM ($n = 3$ independent experiments). $*p < 0.05$, significantly different from control.

(Fig. 6). The production of these pro-inflammatory compounds was higher under LPS conditions compared to IFNγ or Aβ exposures.

LPS, IFNγ and Aβ exposures induced an increase of nitrite levels in MGC1 at 24 h and 48 h (Fig. 6A). In AEC-M1, nitrite release levels were lower than in MGC1. A LPS treatment slightly increased the production of nitrites after 24 h (2-fold), but not after 48 h. IFNγ or Aβ exposures did not promote nitrites production at both 24 and 48 h in AEC-M1.

An increase of PGE$_2$ (Fig. 6B and C) and TNFα release (Fig. 6D and E) was observed in MGC1 after 24 h of LPS, IFNγ and Aβ exposure. In AEC-M1, the release of prostaglandins and TNFα induced by a LPS treatment was strongly lower (respectively −95% and −99%) than in MGC1. An IFNγ or Aβ exposure was unable to promote PGE$_2$ and TNFα releases in AEC-M1.

In addition, secreted amounts of nitrite, PGE$_2$ and TNFα were similar when cells were treated for 24 h with IFNγ or Aβ in MGC1 or AEC-M1.

3.6. Time dependent effects of a microglial contamination

AEC-M1 were studied until 3 weeks after plating (Fig. 7). As shown in Fig. 7A, the number of microglial cells increased from 7 to 21 days of culture. As previously described, the GFAP staining was low at 7 days but higher after 21 days when microglial cells (Iba1 staining) were also numerous. A 6 h LPS exposure (Fig. 7B) increased Nos2, Ptgs2, Tnf and Il1β expression in a time dependent manner. Thereby, the pro-inflammatory state was higher after 21 days, which coincided with an elevated number of Iba1-positive cells.

3.7. Comparison between single- and double-sorted glial cultures

MGC1 were also compared to twice-sorted glial cultures, called AEC-M2 (Fig. 8). Itgam and Aif1 are genes coding respectively for the two proteins CD11b and Iba-1, exclusively expressed in microglia but not in astrocytes. AEC-M2 presented no Iba1-immunostaining (Fig. 8A) as well as no detectable microglial markers (Itgam and Aif1) mRNA expression (Fig. 8B). Compared to MGC1, AEC-M2 expressed lower GFAP expression levels (Fig. 8A and B). As a result of the absence of microglial cells, a pro-inflammatory LPS exposure had only minor effects on AEC-M2 and was subsequently not able to induce a real pro-inflammatory state (Fig. 8C).

4. Discussion

More than 35 years ago, Booher and Sensenbrenner developed *in vitro* methods for brain cells cultures (Booher and Sensenbrenner, 1972). For decades, these methods have been used by the research community. However, in the last few years, scientists have noticed that, due to their high reactivity potential, microglial cell contamination could alter the experimental response obtained from primary astrocytic cultures. For the purification of glial cells, several protocols are frequently found in the literature, like coating, sub-culturing or shaking methods. To reduce the presence of microglia or other contaminating cells, cytosine arabinoside, a specific toxin, anti-mitotic or lysosomotropic agents can also be employed (Saura, 2007; Hamby et al., 2006a). The mechanical shake-off method is the most widely used, which allows recovering microglial cells that are present above the astroglial monolayer. However, with this procedure, the exclusion of microglial cells from mixed glial cell cultures is incomplete. Indeed, this shaking method appears to be inefficient to remove microglial cells located below or inside the astroglial monolayer.

