A Simplified Approach for Efficient Isolation of Functional Microglial Cells: Application for Modeling Neuroinflammatory Responses *In Vitro*

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Purified microglial cells in culture are frequently used to model brain inflammatory responses but obtaining large yields of these cells on a routine basis can be quite challenging. Here, we demonstrate that it is possible to achieve high-yield isolation of pure microglial (MAC-1$^+$/Fcrls$^+$/Ccr2$^-$) cells from postnatal brain tissue through a simple culture procedure that mainly relies on the adhesion preference of these cells to the polycation polyethyleneimine (PEI) in serum-supplemented DMEM medium. Accordingly, other synthetic or biological substrates failed to mimic PEI effects under the same culture conditions. Replacement of DMEM by DMEM/F12 nutrient mixture did not permit microglial cell isolation on PEI coating, indicating that PEI effects were context-dependent. Remarkably, the lack of culture feeding during progression of microglial cell isolation strongly improved cell yield, suggesting that nutritional deprivation was required to optimize this process. When generated in large culture flasks coated with PEI, cultures of microglial cells were easily recovered by trypsin proteolysis to produce subcultures for functional studies. These cultures responded to lipopolysaccharide (LPS, 1–10 ng/ml) treatment by secreting pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and by generating nitric oxide and reactive oxygen species. Most interestingly, this response was curtailed by appropriate reference drugs. Microglial cells were also strongly responsive to the mitogenic cytokine GM-CSF, which confirms that the functional repertoire of these cells was well preserved. Because of its high yield and simplicity, we believe that the present method will prove to be especially convenient for mechanistic studies or screening assays.

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Key words: cell culture, cytokines, lipopolysaccharide, polyethyleneimine, nitrogen and oxygen reactive species

Introduction

Microglial cells, the resident immune cell population in the central nervous system (CNS), originate from immature phagocytes that migrate from the yolk sac early in embryonic development to enter the CNS through the primitive vascular system (Ginhoux et al., 2010; Kierdorf et al., 2013). Besides established functions of immune surveillance and phagocytic clearance in the brain, microglial cells have been implicated in facilitating neurogenesis and neural repair (Aarum et al., 2003; Perry and O'Connor, 2010; Walton et al., 2006), as well as in participating in the modulation of neuronal functions (Béchade et al., 2013; Parkhurst et al., 2013). However, in chronic neurodegenerative pathologies such as Alzheimer's or Parkinson's diseases (Akiyama et al., 2000; Barcia et al., 2011; Noelker et al., 2014), in psychiatric disorders such as schizophrenia (Aguzzi et al., 2013; Najjar

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and Pearlman, 2015) and in states of chronic pain (Ji et al., 2014) there is compelling evidence that persistent reactive microgliosis participates actively to disease progression.

In this context, purified cultures of microglial cells present a great interest as they can be used (i) for improving our understanding of how microglial cells become chronically activated in pathological conditions and evade intrinsic mechanisms of regulatory control (Block and Hong, 2007; Glass et al., 2010) and (ii) for discovering molecules of potential clinical interest. These studies, however, have been often delayed due to inherent difficulties to obtain large yields of microglial cells on a routinely basis. Immortalized cell lines, such as the BV-2, HAPI, and N9 cells, have been widely used to study microglial responses (Henn et al., 2009; Horvath et al., 2008). However, they possess a far more limited cytokine and chemokine expression profile in comparison with primary microglia, among other functional differences (Gresa-Arribas et al., 2012; Henn et al., 2009; Horvath et al., 2008; Stansley et al., 2012). Thus, the use of primary microglial cultures to validate the results obtained from microglial cell lines is generally required (Gresa-Arribas et al., 2012).

In response to this need, a number of methods have been established to produce purified primary microglial cells in culture. The most widely used method consists in separating by vigorous shaking loosely adherent microglial cells from confluent primary mixed glial cultures from newborn rodent cerebral cortices (Chamak and Mallat, 1991; Giulian and Baker, 1986). This method is not only tedious and time-consuming, but it also produces a relatively low yield of microglial cells. A number of modifications have been therefore proposed to improve the yield in microglial cells and also to eliminate the shaking step from the procedure (Floden and Combs, 2007; Saura et al., 2003). One of these methods consists in separating microglial cells that are directly adherent to plastic cell surfaces from an upper layer of cells consisting essentially of astrocytes and microglial cells (Saura et al., 2003). The detachment of the upper layer can be achieved in one piece through mild trypsinization of the cultures followed by aspiration. Of interest, this technique provides a much higher yield of microglial cells than the previous one. However, it requires great expertise in the manipulation of the cultures. More recently, the purification of microglial cells in culture has also been made possible by positively selecting cells expressing the pan-microglial marker CD11b (Gordon et al., 2011; Marek et al., 2008). While this approach is also quite effective, it may turn to be costly when used routinely for screening as it requires CD11b antibodies.

Here, we propose a simplified procedure to obtain highly purified primary microglial cells in culture from the brain of postnatal pups. This technique arises from the serendipitous observation that there is a progressive eradication of astrocytes and the preservation of a virtually pure and dense population of microglial cells in postnatal mouse brain cultures that are seeded in culture vessels coated with the polycation polyethyleneimine (PEI) and then maintained in DMEM culture medium supplemented with fetal calf serum (FCS). Of interest, we found that microglial cells isolated by this technique possess a large functional repertoire. In particular, they were sensitive to low concentrations of the bacterial inflammogen lipopolysaccharide (LPS) and also demonstrated a typical mitogenic response after treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF).

List of abbreviations

APO	apocynin
Ccr2	Chemokine (C-C Motif) Receptor 2
CNS	central nervous system
DAPI	4′,6-Diamidino-2-phenyindole
DEX	dexamethasone
DIV	days in vitro
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
Fcrls	Fc receptor-like S, scavenger receptor
FCS	fetal calf serum
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
Iba-1	Ionized calcium binding adaptor molecule-1
IL	interleukin
LPS	lipopolysaccharide
MAC-1	macrophage antigen-1
NFκB	nuclear factor Kappa-B
NO	nitric oxide
PEI	polyethyleneimine
POR	poly-l-ornithine
ROS	reactive oxygen species
Rplp0	ribosomal protein, large, P0
RT	reverse transcription
TNFα	tumor necrosis factor alpha
TRO	trolox

Materials and Methods

Leibovitz's L-15 medium, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 nutrient mixture, Trypsin-EDTA 0.05%, and penicillin/streptomycin cocktail were all purchased from Invitrogen Life Technologies (Saint Aubin, France). FCS was obtained from Biowest LLC (Eurobio, Les Ulis, France). PEI (average molar mass 750,000, #P3143), poly-L-ornithine (POR; molecular weight 30,000-70,000, #P4957), the RGD tripeptide (#A8052), laminin from mouse Engelbreth-Holm-Swarm sarcoma (#11243217001), LPS (*Escherichia coli* strain O26:B6; #L8274), 4′,6-DiAmidino-2-PhenyIndole (DAPI), trolox (TRO), and dexamethasone (DEX) were all purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). Interleukin (IL)−6 and TNFα enzyme-linked immunosorbent assay (ELISA) kits were obtained from Life Technologies. The mouse IL-1β ELISA kit, recombinant endotoxin-free mouse GM-CSF (#415-ML-010), mouse anti-GM-CSF (#MAB415-500), and apocynin (APO) were purchased from R&D Systems Europe (Lille,

France). Rat anti-macrophage antigen-1 (MAC-1; clone M1/70.15), also named CD11b, was from Bio-Rad (Colmar, France), rabbit anti-ionized calcium binding adaptor molecule-1 (Iba-1, #019-19741) from Wako (Neuss, Germany), rabbit anti-glial fibrillary acidic protein (GFAP, #Z0334) from Dako (Glostrup, Denmark) and rabbit monoclonal anti-Ki67 antibody (#ab16667) from Abcam (Cambridge, UK). CellROX Deep Red Reagent was obtained from Invitrogen Life Technologies. The Griess reagent system was purchased from Promega (Charbonnières, France).

