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Key Words:	Cip/Kip in inflammation, glia, neuroinflammation, astrocytes, microglial cell cultures



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Upregulation of p21^{Cip1} in activated glial cells

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Abstract

The cdk inhibitor p21^{Cip1}, also named p21^{Cip1/Waf1}, is intimately involved in coupling growth arrest to cellular differentiation in several cell types. p21^{Cip1} is a multifunctional protein that might regulate cell-cycle progression at different levels. In a recent study we found no differences in the rate of proliferation between glial cells from wild-type and p21^{Cip1^{-/-}} mice. In the present study we examined differences in glial activation between glial cells from wild-type and p21^{Cip1^{-/-}} mice, using mixed glial cultures, microglia-enriched cultures and astrocyte-enriched cultures. We compared the effect of lipopolysaccharide and two forms (oligomeric and fibrillar) of the 1-42 β -amyloid peptide on glial activation. We observed an attenuation of nuclear translocation of the nuclear factor kappa-B in p21^{Cip1^{-/-}} glial cells, when compared with glial cells from wild-type mice. In contrast, tumour necrosis factor- α release was enhanced in p21^{Cip1^{-/-}} microglial cells. In addition glial activation induced by lipopolysaccharide and the fibrillar form of the 1-42 β -amyloid peptide upregulated p21^{Cip1}. Our results support a role for p21^{Cip1} in the activation of glial cells, particularly in microglia.

Introduction

Glial cells play a key role in the normal function of the CNS. Under physiological conditions, brain glial cells are involved in the production of trophic factors, the maintenance of ionic homeostasis, buffering the action of neurotransmitters, immune surveillance and neural regeneration in the CNS (Kreutzberg, 1996; Anderson and Swanson, 2000, Farina et al., 2007). Microglia are the primary immune cells responsible for detecting invading pathogens and neuronal injury (Aloisi, 1999; Streit et al., 1999; Su and de Vellis, 2005; Hanisch and Kettenmann, 2007). Astroglial and microglial cells respond to neuronal damage, lesions, alterations in neuronal function, toxic agents and infectious agents. Their response involves morphological and functional changes, and is known as glial activation. Reactive astroglial and microglial cells upregulate the expression of a variety of factors, including growth factors, cytokines, chemokines, adhesion molecules, receptors, nitric oxide (NO), etc. These factors have neurotoxic or neuroprotective properties (Chao et al., 1995, Streit et al., 1999, Vitkovic et al., 2000, Herdegen and Waetzig, 2001, Hauwell et al., 2005, Butovsky et al., 2005, Block et al., 2007, Kaushal and Schlichter, 2008). Several transcription factors participate in glial activation, including nuclear factor kappa-B (NF κ -B) (O'Neill and Kaltschmit, 1997; Tak and Firestein, 2001), peroxisome proliferator-activated receptors (PPARs) (Kielian and Drew, 2003), activated protein-1 (AP-1) (Herdegen and Waetzig, 2001) and some members of the CCAAT/enhancer binding proteins (C/EBPs) family of transcription factors (Pérez-Capote et al., 2006; Ejarque-Ortíz et al., 2007a and b). The cascade of microglial activation is a fine-tuned process that is also regulated by factors derived from neurons and other glial populations, in particular astrocytes. Astrocytes are local sources of transforming growth factor- β (TGF- β) and other immunomodulatory or neurotrophic factors, and the prominent role of astrocytes in inducing an immunologically silent state in microglial cells has been demonstrated in a number of experiments *in vitro*. Astrocytes can induce the transformation of ameboid into ramified microglial cells, and reduce proliferative activity (Wilms et al., 1997; Jones et al., 1998; Schilling et al., 2001; Kalla et al., 2003).

In a previous study (Tusell et al., 2005), we examined the role of the cell cycle inhibitor p21^{Cip} in glial activation, using mixed glial cultures (approximately 75% astrocytes and 25% microglia). This protein, also called WAF1, CAP20 or Sd1, is the founding member of the Cip/Kip family of CdKIs, which also includes p27 (Toyoshima and

Hunter, 1994) and p57 (Lee et al., 1995). p21^{Cip1} plays an essential role in growth arrest after DNA damage (Brugarolas et al., 1995), and its overexpression leads to G1 and G2 (Niculescu et al., 1998) or S-phase arrest (Ogryzko et al., 1997). In addition, a variety of transcription factors (including STATs, C/EBP α and C/EBP β) that are induced by different stimuli activate p21^{Cip1} transcription.

Here we examined whether p21^{Cip1} is involved in the modulation of glial activation produced by LPS and fibrillar or oligomeric 1-42 β -amyloid (A β) peptide. We analysed the release of tumour necrosis factor- α (TNF- α), and the nuclear translocation of NF- κ B. We also studied the expression of p21^{Cip1} after inducing glial activation. In summary, we show for the first time that LPS and fibrillar A β upregulates p21^{Cip1} in cultured glial cells and taking into account all the results presented here, we conclude that p21^{Cip1} participates in the neurotoxic or neuroprotective effects associated with glial activation.

Material and Methods

Materials

Trypsin-EDTA solution (#25200-072), Dulbecco's modified Eagle medium-F-12 nutrient mixture (#31330-038), foetal bovine serum (#10270-106), penicillin-streptomycin (#15140-114) and Fungizone® (#15290-018) were from Invitrogen Ltd. (Carlsbad, CA, USA). Deoxyribonuclease I (#D-5025), extravidin peroxidase (#E-2886), laminin (#L-2020), cytosine arabinoside (#C-1768), hexafluoroisopropanol (#H-8505), dimethyl sulfoxide (#D-4540), cycloheximide (#4859) and E. coli LPS serotype 055:B5 (#L-6529) were from Sigma (St. Louis, MO, USA). Alexa Fluor 488 goat anti-mouse (#A11017) and Alexa Fluor 546 (#11010) goat anti-rabbit antibodies were from Molecular Probes (Eugene, OR, USA). PVDF membranes (#IPVH00010) were from Millipore (Bedford, MA, USA). HRP-labelled anti-rabbit antibody (#NA934) and ECL-Plus (#RPN2132) were from Amersham (Buckinghamshire, UK). Protease inhibitor cocktail Complete® (#1836145) was from Roche Diagnostics (Mannheim, Germany). Sensiscript RT enzyme was from Quiagen (Frankfurt, Germany). Absolutely RNA Miniprep kit (#400800) was from Stratagene (La Jolla, CA, USA). Rabbit anti-NF- κ B p65 antibody (#sc-372) was from Santa Cruz Biotechnology (Temecula, CA, USA). Mouse anti-p21^{Cip1} antibody (#OP76) was from Calbiochem (Darmstadt, Germany). Rabbit anti-TLR4 (#ab13556) was from Abcam (Cambridge, UK). Rabbit anti-GFAP

antibody (#Z 0334) was from DakoCytomation (Glostrup, Denmark). Biotinylated anti-rabbit antibody (#31820) was from Pierce (Rockford, IL, USA). Rat anti-CD11b (#MCA711G, clone 5C6) was from Serotec (Oxford, UK). A β 1-42 peptide (#H-1368) was from Bachem AG (Bubendorf, Switzerland). VersaDoc system and Quantity One 5.4.1 software were from Bio-Rad Laboratories (Hercules, CA, USA). Rabbit anti-p36 antibody was a gift from Dr. Oriol Bachs (Faculty of Medicine, University of Barcelona, Spain).