Here, we have compared mouse MGC1 (twice shaken and washed glial cultures), AEC-M1 (magnetic cell sorted cultures), AEC-M2 (twice-sorted cultures), neurosphere-derived astrocyte cultures and primary microglia cultures. From the mixed glial cultures, magnetic cell separation allows a single-step isolation of microglial cells (CD11b-positive fraction), but also AEC-M1 (CD11b-negative fraction). In this study, these different types of culture methods allowed us to evaluate the importance of the presence of microglial cells in the establishment and analysis of an inflammatory condition in glial cultures.
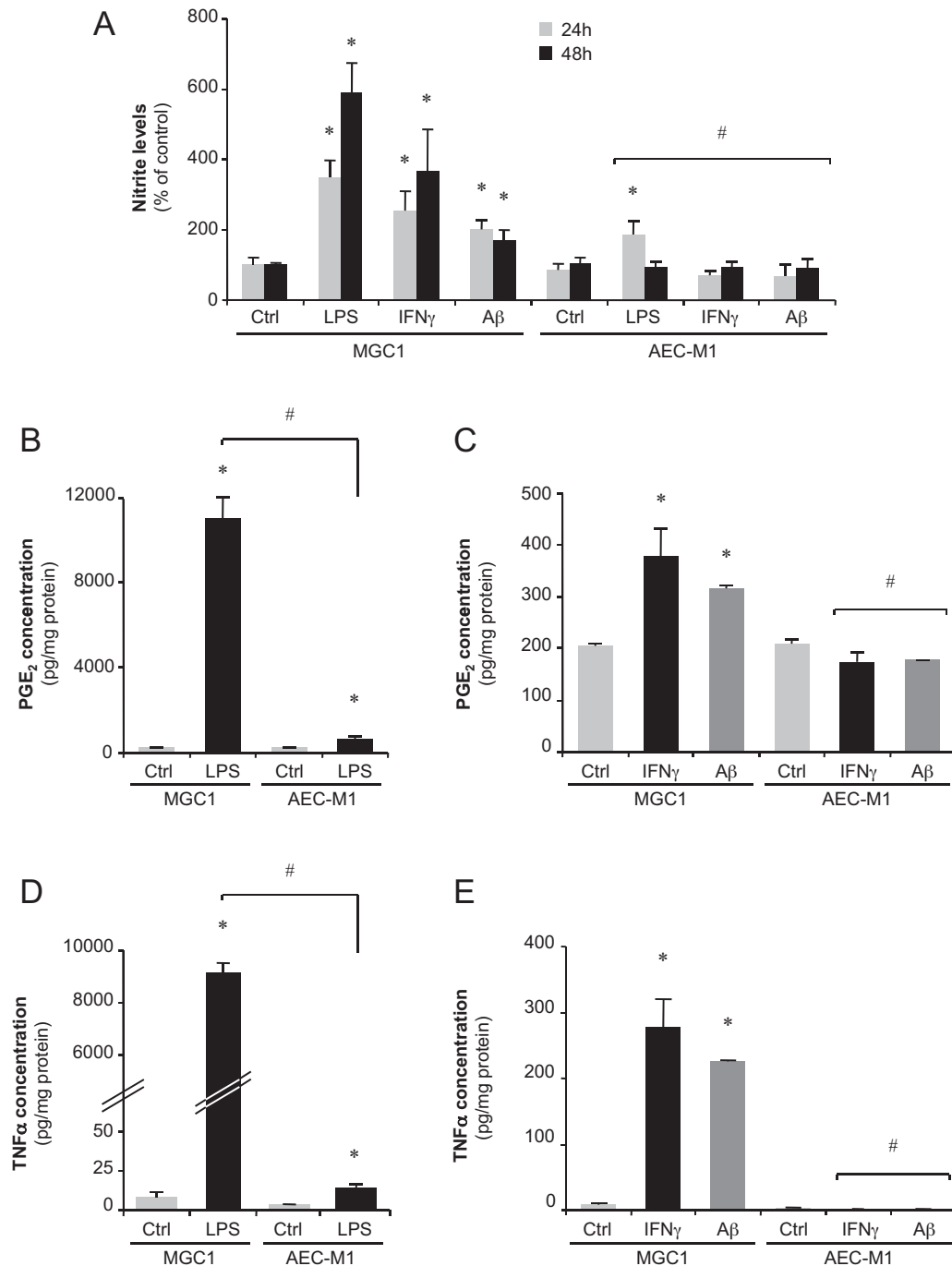
Fig. 6. Measurement of pro-inflammatory mediator releases. Nitrite (A), PGE$_2$ (B and C) and TNFα (D and E) releases were quantified after 24 h and 48 h (for nitrites only) of LPS (1 μg/ml), IFNγ (50 U/ml) or Aβ (5 μM) exposure in supernatant of MGC1 and AEC-M1. Results are given as mean ± SEM (n = 3 independent experiments). *$p < 0.05$, significantly different from control; #$p < 0.05$, significantly different between MGC1 and AEC-M1.