Coating Procedures

Polycation coating solutions consisted of either 1 mg/ml PEI diluted in a pH 8.3 borate buffer (Michel et al., 1997; Rousseau et al., 2013) or 1.5 μg/ml POR diluted in PBS (Xiao et al., 2007). These solutions were applied to culture vessels for 1 h at 37°C, then washed 4 times with PBS before application of culture medium and cell seeding. The RGD tripeptide and laminin were also used as coatings in order to mimic cell attachment to extracellular matrix (ECM) substrates. The tripeptide was diluted in distilled water at a concentration of 5 μM. It was applied for 1 h at 37°C, then washed 4 times with H_2O before addition of culture medium. Laminin coating was carried out according to the manufacturer's instructions, applying 1.1 μg of laminin per well diluted in PBS. PEI-RGD and PEI-laminin coatings were performed by sequential application of PEI and RGD or PEI and laminin. For 48 or 96 multiwell plates and T-75 culture flasks, we used 0.3, 0.05, and 10 ml of coating solutions, respectively.

Microglial Cell Isolation

Animals were housed, handled, and taken care in accordance with recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union Council Directives (86/609/CEE). Experimental procedures were authorized by the ethical committee on animal experiments Charles Darwin No. 5.

Microglial cultures were prepared from the brains of postnatal day 1 C57BL/6J mouse pups (Janvier LABS, Le Genest St Isles, France). Whole brains were harvested, meninges stripped away and brain tissue pieces placed in a 15 ml polypropylene test tube containing 4 ml of Leibovitz's L-15 medium. Cells were then mechanically dissociated by repeated pipetting (50–60 strokes) using a sterile blue tip fitted to a Gilson pipette set to 900 μl. DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (defined as complete medium) was added to dissociated cells to complete the total volume to 12 ml. Subsequently, cells were gently mixed by inversion of the test tube and debris allowed to settle down at room temperature for 5 min. The supernatant containing dissociated cells in suspension was transferred to a precooled 50 ml sterile polypropylene conical tube, while tissue pieces that remained not dissociated at this stage were taken for another round of trituration. The final supernatant was then centrifuged at 1,000 rpm for 5 min at 4°C and the resulting pellet triturated before plating.

To determine how the isolation of microglial cells was impacted by different culture conditions, we plated the equivalent of 0.1 mouse brain tissue/well using 48-well culture plates (Nunc, Thermofischer Scientific, Illkirch, France) that were coated or not with synthetic or biological substratum. Cultures were then maintained in complete medium or when specified in DMEM/F12 nutrient mixture supplemented with 10% FCS and antibiotics for time periods indicated in the text. When required, a fraction (i.e., 2/5) of complete DMEM medium was renewed or an equivalent amount of FCS was added to the cultures without culture medium change, as indicated in the text. The incubation was performed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

To produce microglial cells in bulk quantities, the equivalent of two mouse brains were plated onto PEI-coated Corning T-75 flasks (Sigma-Aldrich) containing 10 ml of complete medium. Eight milliliters of culture medium was removed 48 h after plating to eliminate floating debris and 12 ml of fresh medium was added. No additional medium change was done until the total disappearance of astrocytes, i.e., after 16-18 DIV. If the cultures had to be maintained for longer periods of time before processing, 6 ml of complete medium were added to culture flasks.

Functional Characterization of PEI-Isolated Microglial Cells

Microglial cells isolated through the PEI procedure in T-75 culture flasks were taken for trypsinization in order to seed cells into culture wells, at densities that were suitable for experimental protocols and functional characterization. Briefly, after three gentle washes with 10 ml serum free-DMEM, microglial cells were detached from their culture support by adding 5 ml of a trypsin (0.05%)-EDTA (2 mM) solution. After 5 min of incubation at 37°C, 10 ml of DMEM complete medium was added to neutralize the trypsin solution. Cells recovered by centrifugation at 1,000 rpm for 5 min (4°C), were then seeded onto PEI precoated Nunc 48, at density of 100,000 living cells per well, unless otherwise specified.

Microglial cells were then treated with LPS (1 or 10 ng/ml) for 24 h to study inflammatory responses. Compounds susceptible to interfere with the inflammatory process, the glucocorticoid receptor agonist DEX (10 μM), the NADPH oxidase inhibitor APO (500 μM) and the water soluble analog of vitamin E TRO (10 μM), were added 4 h before initiating the stimulation with LPS and were left thereafter in contact with the cells.

For the proliferation assays with GM-CSF, 60,000 living microglial cells were seeded onto PEI precoated 48-well plates. Cultures were then stimulated or not with 2 ng/ml mouse recombinant GM-CSF for 72 h. An antibody against mouse GM-CSF (5 μg/ml) was also tested to neutralize the effects of GM-CSF. To study the possible involvement of GM-CSF during the phase of isolation of microglial cells onto PEI coating, we added 5 μg/ml of the same antibody to mixed cultures every 3 days until 21 DIV.

Protein Detection by Immunofluorescence

After termination of treatments, cultures were fixed with 4% formaldehyde (12 min, 4°C), washed with PBS, and incubated overnight with antibodies against MAC-1 (1:100 in PBS) or Iba-1 (1:500 in PBS) to detect microglial cells, or with an antibody against GFAP (1:200 in PBS with 0.2% Triton X-100) to identify astrocytes. Ki67 immunolabeling of proliferating cells (Muskhelishvili et al., 2003)

was performed with a rabbit monoclonal antibody (1:200 in PBS Triton X-100) applied for 6 h to the cultures. When double immunostainings Iba-1/MAC1 or Ki67/MAC-1 were performed, the detection of MAC-1 was performed prior to that of Iba-1 or Ki67. As secondary antibodies, we used a goat anti-rat-Alexa Fluo 488 or a goat anti-rabbit Alexa Fluo 488 (Invitrogen). When needed, nuclei of labeled cells were counterstained with DAPI (1 μg/ml). Phase contrast and fluorescent images were acquired using a Nikon TE 2000 inverted microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital camera and the HCimage Imaging software (Hamamatsu, Corp., Bridgewater, NJ).

To assess changes in Iba-1 immunosignal intensities upon test treatments, we measured changes in fluorescence levels in regions of interest delimited by MAC-1^+ immunostaining. Fluorescent images of 10 randomly chosen fields were acquired with identical acquisition parameters using a 20× fluorescence objective. Specific fluorescence intensities were corrected by subtracting local background intensities after omission of the primary Iba-1 antibody. The ImageJ software (W.S. Rasband, ImageJ, US National Institutes of Health, Bethesda, MD) was used for quantification of fluorescent signals. Results were expressed in fold increase of control values.

Cytokine Level Measurements

Conditioned media were collected at the end of treatment periods and frozen at −20°C before further processing. Cytokine concentrations were measured using ELISA kits (IL-6, TNFα, and IL-1β). The absorbance of each sample was measured according to the manufacturer's instructions using a spectrophotometer SpectraMax M4 (Molecular Devices, Sunnyvale, CA). ELISA standard curves were generated using a four parameter logistic curve model (SigmaPlot 12.0 Systat Software, San Jose, CA).