Cell cultures and treatments

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), and the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals. The study was approved by the Ethics and Scientific Committees of the Hospital Clínic de Barcelona. Mixed glial cultures were prepared from 1- or 2-day-old neonatal p21^{Cip1+/+} or p21^{Cip1-/-} mice both from the same C57BL/6 genetic background (Brugarolas et al., 1995). These mice were generously provided by Prof. Oriol Bachs (University of Barcelona, Spain) and were kept at the Animal facilities of the University of Barcelona. To obtain mixed glial cultures, mice cerebral cortices were dissected, carefully stripped of their meninges and digested with 0.25% trypsin for 25 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium (Dulbecco's modified Eagle medium-F-12 nutrient mixture, fetal bovine serum 10%, penicillin 100 U/mL, streptomycin 100 μ g/mL and amphotericin B (Fungizone®), 0.5 μ g/mL) to which 0.02% deoxyribonuclease I was added. The solution was pelleted (5 min, 200 x g), resuspended in culture medium and brought to a single cell suspension by repeated pipetting followed by passage through a 105 μ m pore mesh. Cells were seeded at a density of 250,000 cells/ml (=62,500 cells/cm²) and cultured at 37°C in humidified 5% CO₂-95% air. The medium was replaced every 5-7 days. Mouse mixed glial cultures reached confluence after 7-10 days *in vitro* and were used between 19 and 21 days *in vitro*. At this point, they typically consisted of 75% type-I astrocytes and 25% microglia.

Mouse microglial cultures were prepared by mild trypsinization as described (Saura et al. 2003). Briefly, after 19-21 days *in vitro* mixed glial cultures were treated for 30 min with 0.06% trypsin in the presence of 0.25 mM EDTA and 0.5 mM Ca²⁺. This resulted in the detachment of an intact layer of cells containing virtually all the astrocytes,

leaving a population of firmly attached cells identified as >98% microglia. The microglial cultures were used twenty-four hours after isolation by this procedure.

Mouse astroglial cultures were prepared by seeding cortical cells prepared as for mixed glial cultures on laminin (20 $\mu\text{g}/\text{mL}$)-coated plates. Laminin-coating favours astroglial growth and inhibits microglial growth (Milner and Campbell 2002). When confluent, cultures were treated with 10 μM cytosine arabinoside for 4 days. Cultures were used one day after the end of cytosine arabinoside treatment. The vast majority of cells were type-I astrocytes. Less than 2% were microglia.

For immunocytochemistry studies cells were seeded in 48 well plates using a volume of 300 $\mu\text{L}/\text{well}$. For the isolation of nuclear proteins from glial cultures, three, five or six 950- mm^2 wells of mixed glial, astroglial or microglial cultures, respectively, were used per condition using a total volume of 2000 $\mu\text{L}/\text{well}$.

Cell treatments: control cells were treated with culture medium. LPS was treated at 0.1 $\mu\text{g}/\text{mL}$. The oligomeric and fibrillar A β forms were used at a final concentration of 5 μM .

Isolation of nuclear proteins

Cells were scrapped in cold PBS, and centrifuged for 4 min at 7000 x *g*, and the resulting pellet was resuspended in 400 μL of buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol and 0.1 mM EGTA. Cells were then swollen on ice for 15 min. After the addition of 25 μL of 10% Nonidet-40 (buffer B), cells were vigorously vortexed for 10 s. After 10 min incubation on ice, the broken cells were successively spun for 10 min at 3000 x *g* and for 5 min at 14,000 x *g*. The pellet was resuspended in 50 μL of buffer C consisting of 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol. Solutions A, B, C and PBS were supplemented with the protease inhibitor cocktail Complete®. After a 90-min of gentle shaking at 4°C, the nuclei were pelleted by a 5 min spin at 2000 x *g*. The supernatant containing nuclear proteins was collected and the protein amount was determined by the Lowry assay.

TNF- α release determination

The amount of TNF- α released into the culture medium was determined with an ELISA kit specific for mouse TNF- α (Diaclone, Besançon, France). The ELISA assay was performed following the instructions supplied by the manufacturer.

Western blot

15-20 μ g of protein of denatured (5 min, 100°C) nuclear extracts were subjected to SDS-PAGE on a 15% polyacrylamide gel and transferred to a PVDF membrane. The membranes were processed as follows: 1) 5 min in Tris-buffered saline (TBS, 20 mM Tris, 0.15M NaCl, pH=7.5); 2) 10 s in methanol; 3) air dry; 4) overnight incubation at 4°C on primary antibody (rabbit anti- NF κ -B 1:500 or mouse anti p21 1:2000 or rabbit anti-p36 1:5000) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% nonfat dry milk); 5) 2x 15 s in TBS 0.05% Tween-20; 6) 1 hour in HRP-labelled anti mouse or anti-rabbit antibody diluted 1:5000 in immunoblot buffer; 7) extensive washes in TBS-0.05% Tween-20; 8) 5 min in ECL-Plus. Membranes were exposed to the camera of a VersaDoc system and chemiluminescence was quantified using Quantity One 5.4.1 software. For each antibody used, we verified the linearity between the level of chemiluminescence in de western blots and the amount of protein loaded. Linearity was observed in a range of 10 to 40 μ g of protein.

Immunocytochemistry

For immunocytochemistry, cultured cells were fixed with 4 % paraformaldehyde for 20 min at room temperature. When immunocytochemistry was revealed with diaminobenzidine, the protocol was: 7 min in 0.3% H₂O₂ in methanol, 2 x 15 min in PBS, overnight in primary rabbit anti NF κ -B antibody (1:500) or mouse anti p21 antibody (1:500), 2 x 15 min in PBS, 1 h in biotinylated anti-rabbit or biotinylated anti mouse antibody (1:200), 2 x 15 min in PBS, 1 h in ExtrAvidin-HRP (1:500), 5-10 min in diaminobenzidine (1 mg/ml)- 0.15% H₂O₂ in PBS. All steps were performed at room temperature except the primary antibody incubation (4°C). All antibodies were diluted in PBS containing 10% normal goat serum.