The first part of this work aimed at determining the proportion of microglia in MGC1 and AEC-M1. By immunocytochemistry, we confirmed that Iba1-positive cells are present in mouse MGC1 even after two shaking and washing steps. Conversely, after CD11b magnetic separation, the proportion of Iba1-positive cells in the negative fraction, corresponding to the AEC-M1, was, as expected, much lower than in mixed cultures. The immunostaining of the glial fibrillary acidic protein (GFAP), an intermediate filament component of astroglial cells (Bramanti et al., 2010; Röhl et al., 2007), was slightly higher in MGC1 than in AEC-M1. GFAP is a tightly regulated protein, whose expression is induced by multiple factors such as brain injury and diseases (Middeldorp and Hol, 2011).

Removing microglial cells from culture promoted a decrease of the basal levels of secreted compounds like TNFα. Thus, a decrease of the astrocytic reactivity related to a down-regulation of GFAP expression was subsequently observed. The amount of microglial cells in glial cultures was accurately determined by flow cytometry experiments. A CD11b magnetic separation decreased the Iba1-positive cells from 9 to 10% in MGC1 to 1–1.5% in AEC-M1. Consequently, the GFAP-positive cells increased from 60% in MGC1 to 70% in AEC-M1. These results are in accordance with recent assessments of glial populations in cultures (Marek et al., 2008). Besides, cells that were negative for microglial and astroglial markers in both cultures were probably mainly precursors and differentiated