Measurement of Nitrite Levels in the Culture Supernatants

Nitrite oxide (NO) released in the culture supernatants was assessed using the Griess Reagent protocol that measures nitrite (NO_2^-), one of two primary stable and nonvolatile breakdown products of NO (Tarpey et al., 2004). To optimize nitrite detection, cells were seeded in uncoated 96 multiwell plates at a density of 75,000 cells per well. These cultures were maintained in 100 μl of DMEM complete medium until processing.

Quantification of Intracellular ROS Levels

Intracellular reactive oxygen species (ROS) were assessed using the membrane permeable CellROX Deep Red Reagent, a fluorogenic probe that produces bright near-infrared fluorescence upon oxidation. Briefly, microglial cultures exposed to CellROX (10 μM), 30 min before termination of test treatments, were washed and fixed with 4% formaldehyde in PBS before further analysis. Intracellular ROS quantification was then made using inverted fluorescence microscopy. For each culture condition, fluorescent images of 10 randomly chosen fields were acquired with identical acquisition parameters using a 20× fluorescence objective. Results were expressed in fractional change in fluorescence intensity relative to baseline in control cultures (F/F0) according to protocols described previously for calcium imaging (Toulorge et al., 2011).

RNA Extraction

Total RNA was extracted from PEI-isolated microglial cultures at 21 DIV using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

RT-qPCR Analysis

For reverse transcription (RT), we used 700–900 ng total RNA in 20 μl of reverse transcription reaction mixture (QuantiTect Reverse Transcription Kit, Qiagen). After the RT, qPCRs were performed to analyze the expression levels of mRNA encoding Fc receptor-like S, scavenger receptor (Fcrls), Chemokine (C-C Motif) Receptor 2 (Ccr2), and Nuclear Factor Kappa-B (NFκB) subunit 2. mRNA levels were expressed relative to the housekeeping gene encoding the 60S acidic ribosomal protein P0 (Rplp0) using the formula $2^{-\Delta Ct}$, where Ct is the threshold cycle and ΔCt, Ct_X-Ct_{Rplp0}. qPCRs were performed using a QuantiTect SYBR Green PCR Kit (Qiagen). Primer sets detecting murine Fcrls, Ccr2, NFκB2, and Rplp0 were purchased from Qiagen (QuantiTect Primer Assays: murine Fcrls: Mm_Fcrls_1_SG (Amplicon length: 81 bp); murine Ccr2: Mm_Ccr2_3_SG (Amplicon length: 149 bp); murine Nfkb2: Mm_Nfkb2_1_SG (Amplicon length: 115 bp); murine Rplp0: Mm_Rplp0_1_SG (Amplicon length: 125 bp), Qiagen, Hilden, Germany).

Statistical Analysis

The data were analyzed by one-way ANOVA followed by the Tukey post-hoc test. $P < 0.05$ was considered to be significant. All data are presented as the mean ± SEM of at least three-independent experiments except when noted. Statistical analysis was performed with the SigmaPlot 12.0 software.

Results

PEI Coating Promotes Microglial Cell Enrichment in Postnatal Mouse Brain Cultures: Comparison with Other Synthetic Substrata

We characterized cell populations in cultures from postnatal mouse pups grown onto PEI- or POR-coated culture wells or directly onto the polystyrene surface of culture wells (Fig. 1A–D). After 2 days of growth, total cell numbers (DAPI$^+$ cell nuclei) were estimated at about 190,000 cells/10-mm well in PEI coated wells. In POR-coated and in uncoated wells, cell numbers reached about 155,000 cells/10-mm well, at the same culture age. Irrespective to the type of coating used, cultured cells were predominantly GFAP$^+$ astrocytes at this stage of maturation.

PEI coating favored progressive enrichment of the cultures in MAC-1^+ cells having a microglial appearance while allowing gradual and spontaneous eradication of GFAP-positive astrocytes (Fig. 1A,D). With PEI coating, ~70% and 99% of cultured cells were MAC-1^+, at 14 and 21 DIV, respectively. At 21 DIV, we constantly reached a density of