The cellular localization of p21^{Cip1} expression after treatment with A β peptides was determined by double immunocytochemistry, immunofluorescence labelling was carried out in cells where p21^{Cip1} immunoperoxidase labelling had been performed. A rabbit anti-gial fibrillary acidic protein (GFAP) polyclonal antibody (1:2000) was used

to label astroglial cells and an anti-CD11b monoclonal antibody (1:500) was used to label microglial cells. Cells were incubated with the primary antibodies in 1% goat serum overnight at 4°C, and with goat anti-rabbit ALEXA 488 and goat anti-mouse ALEXA 546 secondary antibodies (1/500) in 1% goat serum for 1 h at room temperature. After extensive washing in PBS, cells were stored at 4°C. All samples were photographed on an Olympus fluorescence microscope equipped with a digital camera Color View 12 powered by a HBO 100 lamp.

Quantitative Real-Time PCR

Total RNA was isolated with Absolutely RNA Miniprep kit and reverse transcribed with random primers using Sensiscript-RT. The primers used to amplify mouse p21^{Cip1} mRNA were 5'-ATGTCCAATCCTGGTGATGT-3' (forward) and 5'-TGCAGCAGGGCAGAGGAAGT-3' (reverse). The primers used to amplify mouse the toll-like receptor 4, TLR4 mRNA were 5'-CCTCTGCCTTCACTACAGAGACTTT-3' (forward) and 5'-TGTGGAAGCCTTCTGGATG-3' (reverse). To normalise of Ct values to an endogenous control the following mouse actin mRNA primers were used: 5'-CAACGAGCGGTTCCGATG-3' (forward) and 5'-GCCACAGGATTCCATACCCA-3' (reverse). Real time PCR was carried out with IQ SYBRGREEN SuperMix and iCycler IQ equipment. Primer efficiency was estimated from standard curves generated by dilution of a cDNA pool. Samples were run for 40 cycles (30 sec 95°C, 1 min 55°C, 30 sec 72 °C). Two samples per condition were analyzed in triplicate in each experiment and 3 independent experiments were performed.

Preparation of fibrillar and oligomeric A β peptides

A β peptide was prepared according to the protocol described by (Dahlgren et al. 2002). Briefly, A β was dissolved in hexafluoroisopropanol, desiccated and dissolved in dimethyl sulfoxide to 5 mM. This solution was brought to 100 μ M by dilution in 10 mM HCl, aged for 24 hours at 37°C (Fibrillar) or diluted in Ham-phenol free and aged for 24 hours at 4°C (Oligomeric). After 24 h of aging, the two A β forms were immediately applied to the cells at the required concentration.

Cell viability

Cell viability was routinely estimated by fluorescent staining. Twenty-four hours after the different treatments cells were incubated with propidium iodide (4.6 $\mu\text{g/ml}$) for 5 min and then examined under a fluorescence microscope (Olympus IX70). Propidium iodide-positive dead cells showed red nuclei. Images of 4 microscopic fields in each well were obtained with a digital camera (ColorView 12) using a 20X objective. Three wells per experimental condition were processed and three independent experiments were performed.

Total cell number was measured by Hematoxylin nuclear staining. Twenty-four hours after the different treatments, fixed cells were washed with PBS before incubation with Hematoxylin for 1 min. After three washes with tap water, cells were examined under a microscope (Olympus IX70). Images of 3 microscopic fields in each well were obtained with a digital camera (ColorView 12) using a 20X objective. Three wells per experimental condition were processed and three independent experiments were performed. Visual counting of nuclei was performed with the imaging analysis software “analy-SIS” (Soft Imaging System GmbH, Germany).

Statistical analysis

The statistical analysis was performed using one-way ANOVA followed by Dunnett’s post-hoc test (TLR4 and p21^{Cip1} mRNA determination) or Student’s t-test (Western blot quantification and TNF- α release determination). Differences were considered significant if $p < 0.05$.

Results

Cell viability and cell number

Propidium iodide staining showed that cell viability was $\geq 95\%$ after 24 hours of any cell treatment in each type of glial culture. In addition, the number of cells in each glial cell culture was estimated after the different treatments. There were not significant differences between the number of control cells and the number of treated cells in any glial culture type after 24 hours of treatments (Table I).

TNF- α expression is dependent on p21^{Cip1} in microglial cells

Figure 1A shows the results obtained in microglia enriched cultures. The increase in TNF- α released by microglia in the culture medium 24 h after LPS treatment was higher

($p < 0.05$, Student's *t* test) in $p21^{Cip1-/-}$ than in wild-type cultures (13.2 pg/mL in wild-type mice microglia and 26.3 pg/mL in $p21^{Cip1-/-}$ mice microglia). However, in astrocyte-enriched cultures, there were no significant differences between wild-type astrocytes and $p21^{Cip1-/-}$ astrocytes (Figure 1B). These results suggest that, microglial cells are responsible for the higher increase in TNF- α release in glial cells from $p21^{Cip1-/-}$ mice.

The increase in the NF- κ B expression is reduced in $p21^{Cip1-/-}$ glial cells

We observed NF- κ B nuclear translocation both in wild-type and $p21^{Cip1-/-}$ mixed glial cultures 24 hours after LPS treatment (Figure 2). However, NF- κ B nuclear translocation was reduced in $p21^{Cip1-/-}$ cultures. Figure 2A shows a representative Western blot from nuclear extracts of mixed glial cultures. The quantification of Western blots shows that p65 increased 2.3 ± 0.8 fold (1.22 ± 0.22 in LPS treated cells versus 0.51 ± 0.09 in control cells, $n = 3$, $p < 0.01$ Student's *t* test) after LPS treatment in wild-type cultures and 1.2 fold (not significant, n.s.) in $p21^{Cip1-/-}$ cultures. Similar results were also given by immunocytochemistry (Figure 2B) after LPS treatment: the increase in NF- κ B nuclear translocation was lower in $p21^{Cip1-/-}$ glia than in wild-type glia.