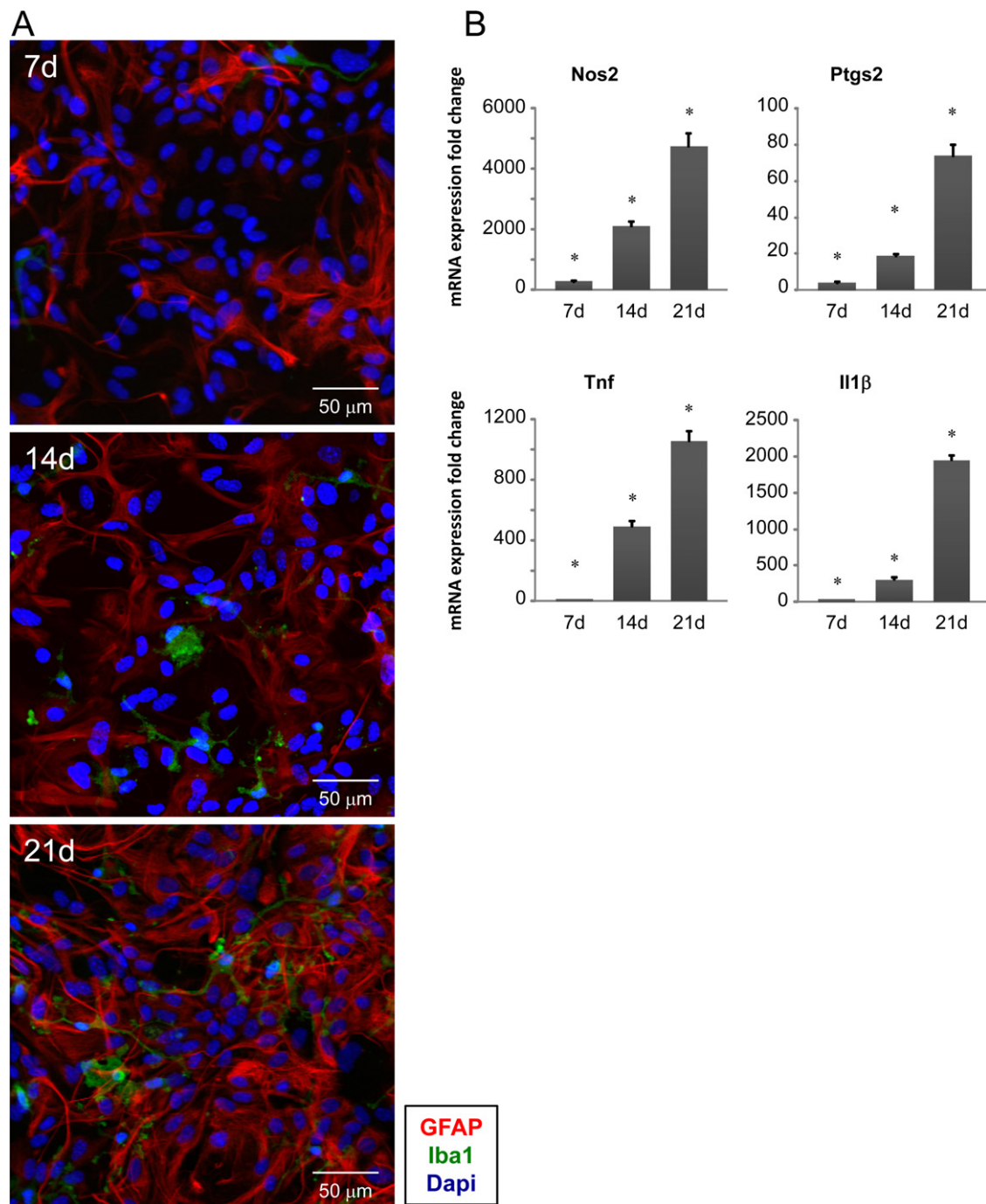
Fig. 7. Time-dependent effect of a microglial contamination. AEC-M1 were cultured for 7, 14 and 21 days after CD11b-magnetic cell sorting. These cells were labeled with anti-GFAP antibody (red) in combination with the microglial marker Iba1 (green) and counterstained with nuclear stain DAPI (blue) at the different time points (A). Pro-inflammatory gene Nos2, Ptgs2, Tnf and Il1β expressions (B) were also determined in these cultures after 6 h of LPS (1 μg/ml) exposure. The transcription levels were analyzed by real-time PCR. Control expression levels were fixed at 1.0. All gene expressions were normalized to Rpl27 expression level. Results are given as mean ± SEM ($n = 3$ independent experiments). *$p < 0.05$, significantly different from control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

oligodendrocytes, but also GFAP-negative astrocytes. In fact, it has been reported that some astrocytes populations in healthy CNS do not express, or express at low level, GFAP (Sofroniew and Vinters, 2010; Sofroniew, 2009; Buffo et al., 2008).

Microglia, the most reactive cells against pro-inflammatory stimuli, detect and respond rapidly to pro-inflammatory triggers (Lull and Block, 2010; Kim and Joh, 2006). Based on previous papers, astrocyte-mediated effects are completely masked when cultures contain a large population of microglia. For this reason,