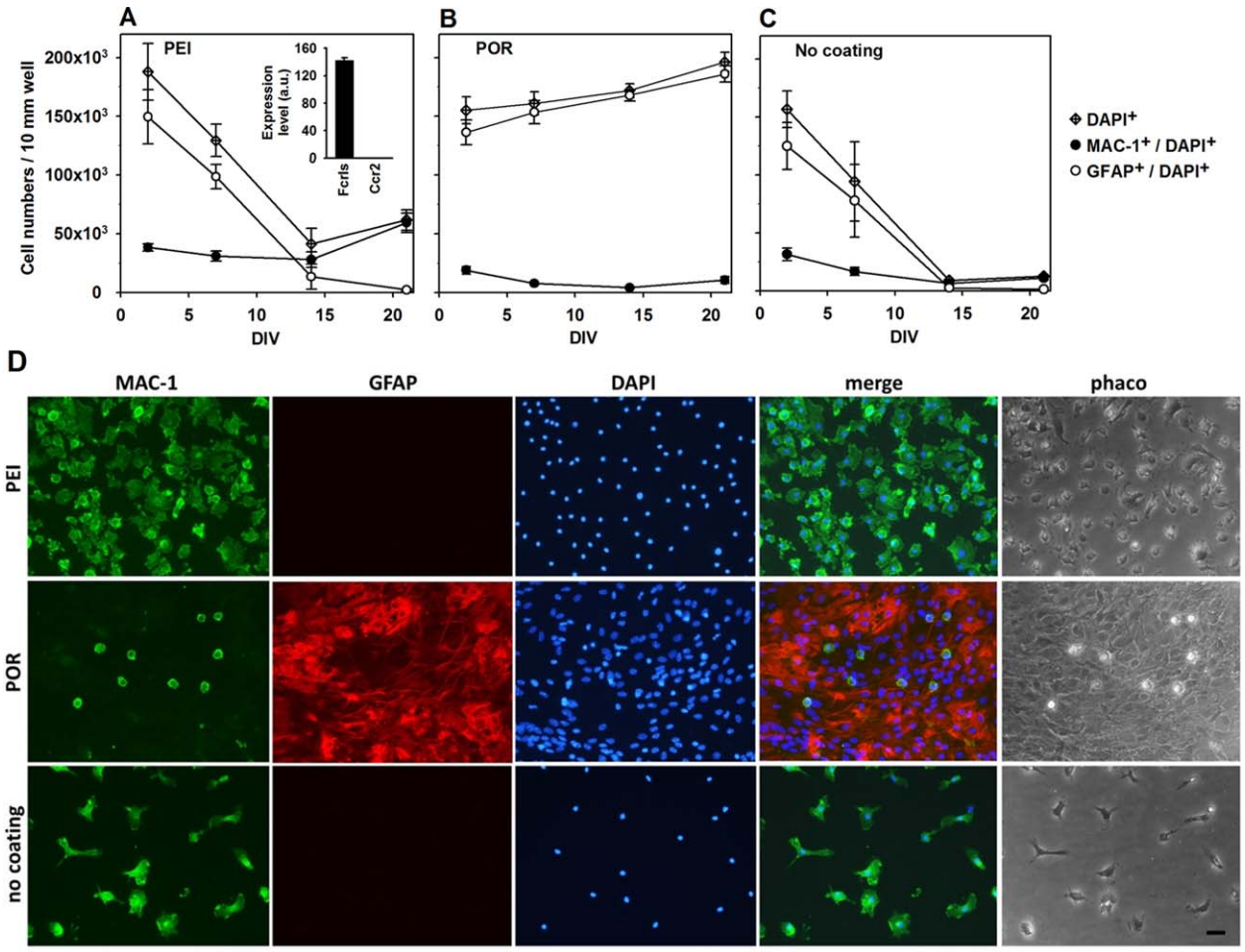

FIGURE 1: PEI coating is permissive for microglial cell isolation. (A–C) Counts of microglial (MAC-1$^+$/DAPI$^+$) cells, astrocytes (GFAP$^+$/DAPI$^+$) and DAPI$^+$ nuclei in brain cultures from P1 mouse pups seeded in culture wells coated with PEI, POR or no specific substrate. Number of cells are expressed as a function of culture time after plating (0 to 21 DIV). *Insert in A*: RT-qPCR analysis of Fcrls and Ccr2 relative to the housekeeping gene Rplp0 in 21 DIV cultures isolated onto PEI. (D) Visualization of MAC-1$^+$ (green), GFAP$^+$ (red) cells and DAPI$^+$ (blue) nuclei in the same visual fields of 21 DIV cultures initially seeded in culture wells coated or not with PEI or POR. Merged and phase contrast (Phaco) images are shown for each culture condition. Scale bar: 20 μm.

50,000–60,000 MAC-1$^+$ cells per 10-mm well (Fig. 1A). Note that neuronal cells and oligodendrocytes characterized by the presence of the pan-neuronal marker microtubule associated protein-2 and the oligodendrocyte marker O4, respectively, were virtually absent from PEI isolated cultures (not shown). To confirm that MAC-1$^+$ cells isolated through the PEI coating procedure were microglial cells, we used RT-qPCR to measure the expression of Fcrls, a biomarker gene that specifically distinguishes microglial cells from other closely related myeloid subsets (Butovsky et al., 2014). We also looked at Ccr2, a chemokine receptor gene typically expressed by peripheral inflammatory monocytes/macrophages (Hellwig et al., 2013; Wermuth and Jimenez, 2015). We found that Fcrls transcripts were expressed in abundance in PEI-isolated cultures when normalized relative to the housekeeping gene Rplp0 (Fig. 1A, insert). At variance, Ccr2 transcripts were virtually undetectable in these cultures ($2^{-\Delta Ct} = 1.8 \times 10^{-4}$), thus confirming that MAC-1$^+$ cells isolated onto PEI coating had a microglial phenotype.

Unlike PEI, POR favored the attachment and proliferation of GFAP$^+$ astrocytes at the expense of MAC-1$^+$ cells so that after 21 DIV <5% of the cells were positively labeled with MAC-1 (Fig. 1B). Note that MAC-1$^+$ cells persisted essentially on the top of an astrocytic cell layer that reached almost confluence in this condition. Finally, in the situation where cells were plated directly onto the polystyrene surface of culture vessels, we also observed an enrichment of the cultures in MAC-1$^+$ cells with a progressive elimination of astrocytes. The overall quantity of microglial cells/culture well was, however, much lower than with PEI (Fig. 1C,D).

When microglial cells were derived from cultures grown in T-75 culture flasks, the time course of their isolation was monitored routinely through phase contrast microscopy. This time course was quite similar to what was observed in

multiwell plates. Typically, we were able to recover 4×10^6 cells/flask after 18-21 DIV, which also corresponds to $\sim 2 \times 10^6$ cells/brain.

Noticeably, coating conditions seemed to not only profoundly influence the fate and density of microglial cells but also their morphology. Microglial cells growing onto PEI-coated surfaces presented a unipolar or bipolar elongated shape with few ramifications or an amoeboid form with spinous processes (Fig. 1D). When seeded onto POR-coated culture vessels, microglial cells had a more compact morphology and they grew atop a monolayer of astrocytes, as revealed by double immunofluorescent staining for MAC-1 and GFAP (Fig. 1D). Purified microglia grown directly onto polystyrene surfaces showed a branched morphology, with multiple spinous processes. This feature distinguished them from microglial cells grown onto PEI. Overall these results suggest that PEI coating allows the isolation of microglial cells by favoring their adhesion and growth, at the expense of astrocytes. Note that regardless of the coating condition we used, nuclei of microglial cells were smaller in size that those of astrocytes and they also contained a very bright and densely compacted chromatin (Fig. 1D).

PEI Effects are Neither Reproduced Nor Amplified by Biological Substrates that Mimic cell-ECM Adhesive Interactions

We also tested whether biological substrates that mimic cell-ECM adhesion were able to reproduce or amplify the interactions that PEI exerts toward microglial cells. We were more specifically interested in evaluating the effects of the tripeptide RGD that acts a common recognition site for integrins in several ECM glycoproteins (Hayman et al., 1985; Xu and Mosher, 2011). Figure 2 shows that the capacity of PEI to promote microglial cell isolation remained unchanged when a double coating with PEI and RGD was carried out. Note that a simple coating with RGD promoted astrocyte growth at the expense of microglial cells (Fig. 2), indicating that proteins with a RGD integrin-binding motif cannot mimic PEI effects in present experimental conditions. Laminin, another ECM glycoprotein which does not have a RGD motif (Xu and Mosher, 2011), prevented the effect of PEI on microglial cell enrichment and also promoted astrocyte isolation on its own (Fig. 2).

Isolation of Microglial Cells onto PEI Coating: Influence of Feeding Procedures and Culture Medium Composition

We tested whether feeding of the cultures had an impact on microglial cell isolation onto PEI coating. To this aim, we quantified MAC-1^+ cells in cultures where 2/5 of complete culture medium was replaced every 3 days until 21 DIV. Using the same paradigm of treatment, we supplemented

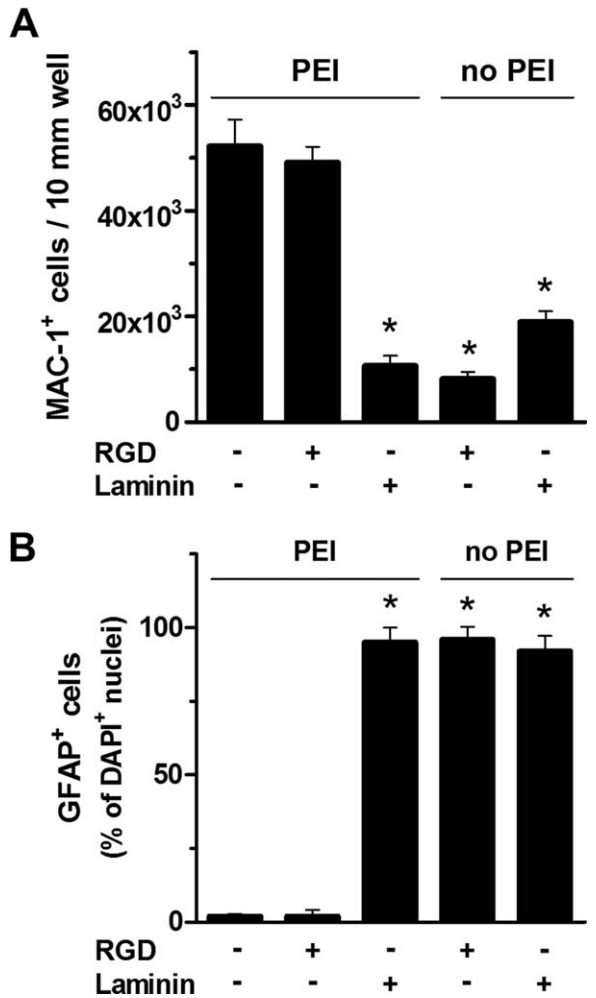

FIGURE 2: Biological ECM components neither improve nor mimic the effects of PEI. (A) Number of microglial (MAC-1^+) cells in 21 DIV mouse brain cultures seeded onto culture wells coated with PEI, RGD, laminin, PEI/RGD or PEI/laminin. Data are expressed in number of MAC-1^+ cells in PEI-coated cultures. (B) Number of astrocytes (GFAP$^+$ cells) expressed in percent of DAPI$^+$ nuclei in the same culture conditions as above. *P <0.05 vs controls.