In microglia-enriched cultures, an increase in NF- κ B nuclear translocation was observed in wild-type cultures after LPS treatment. This was revealed by both Western blot and immunocytochemistry (Figure 3). The increase in NF- κ B nuclear translocation was lower in $p21^{Cip1-/-}$ microglia. The increase was 1.89 ± 0.14 (1.92 ± 0.12 in LPS treated cells versus 0.91 ± 0.06 in control cells, $n = 3$, $p < 0.001$) times the control value in wild-type microglia and 1.4 (n.s) times in $p21^{Cip1-/-}$ microglia after 24 h of LPS treatment. Figure 3B show p65 nuclear translocation in the different experimental conditions, as revealed by immunocytochemistry. The effect was similar to that shown by Western blot.

In astrocyte-enriched cultures, the increase in NF- κ B nuclear translocation after LPS was lower in $p21^{Cip1-/-}$ than in wild-type cultures (Figure 4). Quantification of Western blots after LPS treatment (Figure 4A) showed 1.71 ± 0.36 fold increase in the control value in wild-type astrocytes (1.53 ± 0.27 in LPS treated cells versus 0.90 ± 0.06 in control cells, $n = 3$, $p < 0.05$), while there was no detectable increase in $p21^{Cip1-/-}$ astrocytes. The same effect was observed by immunocytochemistry (Figure 4B).

The study of both cell types together (mixed cultures) or the study of each cell type individually (either astrocytes or microglia) showed that, either way, p21^{Cip1-/-} glial cells had a reduced capacity to translocate NF- κ B into the nucleus.

TLR4 expression

The toll-like receptor 4 (TLR4) is the receptor for LPS-dependent signalling (Heine et al., 1999). For this reason, we studied whether the LPS treatment produced different expression of TLR4 in glial cells from wild-type and p21^{Cip1-/-} mice. Figure 5 shows the results obtained by quantitative real time PCR. No differences in the basal expression of TLR4 were observed between wild-type and p21^{Cip1-/-} glial cells. LPS treatment caused a similar decrease in TLR4 mRNA expression in wild-type and p21^{Cip1-/-} glial cells. We did not observe any differences in TLR4 protein expression by immunocytochemistry (data not shown). These results suggest that TLR4 expression is not modified in p21^{Cip1-/-} glial cells.

p21^{Cip1} expression increases during glial activation

We were interested to study whether LPS induced changes in p21^{Cip1} expression in glial cells. In mixed glial cultures LPS significantly increased p21^{Cip1} mRNA levels 2h after treatment (1.8-fold increase, $P < 0.01$). P21^{Cip1} returned to control levels 4 h after LPS (Figure 6A). The observed increase was accompanied with an increase in p21^{Cip1} protein expression. A time-course experiment revealed that nuclear p21^{Cip1} increased from 4 h post-treatment and the increase was maximal after 16-24 h (data not shown). Cycloheximide pre-treatment (50 μ M, 30 min before LPS) completely prevented the protein increase (Figure 6B). We also studied the effect of LPS on p21^{Cip1} protein expression 24 h after treatment in the three different types of glial cultures. Figure 7A shows the results obtained in mixed glial cultures. Western blots show that LPS treatment caused an increase of 2.51 ± 0.81 times the control value (2.03 ± 0.49 in LPS treated cells versus 0.82 ± 0.23 in control cells, $n = 3$, $p < 0.05$). In Figure 7D, a and d panels show the results obtained by immunocytochemistry: a strong increase in p21^{Cip1} immunolabeling after LPS treatment was observed.

In microglia-enriched cultures, the increase of p21^{Cip1} produced by LPS treatment was also very clear. LPS treatment resulted in an increase that was 2.62 ± 0.33 times the control value (1.59 ± 0.02 in LPS treated cells versus 0.61 ± 0.08 in control cells, $n = 3$,

$p < 0.001$) (Figure 7B). The images obtained by immunocytochemistry were consistent (figure 7D, panels b and e) with the results obtained by Western blot.

In astrocyte-enriched cultures, there was an increase of p21^{Cip1} protein after LPS treatment that was 1.54 ± 0.36 times the control value (2.76 ± 0.51 in LPS treated cells versus 1.80 ± 0.08 in control cells, $n=3$, $p < 0.05$) (Figure 7C). The images obtained by immunocytochemistry (Figure 7D, panels c and f) are consistent with those obtained by Western blot.

Treatment with A β increases p21^{Cip1} expression in glial cultures

In addition to the LPS treatment, we also examined the effect of A β treatment on NF- κ B nuclear translocation, TNF- α release and p21^{Cip1} expression in glial cells. Both oligomeric and fibrillar A β were used.

Figure 8A shows the results obtained by Western blot on NF- κ B translocation after treatment with LPS or the two types of A β . In this group of experiments LPS treatment of wild-type glial cell cultures (used as a positive control of glial activation) produced a strong increase of 5.61 ± 1.02 times the control value in nuclear NF- κ B content (2.83 ± 0.38 in LPS treated cells versus 0.40 ± 0.08 in control cells, $n=4$ $p < 0.001$). Fibrillar A β produced an increase of 3.78 ± 1.69 fold (1.55 ± 0.46 in A β treated cells versus 0.40 ± 0.08 in control cells, $n=4$, $p < 0.01$). Oligomeric A β did not produce any effect. In glial cultures from p21^{Cip1}^{-/-} mice, LPS produced a lower increase than in wild-type: 2.3 ± 0.57 times the control value (2.22 ± 0.51 LPS treated cells versus 0.96 ± 0.21 in control cells, $n=4$, $p < 0.01$). After treatment with A β forms, no NF- κ B nuclear translocation was observed.

Figure 8B shows a mixed culture by phase contrast microscopy 24 h after the different treatments. Panels a, and b correspond to control cells from wild-type and p21^{Cip1}^{-/-} mice, respectively. These images are very similar to those from cultures 24 h after treatment with oligomeric A β (Figure 8B, c and d). However, treatment with fibrillar A β (Figure 8B, e and f) induced a change in the morphology of the cultures. The smaller refringent cells, corresponding to microglial cells, were not observed. This effect was detected both in wild-type and in p21^{Cip1}^{-/-} cultures. After LPS treatment, the two cultures (figure 8B, g and h) had the same appearance as the control cells or the cells treated with oligomeric A β .

We also analysed the effect of LPS and A β peptide treatments on TNF- α release into the culture medium. There were no significant differences between the control and treatment values either type of mixed glial cultures (wild-type and p21^{Cip1-/-} mice). LPS induced a TNF- α release in wild-type which was significantly higher in p21^{Cip1-/-} glial cultures. A β did not induce TNF- α release in wild-type or p21^{Cip1-/-} (results not shown).