we agree with the authors who have underlined the necessity to minimize the proportion of microglial cells in glial cultures (Marek et al., 2008; Saura, 2007; Hamby et al., 2006a,b; Hewett et al., 1999). Thus, in this work, we have made a direct comparison between cultures with a known percentage of microglia contamination and cultures with minimal or no microglia. To induce an inflammatory environment, we treated our glial cultures with pro-inflammatory compounds, known to promote cellular activation by distinct biological pathways. Thus, we have used a nuclear
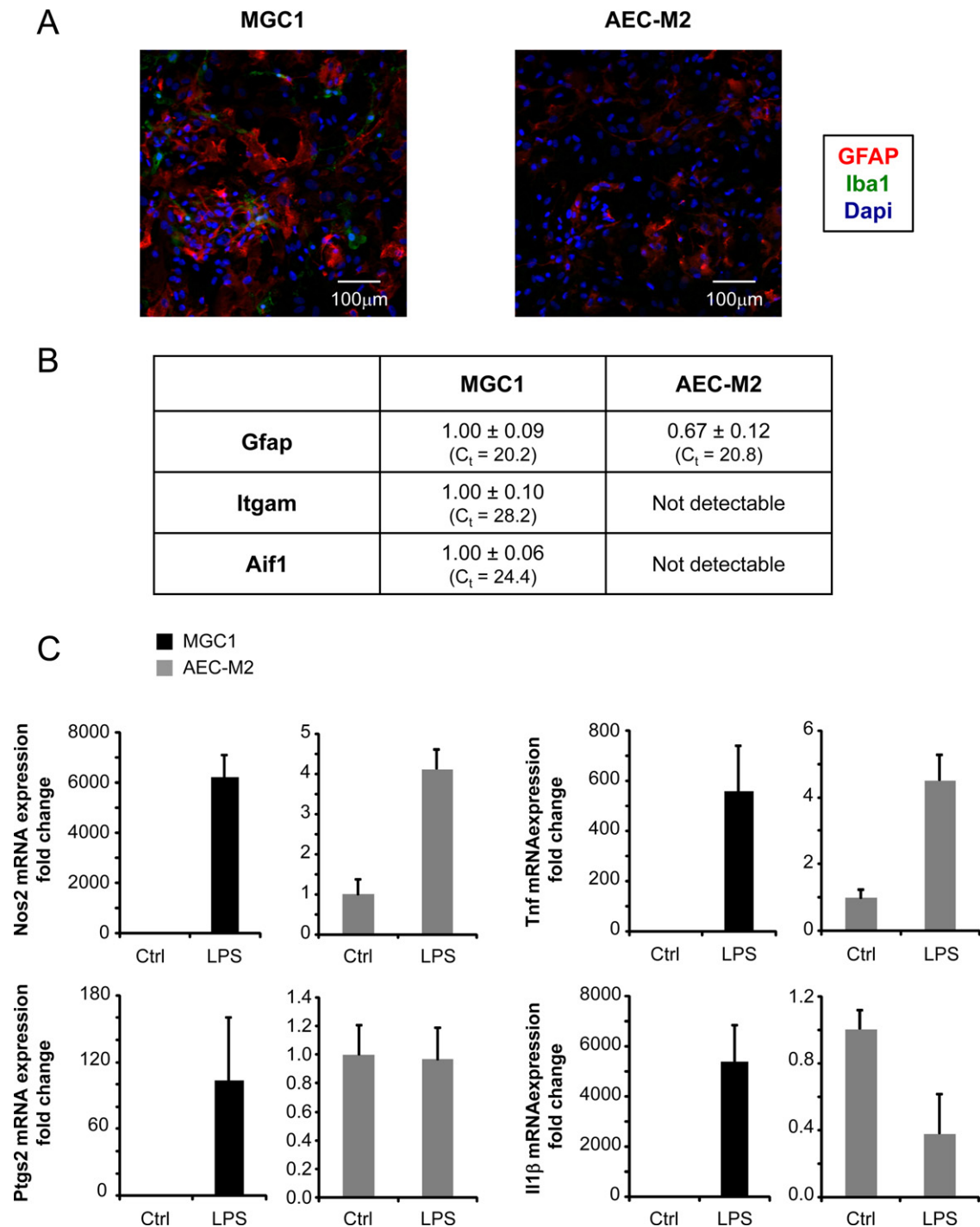
Fig. 8. Comparison of MGC1 with AEC-M2. Both glial cells cultures were labeled with anti-GFAP antibody (red) in combination with the microglial marker Iba1 (green) and counterstained with nuclear stain DAPI (blue) (A). Transcription levels and C_t values of the astrocytic marker (Gfap) as well as microglial markers (Itgam and Aif1) were evaluated by real-time PCR in control condition in both glial cultures (B). Cultures were also treated with LPS (1 μg/ml) for 6 h. The transcription levels of the pro-inflammatory genes Nos2, Ptgs2, Tnf and Il1β were analyzed by real-time PCR (C). Control expression levels were fixed at 1.0. All gene expressions were normalized to Rpl27 expression level. Results are given as mean ± SEM ($n = 3$ independent experiments). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