some cultures with FCS in the amount contained in the fraction of medium that was renewed. At 21 DIV, we observed that MAC-1^+ cells represented >98% of all cultured cells in either conditions, suggesting that culture feeding had no significant impact on astrocyte elimination. Microglial cell yield was, however, drastically reduced in 21 DIV cultures receiving serum or culture medium supplementation (Fig. 3A, left panel). The impact of culture feeding on microglial cell numbers was already evident at 14 DIV, i.e., at a stage where the elimination of astrocytes is still partial in all test conditions (Fig. 3A, left panel).

In an attempt to explain why culture feeding had such a negative impact on microglial cell yields, we compared the proliferation rate of these cells in 14 DIV and 21 DIV

cultures maintained under conditions described before (Fig. 3A, right panel). We observed that in unfed cultures the proliferation rate of microglial cells (% of MAC-1$^+$ cells with Ki67$^+$ nuclei) peaked at 14 DIV while remaining substantial at 21 DIV (Fig. 3A, right panel). At the same time points, this rate was much lower in cultures receiving supplementation with serum or complete medium, suggesting that feeding had a negative impact on microglial cell proliferation. Digitized images depict the expression of Ki67 (green) in DAPI$^+$ nuclei (blue) of MAC-1$^+$ cells (presented as inverted fluorescence images) in 14 DIV control cultures (Fig. 3B).

Interestingly, we also observed that medium composition strongly influenced microglial cell isolation by PEI coating. Indeed, when mixed glial cultures grown on PEI-coated vessels were maintained in complete medium prepared with DMEM/F12 instead of DMEM, the cultures were predominantly enriched in GFAP$^+$ cells after 21 days (>90%, Fig. 3C,D) and they contained a relatively low number of microglial cells.

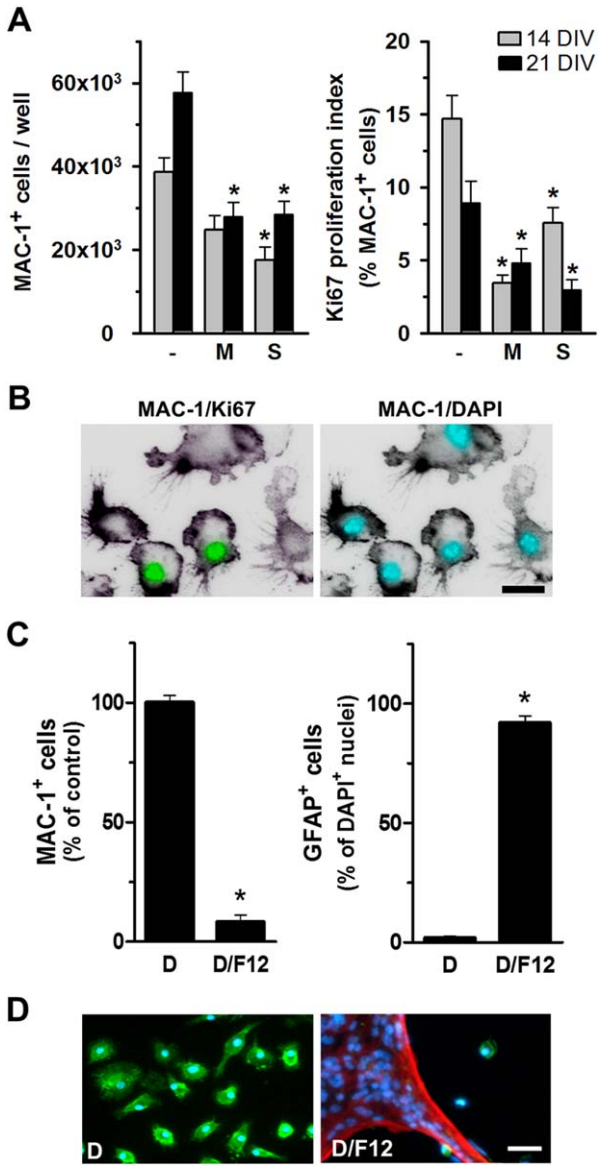

FIGURE 3: Influence of culture maintenance conditions on microglial cell isolation. (A) *Left panel*: Number of microglial (MAC-1$^+$) cells in 14 and 21 DIV mouse brain cultures that were seeded onto PEI-coated wells and then maintained according to the following paradigms: no change of culture medium throughout the culture time (–), partial renewal (2/5) of complete DMEM medium (M) every 3 days or addition of a corresponding amount of FCS with the same frequency (S). *Right panel*: Percentage of Ki67$^+$/DAPI$^+$ nuclei in MAC-1$^+$ microglial cells (Ki67 proliferation index) in the same culture conditions as before. (B) Digitized images illustrating the presence of Ki67 (green) in DAPI$^+$ nuclei (blue) from MAC-1$^+$ cells (shown under an inverted fluorescent format) in 14 DIV control cultures. Scale bar: 15 μm. (C) Number of MAC-1$^+$ microglial cells (*left panel*) and GFAP$^+$ astrocytes (*right panel*) in cultures seeded onto PEI and maintained with either DMEM (D) or DMEM/F12 (D/F12), both supplemented with 10% FCS. (D) Visualization of MAC-1$^+$ microglial cells, GFAP$^+$ astrocytes, and DAPI$^+$ nuclei in 21 DIV mouse brain cultures seeded onto PEI-coated wells and maintained with either complete DMEM or complete DMEM/F12. Note that a large proportion of astrocytes remained attached to PEI-coated wells in cultures that were maintained with DMEM/F12 instead of DMEM. In the meantime, the density of adherent microglial cells was dramatically reduced with DMEM/F12. Scale bar: 30 μm.

Microglial Cells Isolated by Differential Adhesion to PEI Are Highly Responsive to the Bacterial Inflammogen LPS

We wished to functionally characterize microglial cells obtained through the PEI differential adhesion procedure. For that, microglial cells isolated in T-75 culture flasks were taken for trypsinization and seeded into culture wells coated with PEI, at densities that were suitable for experimental protocols. More specifically, we studied the response of microglial cells that were challenged for 24 h with the bacterial endotoxin LPS used at 1 or 10 ng/ml.

First, we observed that the expression of Iba-1, a macrophage/microglia activation marker, was strongly increased in the population of MAC-1$^+$ cells undergoing LPS stimulation (Fig. 4), which was the first sign that microglial cells purified with PEI coating were highly responsive to the bacterial inflammogen. Typical morphological changes were also observed in activated microglia, which displayed hypertrophic cell bodies with ramified projections. Of interest, the synthetic glucocorticoid DEX (10 μM), strongly reduced cellular expression of Iba-1 (Fig. 4A,B). Morphologically, DEX-treated microglial cells had rounded cell bodies with few, if any, processes.