Figure 9 shows the p21^{Cip1} protein expression after treatment with the two types of A β or LPS. Figure 9A shows a Western blot obtained after the different treatments. The quantification of Western blots showed that fibrillar A β treatment resulted in 4.06 \pm 0.83 times more p21^{Cip1-/-} protein expression than in control (5.78 \pm 2.82 in fibrillar A β treated cells versus 1.4 \pm 0.52 in control cells, n=4, p<0.05). There were no differences between treated cultures and the control after A β oligomeric treatment. LPS treatment resulted in an increase of 2.05 \pm 0.08 times more than the control value (2.86 \pm 0.99 in LPS treated cells versus 1.4 \pm 0.52 in control cells, n=4, p<0.05). The Western blots obtained from p21^{Cip1-/-} mixed glial cultures did not present the p21^{Cip1} band. This negative control was used to verify antibody specificity. Figure 9B shows the results of immunocytochemistry. A strong increase in p21^{Cip1} expression was observed after fibrillar A β (Figure 9B, e) or LPS treatment (Figure 9B, g). Oligomeric A β treatment (Figure 9B, c) produced a weaker increase. Panels b, d, f and h in Figure 9 B correspond to treatments carried out with cultures from p21^{Cip1-/-} mice. No immunoreactivity was observed, indicating the specificity of the p21^{Cip1} antibody.

Figure 10 shows a double immunostaining, carried out to find out whether p21^{Cip1} increases after fibrillar A β treatment in the two cell types contained in the mixed culture (microglia and astrocytes). The results show that there was a p21^{Cip1} increase in both microglia (Figure 10, a,b) and astrocytes (Figure 10, c and d).

Discussion

In a preliminary study (Tusell et al., 2005), we examined possible differences in the process of glial activation between mixed glial cultures (microglia and astrocytes) from wild-type and p21^{Cip1-/-} mice. We compared the effect of serum mitogenic stimulation on proliferation rate and on the total number of glial cells. No differences between wild-type and p21^{Cip1-/-} were observed. We also compared the effect of LPS on NO and TNF- α release and nuclear NF- κ B translocation as indicators of glial activation. We observed an attenuation of NO release and NF- κ B activation in p21^{Cip1-/-} glial cells when

compared with glial cells from wild-type mice. In contrast TNF- α was enhanced in p21^{Cip1-/-}. These results suggest that the role of p21^{Cip1} in the glial inflammatory response does not depend on its function as an inhibitor of cell proliferation. In the present study, we further examined the role of p21^{Cip1} in glial activation to determine whether it differentially contributes to astroglial and microglial activation. Thus, we studied the role of p21^{Cip1} in glial activation using mixed, microglial-enriched and astroglial-enriched glial cultures (with above 95% purity for each cell type). In the aforementioned study, we evaluated the effect of LPS, an agent widely used to induce glial activation. In the present study, we extended this work by using two forms of the peptide A β . Finally, we also examined the way in which the different treatments affected p21^{Cip1} protein expression. As glial activation indicators, we measured TNF- α release into the culture medium and NF- κ B nuclear translocation. The results confirm that mixed glial cells from p21^{Cip1-/-} mice had a higher increase of TNF- α release in response to LPS. However we demonstrated that this effect was basically caused by microglial cells (Figure 1A, B) rather than astrocytes.

Mixed, astroglial and microglial cultures from wild-type mice increased NF- κ B translocation after LPS treatment. However, glial cultures from p21^{Cip1-/-} mice had a reduced capacity to activate NF- κ B. In contrast, TNF- α release was increased in LPS-treated p21^{Cip1-/-} glial cells compared with glial cells from wild-type mice, specifically in microglia. Our results show that these changes are not due to differences in glial cell viability between cells from wild-type and p21^{Cip1-/-} mice. **The changes are neither due to differences in TLR4 expression between glial cells from wild-type and p21^{Cip1-/-} mice.** This receptor is required for the transduction of LPS signals in monocytes and macrophages (Qureshi et al., 1999). Microglia is the most important cell type in the resting CNS that expresses TLR4 (Lenhardt et al., 2003). This receptor is responsible, in part, for microglial activation in response to LPS (Kitamura et al., 2001; Qin et al., 2005). Systemic LPS administration to rats and mice induces a robust increase in TLR2 expression in the CNS, whereas TLR4 is reduced (Laflamme et al., 2001, 2003). Our results show that no differences are observed in basal TLR4 expression between glial cells from wild-type or p21^{Cip1} mice. In addition, we detected a similar decrease in TLR4 expression after LPS treatment in mixed glial cultures from wild-type and p21^{Cip1-/-} mice.

As NF- κ B is one of the transcription factors involved in the inflammatory response given by activated glial cells, the reduced capacity to activate NF- κ B we observed in p21^{Cip1-/-} glial cultures suggest that p21^{Cip1} is actively involved in this inflammatory response both in astrocytes and microglia, acting as a positive regulator of NF- κ B activation. Activation of NF- κ B followed by increased TNF- α production has been repeatedly described in reactive glial cells. In addition, as LPS and other cytokines, TNF- α is able to induce NF- κ B translocation and glial activation. However, although we observed an increase in both NF- κ B activation and TNF- α production in wild type cultures in response to LPS, the absence of p21^{Cip1} resulted in a reduced activation of NF- κ B but in an increased production of TNF- α in p21^{Cip1-/-} cultures. These results suggest that p21^{Cip1} acts as an inhibitor of TNF- α in LPS-induced glial activation. The differences observed between microglial and astroglial cells from p21^{Cip1-/-} cultures as regards LPS-induced TNF- α production may be due to a differential regulation of TNF- α production in each glial cell type. In relation to that, TNF- α production in response to different stimuli is usually higher in microglial than in astroglial cells.

We have described for the first time a possible relationship between p21^{Cip1} expression, TNF- α release and NF- κ B translocation in cells of the CNS, and it is difficult to explain the opposite effects observed in NF- κ B and TNF- α production in glial activation in the absence of p21^{Cip1}. There is a previous work showing a relationship between TNF- α , NF- κ B activation and p21^{Cip1} expression in human epithelial cells: TNF- α induced growth arrest in these cells is mediated by NF- κ B-dependent increase in p21^{Cip1} expression (Basile et al., 2003). In our case, the effects observed in p21^{Cip1-/-} cultures do not appear to be related to alterations in cell proliferation (Tusell et al., 2005), suggesting additional roles for p21^{Cip1}. One possibility is that, in reactive glial cells, p21^{Cip1} may be involved both in the inflammatory response and in the resolution of the inflammation by a still unknown mechanism, on the one hand facilitating NF- κ B action and on the other hand limiting glial activation, specially in microglial cells, through the inhibition of TNF- α which otherwise could result in overactivation of NF- κ B. In addition, as TNF- α produced by reactive glial cells has been shown to be neurotoxic to neurons (Feuerstein et al., 1998; Takeuchi et al., 2006; Kaushal and Schlichter, 2008), the presence of p21^{Cip1} in glial cells may be important to prevent neurotoxicity induced

by reactive glia. Further studies are needed to establish the mechanism of action of p21^{Cip1} in glial activation.