factor kappa B (NFκB) pathway-inducer (LPS) (Carmody and Chen, 2007), a janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway-inducer (IFNγ) (Gough et al., 2008) as well as the beta-amyloid peptide (1–42) (Aβ) in an oligomeric conformation (Heurtaux et al., 2010). In recent studies, we have demonstrated that LPS, IFNγ, as well as the oligomeric form of the Aβ peptide were able to strongly activate microglial cells (Heurtaux et al., 2010; Michelucci et al., 2009). At the transcriptional level,

we have observed that 6 h of LPS exposure in MGC1 induced a higher pro-inflammatory state than an IFNγ or Aβ exposure. This pro-inflammatory status appeared to be lower in AEC-M1 where microglial cells were almost absent. In these AEC-M1, a 6 h Aβ exposure was unable to up-regulate Nos2, Ptgs2 and Tnf mRNA expression and only slightly induced Il1β and Il6 expression. Thus, a short single Aβ treatment had poor effects on AEC-M1 activation. In addition, as a result of gene expression modulation, we

have also observed that pro-inflammatory mediators (nitrites, PGE$_2$ and TNFα) releases were strongly decreased in AEC-M1 compared to MGC1 both treated with either LPS, IFNγ or Aβ. Interestingly, results obtained with Aβ clearly showed that a short treatment was unable to activate AEC-M1. This is in accordance with a recent study (Ebenezer et al., 2010). In this work, we have used an oligomeric Aβ preparation, which is known to strongly activate microglial cells. These results support the hypothesis that activation of astrocytes around Aβ deposits in Alzheimer's disease may be due to prior microglial activation inducing finally a chronic inflammatory state. It is well documented that activated microglia represent a major source of inflammatory factors. In this study, we have described that primary microglia strongly reacted to LPS exposure (Table 3). The presence of IFNγ or Aβ peptide also induced a pro-inflammatory phenotype in primary microglial cells. Microglia appeared to be more sensitive to LPS, IFNγ and Aβ treatments than AEC-M1 and MGC1. The strong up-regulation of the pro-inflammatory genes underlined the importance of a microglial contamination even if the percentage of these cells is lower than 10% of the total cells in culture. Thus, to confirm the importance of microglia in the initiation of an inflammatory environment, we have also realized a control by using cultures devoid of microglial cells. These microglia-free astrocyte cultures were obtained after differentiation of neurospheres (Crocker et al., 2008). Challenging these cultures with either LPS, IFNγ or Aβ had only little effect on the up-regulation of pro-inflammatory genes. With the exception of LPS treatment, which is a strong microglial activator, the inflammatory response in AEC-M1 and neurosphere-derived pure astrocyte cultures are obviously very closed. Thus, both cultures represent similar *in vitro* models for astrocyte studies. These results are in accordance with published work (Crocker et al., 2008). In the same way, Hamby et al. (2006a) reported a method for eradicating microglia from primary glial cultures. The authors treated confluent astrocyte monolayers with a mitotic inhibitor (cytosine β-D-arabinofuranoside) followed by a brief exposure to high concentrations of a lysosomotrophic agent (L-leucine methyl ester, LME). By this method, they generated highly purified astrocytes without the need for cell passage. Their results are quite similar to our results and confirm the necessity to eliminate microglia from glial cultures. However, this method using the LME lysosomotrophic agent only permits to obtain near-pure astrocyte cultures when our magnetic separation permits to obtain rapidly both near-pure astrocyte cultures and pure microglial cultures.