Next, we examined whether microglial cells purified through the PEI coating procedure retained their capacity to produce and secrete pro-inflammatory cytokines upon

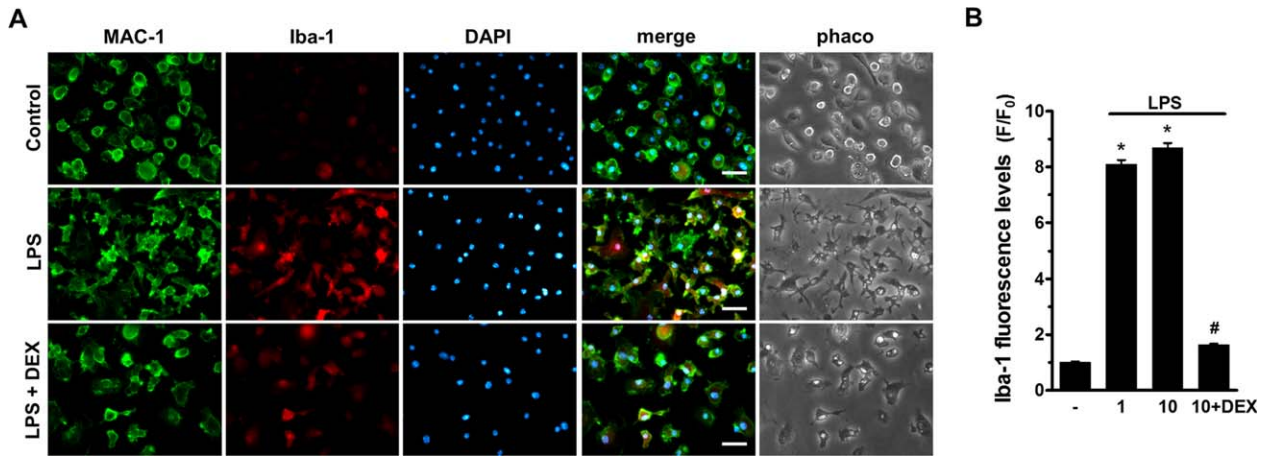

FIGURE 4: Modulation of expression of the microglial marker Iba-1 in PEI-isolated microglial cells exposed to LPS. (A) Immunofluorescent detection of MAC-1 (green) and Iba-1 (red) together with DAPI nuclear staining (blue) in PEI-isolated microglial cell cultures exposed or not to LPS (10 ng/ml for 24 h) in the presence or the absence of the glucocorticoid DEX (10 μM). Corresponding phase contrast (Phaco) images are shown for each conditions. Scale bar: 30 μm. (B) Modulation of Iba-1 fluorescence levels in MAC-1$^+$ cells exposed or not to the same treatments as before. *$P < 0.05$ vs control; $^{\#}P < 0.05$ vs 10 ng/ml LPS.

activation with LPS. More specifically, we assessed the levels of TNFα, IL-1β, and IL-6, i.e., cytokines that are normally induced after LPS challenge (Esen and Kielian, 2007; Horvath et al., 2008; Lee et al., 1994). TNFα, IL-1β, and IL-6 were just above detectable levels in the culture medium of control cultures (Fig. 5A–C), suggesting that the basal state of activation of microglial cells was low in these conditions. Accordingly, we found minimal expression of transcripts encoding NFκB2/p52 ($2^{-\Delta Ct} = 0.24 \pm 0.02$), a subunit of the transcription factor complex NFκB, a master regulator of inflammation (Bennett et al., 2016).

LPS (1 ng/ml) treatment robustly increased the release of all test cytokines when compared with corresponding controls (Fig. 5A–C). This effect was further amplified when LPS was increased to 10 ng/ml, indicating that the response to the inflammogen was concentration-dependent. The effect of LPS was largely reduced when cultures were pretreated with the anti-inflammatory drug DEX (10 μM). These results suggest that microglial cell cultures purified by differential adhesion onto PEI coating retain their potential to produce and release cytokines after activation with LPS.

ROS generation by the enzymatic NADPH oxidase complex is also a critical component of the process of microglial cell activation. Using the CellROX fluorescent probe, we showed that microglial cells purified by PEI coating responded to LPS (10 ng/ml) treatment by generating intracellular ROS (Fig. 6A,B). Noticeably, this effect was almost totally suppressed by DEX (10 μM). APO (500 μM), an inhibitor of NADPH oxidase (Vejrazka et al., 2005), and TRO (10 μM), a soluble vitamin E analog that works as an antioxidant (Nakamura and Hayashi, 1992), also led to a strong reduction in intracellular oxidative stress upon LPS exposure (Fig. 6A,B).

NO produced through activation of the inducible NO synthase is also a key factor in the inflammatory response mediated by microglial cells. Nitrite accumulation in the culture medium which reflects indirectly NO production, was measured 24 h after application of LPS (10 ng/ml) to microglia isolated by PEI coating. As expected, LPS caused a robust increase in nitrite production and DEX (10 μM) largely reduced this effect (Fig. 6C).

Microglial Cells Isolated through Selective Adhesion onto PEI are Responsive to the Mitogenic Cytokine GM-CSF

Microglial cells replated after trypsinization were also challenged with the mitogenic cytokine GM-CSF (2 ng/ml) for 72 h. GM-CSF elicited a strong proliferative response from microglial cells. More specifically, the number of microglial cells was increased by about 230% under these conditions (Fig. 7, left panel). The effect of GM-CSF was totally prevented in cultures treated concurrently with a neutralizing anti-GM-CSF antibody (5 μg/ml). The antibody alone had no effect, however, on microglial cell numbers.

We also assessed the impact of the anti-GM-CSF neutralizing antibody during the process of microglial cell isolation. The yield of isolated microglial cells was not significantly affected when the treatment with the antibody was renewed every 3 days until 21 DIV (Fig. 7, right panel). This excluded the possibility that GM-CSF could favor microglial cell proliferation and isolation onto PEI coating. Coherent with this view, this cytokine remained undetectable in the supernatants of cultures that were obtained with the same protocol (not shown).

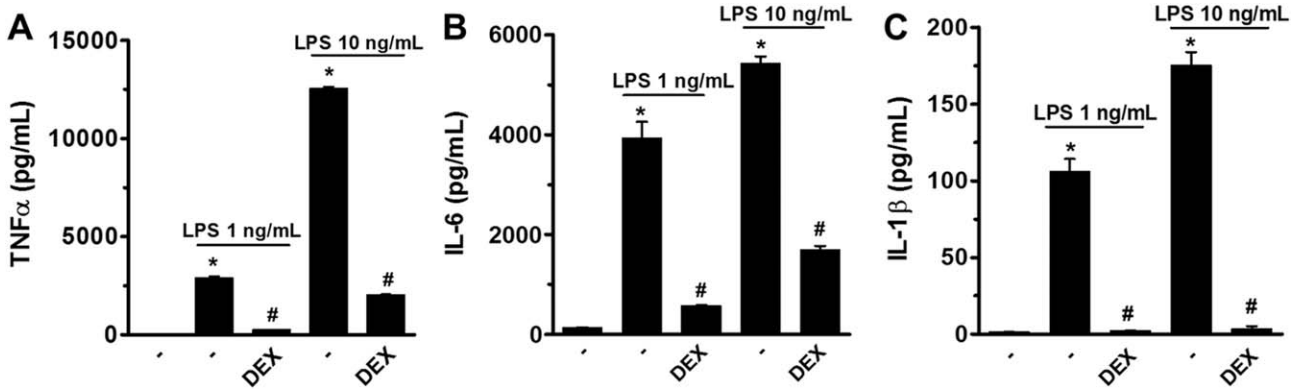

FIGURE 5: Modulation of pro-inflammatory cytokines in PEI-isolated microglial cell cultures exposed to LPS. (A–C) Detection of TNFα, IL-6, and IL-1β in microglial cell cultures submitted or not to LPS treatment (1 or 10 ng/ml for 24 h) in the presence or absence of DEX (10 μM). *$P<0.05$ vs control; #$P<0.05$ vs LPS treatment.