LPS-treated glial cells show an increase in p21^{Cip1} at mRNA and protein levels due to de novo synthesis. These results further suggest that p21^{Cip1} is involved in glial activation. They are in agreement with the results obtained by Ring et al. (2003) in an *in vivo* study that used LPS as a model for acute inflammation. These authors reported that p21^{Cip1} is induced in the mouse CNS as part of the acute response to inflammation, suggesting that this induction is a cytokine signalling event, involving TLR4.

Finally, we observed that neither the oligomeric nor the fibrillar A β form modified TNF- α release in mixed glial cultures of wild-type or p21^{Cip1}^{-/-} mice. Although an increase in NF- κ B translocation was observed in wild-type cultures treated with fibrillar A β , no effect was observed in p21^{Cip1}^{-/-} cultures. However, we did observe a change in the appearance of the glial cultures treated with fibrillar A β both in wild-type and p21^{Cip1}^{-/-} glia. Similarly, we observed an increase in p21^{Cip1} expression in glial cells treated with the fibrillar form, but not in those treated with the oligomeric form. In a previous study, we showed that the effect of A β on microglial activation depended on the aggregation state of the peptides (Casal et al., 2002). It is unclear how the difference in the aggregation of peptides accounts for the difference in bioactivity. However, particular conformations of peptides could determine the receptor activation potential, due to secondary and tertiary peptide conformations. In some cases, these conformations might be incapable of efficient binding and activate glial receptors that induce immune activation and inflammatory cascades. Thus, as we observed after LPS treatment, wild type glial cells treated with fibrillar A β show NF- κ B activation and induction of p21^{Cip1} expression, and NF- κ B activation is inhibited in the absence of p21^{Cip1}. Nevertheless, the magnitude of the effects observed is lower after fibrillar A β treatment, what may account for the absence of effect in TNF- α release observed.

In summary, the present results implicate p21^{Cip1} protein in glial activation. The mechanisms involved in the expression of this protein and TNF- α activation or NF- κ B activation need to be studied further. There are probably other molecules involved. Preliminary studies that are underway in our laboratory suggest that some transcription factors from the C/EBPs family require p21^{Cip1} to be activated during glial activation.

Moreover, the increases in p21^{Cip1} after treatment with fibrillar A β peptide suggest that p21^{Cip1} may be involved in the chronic glial response in Alzheimer's disease.

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	Mixed		Microglia		Astrocytes	
	WT	KO	WT	KO	WT	KO
C	969±138	1032±143	213±12	197±10	363±57	467±23
LPS	943±140	965±149	201±8	198±6	367±61	495±19
OLG	867±98	922±99	NT	NT	NT	NT
FIB	930±106	886±47	NT	NT	NT	NT

Table I. Cell number 24 hours after treatment in mixed, microglial and astrocyte cultures. WT= wild-type cultures. KO= p21^{Cip1-/-} cultures. C= control cells (culture medium). LPS= cells treated with LPS, OLG= cells treated with oligomeric A β form. FIB= cells treated with fibrillar A β form. NT= non tested. Data are means \pm SD from three independent experiments.

Figure Legends

Figure 1. TNF- α release in microglia-enriched cultures (A) and in astrocyte-enriched cultures (B) from wild-type (WT) and p21^{Cip1^{-/-}} (KO) mice 24 h after LPS treatment. Bars represents the means \pm SD of five independent experiments. Data were expressed as a quotient between LPS treated cells versus their control value. * $p < 0.05$, Student's t test.

Figure 2. Nuclear translocation of NF- κ B in mixed glial cultures from WT and KO mice using an anti-p65 subunit antibody. (A) Western blot from nuclear extracts of mixed glial cultures after 24 h of LPS treatment. p36 was used as a loading control. This western blot is representative of three independent experiments. (B) Immunocytochemistry of the mixed glial cultures using the anti-p65 subunit antibody; a and b control glial cells from wild-type and p21^{Cip1^{-/-}} mice, respectively; c and d glial cells after 24 h of LPS treatment. Images are representative of at least three independent experiments. Bar= 50 μ m. Arrowheads point out cell nuclei.

Figure 3. Nuclear translocation of NF- κ B in microglial-enriched cultures from WT and KO mice using the anti-p65 subunit antibody. (A) Western blot from nuclear extracts of microglial cells after 24 h of all treatments. p36 was used as a loading control. This Western blot is representative of three independent experiments. (B) Immunocytochemistry of the microglial cultures using the anti-p65 subunit antibody; a and b control microglial cells from wild-type and p21^{Cip1^{-/-}} mice, respectively; c and d microglial cells after 24h of LPS treatment. Images are representative of at least three independent experiments. Bar= 50 μ m. Arrowheads point out cell nuclei.

Figure 4. Nuclear translocation of NF- κ B in astroglial-enriched cultures from WT and KO mice using the anti-p65 subunit antibody. (A) Western blot from nuclear extracts of astrocytic cells after 24 h of all treatments. p36 was used as a loading control. This western blot is representative of three independent experiments. (B) Immunocytochemistry of the astrocyte cultures using the anti-p65 subunit antibody; a and b control astroglial cells from wild-type and p21^{Cip1^{-/-}} mice, respectively; c and d astroglial cells after 24h of LPS treatment. Images are representative of at least three independent experiments. Bar= 50 μ m. Arrowheads point out cell nuclei.

Figure 5. TLR4 mRNA expression in mixed glial cells. After 6h of LPS treatment no differences between WT and P21^{Cip1} KO were observed. Bars represent the means \pm SD of three independent experiments, normalized versus control values. The statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.05$ versus their control value.

Figure 6. p21^{Cip1} expression in LPS treated mixed glial cultures. (A) Real-time PCR analysis of p21^{Cip1} mRNA expression induced by LPS. Cycle thresholds normalized to actin were used to calculate p21^{Cip1} mRNA levels. Bars represent the means \pm SD from four independent experiments. The statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.01$ versus control. (B) Expression of p21^{Cip1} protein. Cells were treated with LPS \pm cycloheximide (CHX, 50 μ M) for 16 h. This is a representative Western blot of three independent experiments probed for p21^{Cip1} and for p36 as loading control.