Bacterial endotoxin receptors, mainly Toll-like receptor 4 (TLR4), are implicated in LPS bioactivity (Peri et al., 2010). However, in these neurosphere-derived astrocytes, the LPS-TLR4 signaling pathway does not seem to be operational. This observation could be linked to the low level of TLR4 expression in astrocytes (Mishra et al., 2006; Jack et al., 2005). TLR4 receptor is documented to be present on circulating monocytes, macrophages and other systemic immune cells. We have also demonstrated that microglia strongly express TLR4 whereas astrocytes do not express it (data not shown). These data are in accordance with a previous work (Lehnardt et al., 2002). Moreover, due to the high TLR4 expression level in microglia (Buchanan et al., 2010; Mishra et al., 2006; Jack et al., 2005), treating glial cultures with LPS could represent a way to confirm their presence. Furthermore, the basal expression levels of pro-inflammatory genes (Tnf, Il1β and Ccl3), as well as those of specific microglial markers (Itgam and Aif1) are higher in primary microglia than in MGC1 (Table 2). In AEC-M1 and in neurosphere-derived astrocytes, the expression levels are even lower (threshold cycle $C_t > 30$). According to these data, the level of microglial cell contamination could also be evaluated by a simple comparison of the threshold cycle of these pro-inflammatory genes or microglial markers in basal conditions in cultures.

We have also described time-dependent effects of a microglial contamination. AEC-M1 were studied up until 3 weeks after plating. The percentage of Iba1-positive cells increased in a time dependent manner in these non-treated confluent glial cultures. Possibly as a result of the increased number of microglia, the GFAP expression level was also enhanced. This is in accordance with our observations, where we described that astrocytic reactivity and GFAP expression were increased in the presence of microglial cells (Fig. 2). Moreover, a LPS exposure induced a time dependent pro-inflammatory state, which was higher after 3 weeks of culture, and coincided with the presence of a high number of contaminating microglial cells. Finally, we have compared MGC1 and AEC-M2 (twice-sorted glial cultures). The AEC-M2 presented a lower GFAP expression level and no detectable microglial markers expression. Furthermore, a LPS exposure was not able to induce a real pro-inflammatory state, thus confirming the absence of microglial cells with this culturing procedure.

In our conditions, we clearly show that microglia-depleted cultures (AEC-M1/M2 and neurosphere-derived astrocytes) are weakly activated by pro-inflammatory agents. The presence of microglia appears to be crucial in the establishment of an inflammatory state. There is growing interest about the role of glial cells in neurodegenerative pathologies (Amor et al., 2010; Hamby and Sofroniew, 2010; Perry et al., 2010). On one hand, the isolation of glial cells allows studying the responsiveness of each cell type to pro-inflammatory agents. On the other hand, the use of mixed cultures could reflect the physiological condition and could be useful to observe global modifications and cellular interactions characterizing CNS disorders. Microglial cells rapidly and strongly react to brain insults. Due to their high reactivity to various aggressions or pro-inflammatory stimuli, microglia-derived effects might be credited to astrocytes in mixed cultures. In these conditions, the subsequent reactive astrocytes interact in a complex manner with microglia and can exert pro-inflammatory but also anti-inflammatory effects (Fuller et al., 2010; Zhang et al., 2010).

In conclusion, our results suggest the necessity to rigorously check the purity of glial cultures. Attention should be paid to the presence of microglia in glial cultures because of their potential alteration of the results, especially when inflammatory processes are investigated. For that reason, the magnetic cell sorting technology appears to be an efficient and rapid method to limit microglial contamination in glial cultures. In addition, it allows to rapidly obtain near-pure astrocyte cultures but also pure microglial cultures.

Conflict of interest statement

No conflicts of interest exist.

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