Discussion

Present data demonstrate that the isolation of microglial cells from the brain of newborn mouse pups can be achieved through a simple cell culture procedure that requires the coating of culture vessels with the polycation PEI and the maintenance of the cultures with FCS-supplemented DMEM. Microglial cells isolated through this procedure were easily dislodged from their substrate by mild trypsinization and seeded at densities suitable for functional characterization. Importantly, PEI-isolated microglial cells exhibited typical responses to the bacterial inflammogen LPS and to the mitogenic cytokine GM-CSF.

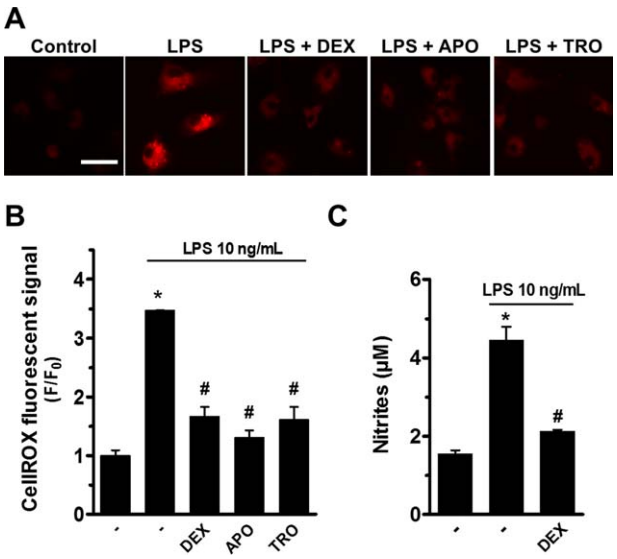

FIGURE 6: Modulation of ROS and NO in PEI-isolated microglial cell cultures exposed to LPS. (A) Intracellular ROS production visualized with the fluorogenic probe CellROX Deep Red in microglial cells treated or not with LPS (10 ng/ml for 24 h) in the presence or the absence of DEX (10 μM), APO (500 μM) or TRO (10 μM). Scale bar: 25 μm. (B) CellROX fluorescence intensities in the same culture conditions as in A. (C) Nitrite production measured in the supernatants of microglial cell cultures treated or not with LPS (10 ng/ml for 24 h) in the presence or absence of DEX (10 μM) (C). *$P<0.05$ vs control; #$P<0.05$ vs LPS treatment.

PEI Coating Allows the Isolation of Pure Microglial Cell Cultures

PEI is a positively charged polymer that has been used as a transfection reagent (Horbinski et al., 2001; Vancha et al., 2004). PEI was also used successfully as an attachment factor for cultivating a number of cell lines (Darios et al., 2003; Vancha et al., 2004) and different types of neuronal cells (Lelong et al., 1992; Toulorge et al., 2011). Here, we serendipitously discovered that in the presence of FCS-supplemented DMEM, PEI coating favors the growth and

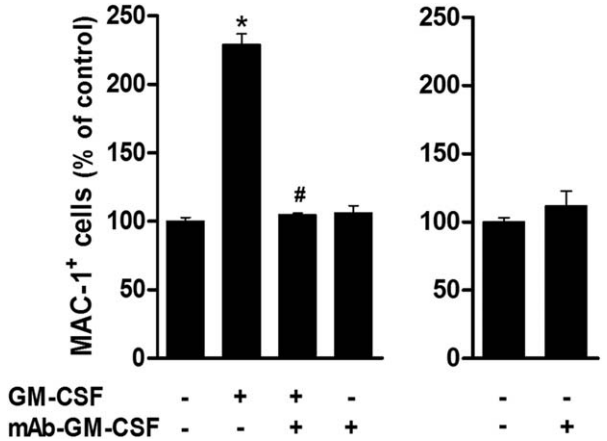

FIGURE 7: Proliferative response of PEI-isolated microglial cells. *Left panel*: Number of MAC-1$^+$ cells in PEI-isolated microglial cultures treated or not with a mouse recombinant GM-CSF (2 ng/ml for 72 h) in the presence or absence of a GM-CSF neutralizing antibody (5 μg/ml). *Right panel*: The same GM-CSF antibody (5 μg/ml) was applied or not, every 3 days to cultures used for microglial cell isolation on PEI coating and MAC-1$^+$ cells were quantified at 21 DIV. *$P<0.05$ vs control; #$P<0.05$ vs GM-CSF treatment.

attachment of MAC-1$^+$ cells having the appearance of microglial cells while causing the spontaneous and gradual elimination of other cell populations; neurons do not survive in these conditions even at early stages of the cultures (not shown) and astrocytes start to detach after 15–16 DIV to form floating aggregates that can be removed by simple aspiration of the culture medium. In any case, the isolation of MAC-1$^+$ cells was complete by 18–21 DIV. Importantly, PEI-isolated MAC-1$^+$ cells expressed the microglial gene marker Fcrls (Butovsky et al., 2014) but not the chemokine receptor gene Ccr2 that is exclusively expressed by pro-inflammatory monocytes/macrophages (Hellwig et al., 2013; Wermuth and Jimenez, 2015). This demonstrates that MAC-1$^+$ cells isolated by differential adhesion onto PEI had a microglial phenotype. Note that seeding of brain cells directly onto uncoated polystyrene culture wells, also led to the isolation of microglial cells at the expense of astrocytes in complete DMEM medium. Yet, the yield in microglial cells was very low in comparison to that observed in PEI-coated conditions suggesting that the polystyrene surface was not optimal by itself for microglial cell growth.

Surprisingly, we could not achieve microglial cell isolation when brain cultures were seeded on POR, another polycationic polymer. So, we may assume that the electrostatic interactions exerted by PEI onto the negatively charged plasma membrane of microglial cells (Tonna et al., 2014) were optimal to selectively retain these cells and favor their growth under present experimental conditions. The exact nature of these interactions remains, however, to be established.

Biological Substrates That Mimic Cell-ECM Adhesion Neither Amplify Nor Reproduce PEI Effects

We also tested whether biological substrates that mimic cell-ECM adhesion could mimic or amplify the ability of PEI to promote the isolation of microglial cells. We were particularly interested in evaluating the effects of the tripeptide RGD that acts a common recognition site for integrins in several ECM glycoproteins (Hayman et al., 1985; Xu and Mosher, 2011). Indeed, RGD mimics the ECM capacity of promoting adhesion in place of fibronectin or vitronectin, which both facilitate microglial cell adhesion through integrin-mediated mechanisms (Hayman et al., 1985; Milner and Campbell, 2002). Our data show that the yield in microglial cells was not improved in culture wells that had been sequentially coated with PEI and RGD. Besides, RGD alone failed to mimic the effect of PEI as it promoted astrocyte rather than microglial cell isolation. Laminin another glycoprotein that operates as a weak substrate for microglial cells (Milner and Campbell, 2002) prevented microglial cell isolation onto PEI and it also promoted astrocyte growth on its own. Overall, these results confirm that the potential of PEI to promote microglial cell isolation is rather unique and not shared by biological substrates that mimic cell-ECM adhesion.