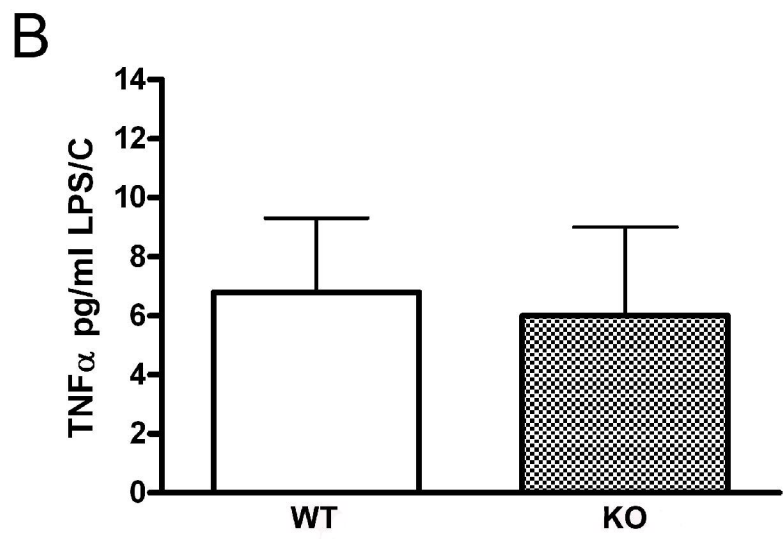
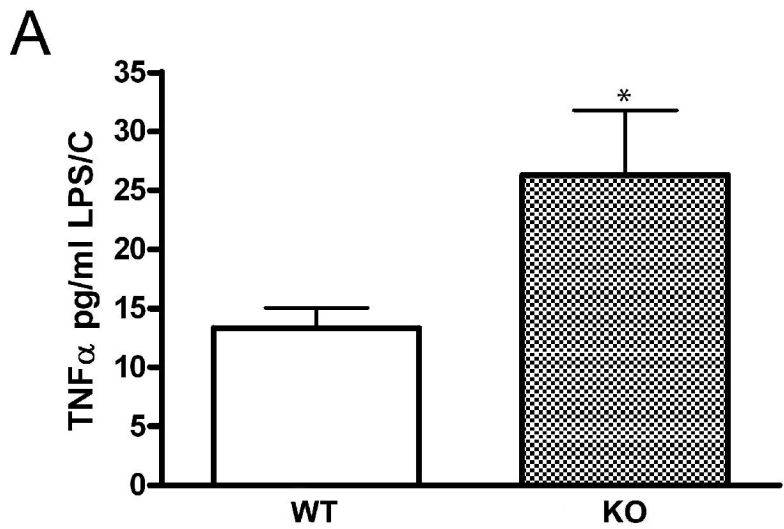
Figure 7 Expression of p21^{Cip1} in glial cells after LPS treatment. A, B and C show the Western blots from mixed glial cultures (A), microglial-enriched cultures (B), and astroglial-enriched cultures (C) from wild-type (WT) and p21^{Cip1}^{-/-} mice (KO) using an anti-p21^{Cip1} specific antibody. Results were obtained after 24h of LPS treatment. Actin was used as a marker of protein loading. The Western blots are representative of three independent experiments. (D) shows the results obtained by immunocytochemistry using the anti-p21^{Cip1} antibody; a, b, and c control cells from mixed glial cultures, microglial-enriched cultures and astroglial-enriched cultures respectively; d, e and f show the images from each cell culture type obtained after 24h of LPS treatment. Images are representative of at least three independent experiments. Bar=50 μ m. Arrowheads point out cell nuclei.

Figure 8. Effect of A β peptide forms in glial cells. (A) shows the western blot of nuclear extracts from mixed glial cultures from WT and KO mice using the p-65 subunit antibody after 24h of treatment with the oligomeric A β form (OLG) and with fibrillar A β (FIB). LPS was used as a positive control of glial activation after 24h of the treatment. p36 was used as a loading control. This Western blot is representative of three different experiments. (B) shows the appearance by phase contrast microscopy of the mixed glial cells 24h after the diverse treatments; a and b show the control mixed glial cells from wild-type and p21^{Cip1} mice respectively; c and d show the glial cells

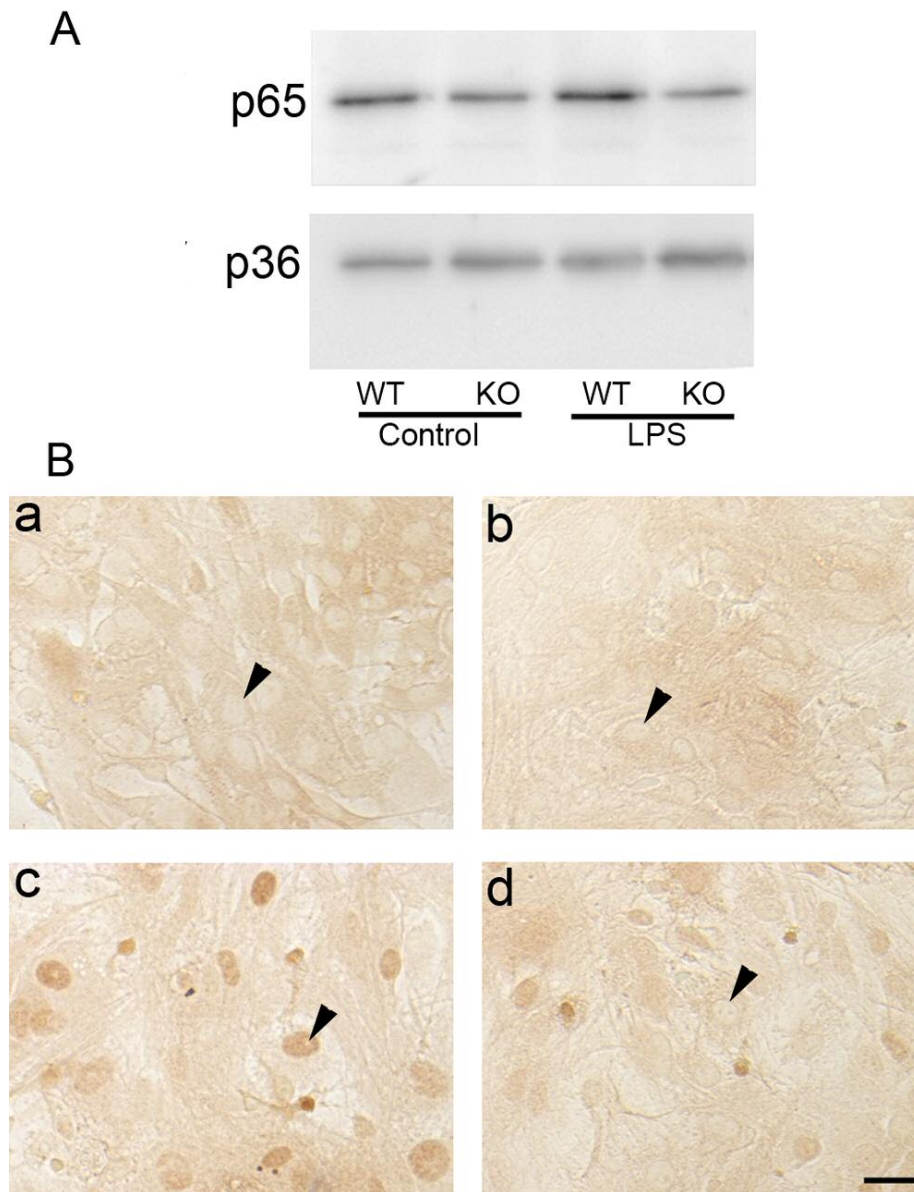
after treatment with the OLG; e and f show the changes in cell appearance after treatment with FIB; g and h show the aspect of the glial cells after LPS treatment. Images are representative of at least three independent experiments. Bar= 50 μ m. Arrowheads point out cell nuclei.