Other Parameters That Influence the Isolation of Microglial Cells Through the PEI Coating Procedure

We observed that the yield in microglial cells was optimal in the absence of culture medium change after 2 DIV and until completion of microglial cell isolation, i.e., at approximately 18–21 DIV. Indeed, when part of the culture medium was renewed periodically (i.e., every 3 days), the yield in microglial cells dropped by about 55%. The reduced yield in microglial cells was unlikely to be caused by mechanical detachment of adherent cells as the result of repeated culture medium changes, since only 2/5 of the culture medium was replaced each time. Alternatively, conditioning factors produced by astrocytes and crucially needed by microglial cells for their survival and proliferation might be lost through culture medium replenishment. Although attractive, this possibility is not supported by data showing that the yield in microglial cells was similarly reduced in cultures receiving only the amount of FCS contained in the fraction of culture medium that was renewed. Thus, we may assume that in the absence of culture medium renewal, serum components were gradually degraded, leading to a state of nutritional deprivation that was favorable for the maintenance and proliferation of microglial cells as suggested earlier in other paradigms (Hao et al., 1991; Saura, 2007; Zhang and Fedoroff, 1996). Still coherent with our present observation, we found that the proliferation rate of microglial cells was by far the highest in cultures that remained unfed throughout the isolation process. Interestingly, astrocytes detached from PEI regardless of whether they received or not fresh DMEM supplemented with FCS or just FCS, suggesting that culture feeding impacted specifically the proliferation rate of microglial cells.

The composition of the culture medium was also playing a key role in the isolation process of microglial cells onto PEI coating. In fact, we found that our cultures contained predominantly astrocytes and only a limited number of microglial cells when DMEM was replaced by DMEM/F12. This is somehow surprising considering that DMEM/F12 has been used frequently in other protocols to isolate microglial cells (Saura, 2007). It is worth noting, however, that no coating or different coatings were used in these conditions. Overall, we can conclude that postnatal mouse brain cultures can give rise spontaneously to a pure and densely packed population of microglial cells on the condition that (i) PEI is used as an attachment factor, (ii) complete DMEM is utilized for cell maintenance, and (iii) cultured cells receive no feeding until completion of isolation.

Comparison of the PEI-Coating Procedure to Other Current Protocols

From each brain pup, we were able to generate approximately 2×10^6 microglial cells in culture when using the PEI isolation method. In comparison, the conventional shaking protocol described initially by Giulian and Baker (1986) yielded ~5 times less microglial cells (Saura et al., 2003). Besides, this technique is relatively tedious. A yield similar to that obtained with PEI was also reported with protocols where the isolation is achieved by mild trypsinization (Saura et al., 2003) or positive selection of cells expressing the pan-microglial marker CD11b (Marek et al., 2008). Yet, the first of these protocols presents several constraints and it is relatively demanding technically and the second one may be costly if large quantities of cells are needed, for instance, for molecular screening.

The Inflammatory Response Is Preserved in Microglial Cells Purified by PEI Coating

In a next step, we wished to demonstrate that this model system was suitable for experimental studies that focus on neuroinflammatory processes. To this aim, PEI-isolated microglial cells were replated and challenged with the bacterial toxin LPS, a prototypical activator of toll-like receptor 4 that operates as inducer of innate immune responses (Lu et al., 2008). More specifically, we used functional assays that measure the expression of Iba-1 at the cellular level, the accumulation of different cytokines and nitric oxide in LPS-conditioned culture medium, and the production of ROS, intracellularly.

Upon stimulation with LPS, PEI-isolated microglial cells exhibited typical morphological changes that were associated with the induction of the calcium-binding protein Iba-1, as already observed in other experimental models of inflammation (Carrillo-de Sauvage et al., 2013; Kettenmann et al., 2011). LPS treatment (1, 10 ng/ml) also led to a concentration-dependent increase in the release of TNFα, IL-6, and IL-1β in the medium of cultures treated with the bacterial inflammogen. The glucocorticoid nuclear receptor agonist DEX, profoundly reduced Iba-1 expression and cytokine release which is in agreement with the known inhibitory effects of this compound on immune responses (Carrillo-de Sauvage et al., 2013; Sierra et al., 2008).

NO produced through the inducible NO synthase and ROS generated through NADPH oxidase, an enzymatic complex that constitutes the main source of superoxide ions (Stefanska and Pawliczak, 2008), represent two other components of the inflammatory response mediated by microglial cells. Each of these markers was robustly induced with 10 ng/ml LPS and this response was efficiently curtailed by DEX. APO, an inhibitor of NADPH oxidase (Chéret et al., 2008; Vejrazka et al., 2005), and TRO, a soluble analog of vitamin E known to operate as an inhibitor of lipid peroxidation (Nakamura and Hayashi, 1992), also potently reduced ROS production in these cultures.

This set of results shows that concentrations of LPS comprised between 1 and 10 ng/ml are sufficient to promote a robust activation of PEI-isolated microglial cells, presumably because these cells are in a relatively quiescent state in control conditions. Accordingly, pro-inflammatoty cytokines were barely detectable in control cultures. Coherent with these observations, we also found that transcripts encoding the canonical activation marker NFκB2/p52 (Bennett et al., 2016) were expressed at low level under control conditions. The concentrations of LPS used, here, appear to be either lower (O'Callaghan et al., 2015; Panicker et al., 2015; Saura et al., 2003) or identical (Gao et al., 2008; Qian et al., 2007) to concentrations used to generate inflammatory responses in other culture systems of microglial cells, which further attests to the validity of the present protocol. Overall, these data clearly indicate that microglial cells obtained through the PEI-coating procedure, exhibit a typical LPS inflammatory response that can be curtailed by adequate reference treatments.

The Mitogenic Response is Well Preserved in Microglial Cells Purified by PEI Coating

Finally, because microglial proliferation is a major component in the evolution of chronic disorders, we wished to determine whether PEI-isolated microglial cells had a capacity to proliferate in response to GM-CSF, a potent mitogenic factor for these cells (Liva et al., 1999; Henze et al., 2005). Microglial cells isolated through the PEI coating procedure were strongly responsive to GM-CSF when replated for functional assays, which further confirms that the functional repertoire of these cells remains totally preserved. Interestingly, an antibody that neutralized the mitogenic potential of GM-CSF in these conditions, failed to reduce microglial cell numbers during the isolation step onto PEI coating. This is a clear indication that microglial cells did not require GM-CSF as a mitogen to proliferate during the isolation process.

In conclusion, the PEI protocol used here for the isolation of microglial cells is easy to implement, inexpensive and its cell yield is considerably high. Microglial cells exposed to LPS exhibited a typical inflammatory response that can be monitored at cellular and biochemical levels. Besides, these cells demonstrated a typical mitogenic response. Therefore, this model system may be useful for mechanistic studies. Because it allows the production of bulk quantities of microglial cells, it may be also of particular of interest for implementing drug screening assays.

Authors' Contribution

JSD, MO, RRV, PPM developed and contributed to the experimental design. SG and PPM contributed resources and

materials. JSD, MO, PPM wrote and edited the manuscript. JSD, MO, SH, SS, PPM performed data acquisition and analysis. All authors have approved the final manuscript.

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