Figure 9. Effect of A β peptide forms on p21^{Cip1} expression in mixed glial cultures. (A) shows the western blot from nuclear cell extracts using the ant-p21^{Cip1} antibody. Mixed glial cultures from KO were used as a negative control to verify the specificity of the p21^{Cip1} antibody. This Western blot is representative of three independent experiments. p36 was used as a loading control. (B) shows the immunocytochemistry after 24h of all treatments. p21^{Cip1}^{-/-} mixed glial cells (b, d, f, h) were used to verify the antibody specificity. a and b show control cells from WT and KO respectively; c and d show glial cells after treatment with OLG; e and f are glial cells after FIB treatment; g and h are glial cells after LPS treatment. Images are representative of at least three independent experiments. Bar= 50 μ m. Arrowheads point out small refringent cells.

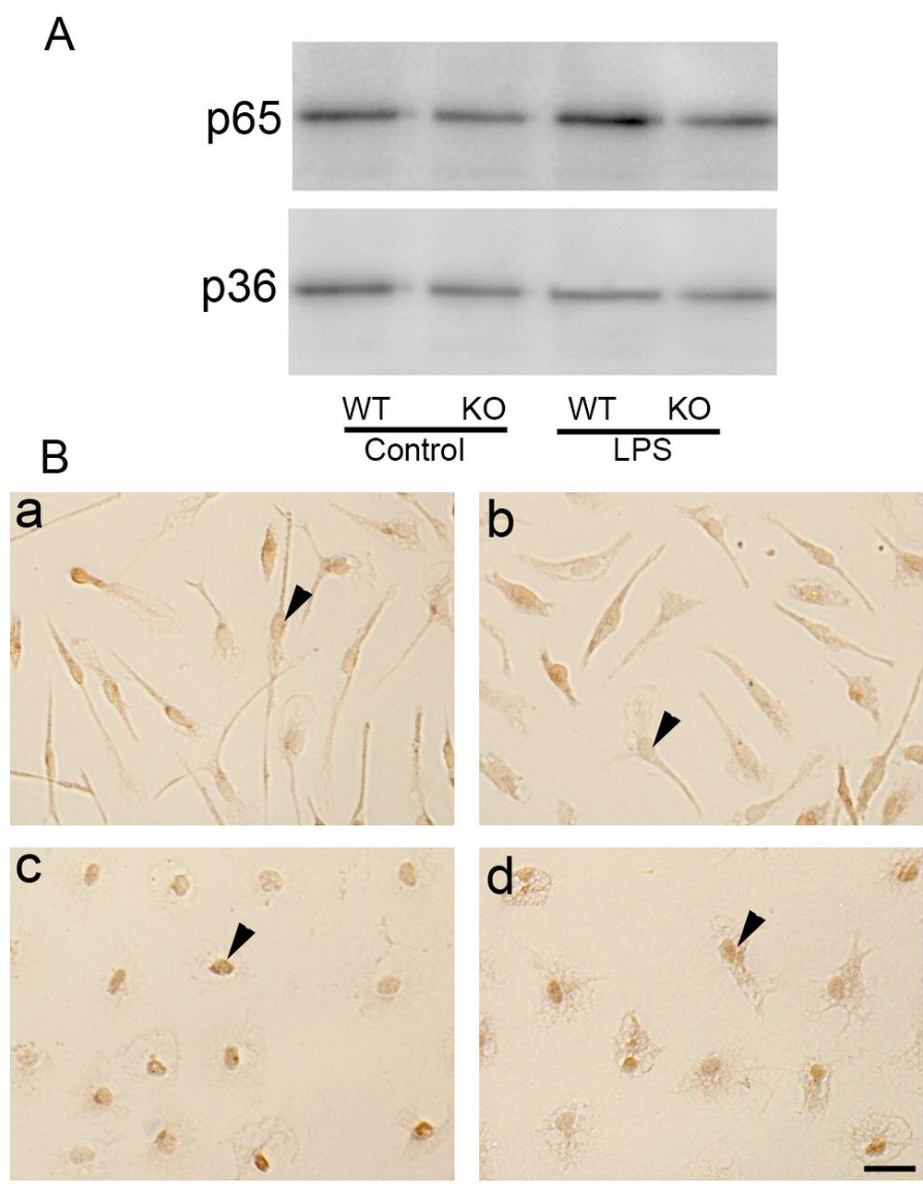
Figure 10. Expression of p21^{Cip1} in mixed glial cultures after 24h of FIB treatment. The p21^{Cip1} expressing cells in mixed glial cultures were detected by double immunocytochemistry. Images show p21^{Cip1} immunolabelling (left column, a and c) and CD11b or GFAP immunofluorescence respectively (right column, b and d). After 24h of treatment with FIB, nuclear expression of p21^{Cip1} was observed in CD11b positive cells (microglia, arrowheads in a, b) and in GFAP positive cells (astrocytes, arrowheads in c, d); a, and b are in the same microscopic field; c and d are in the same microscopic field. Bar=50 μ m.



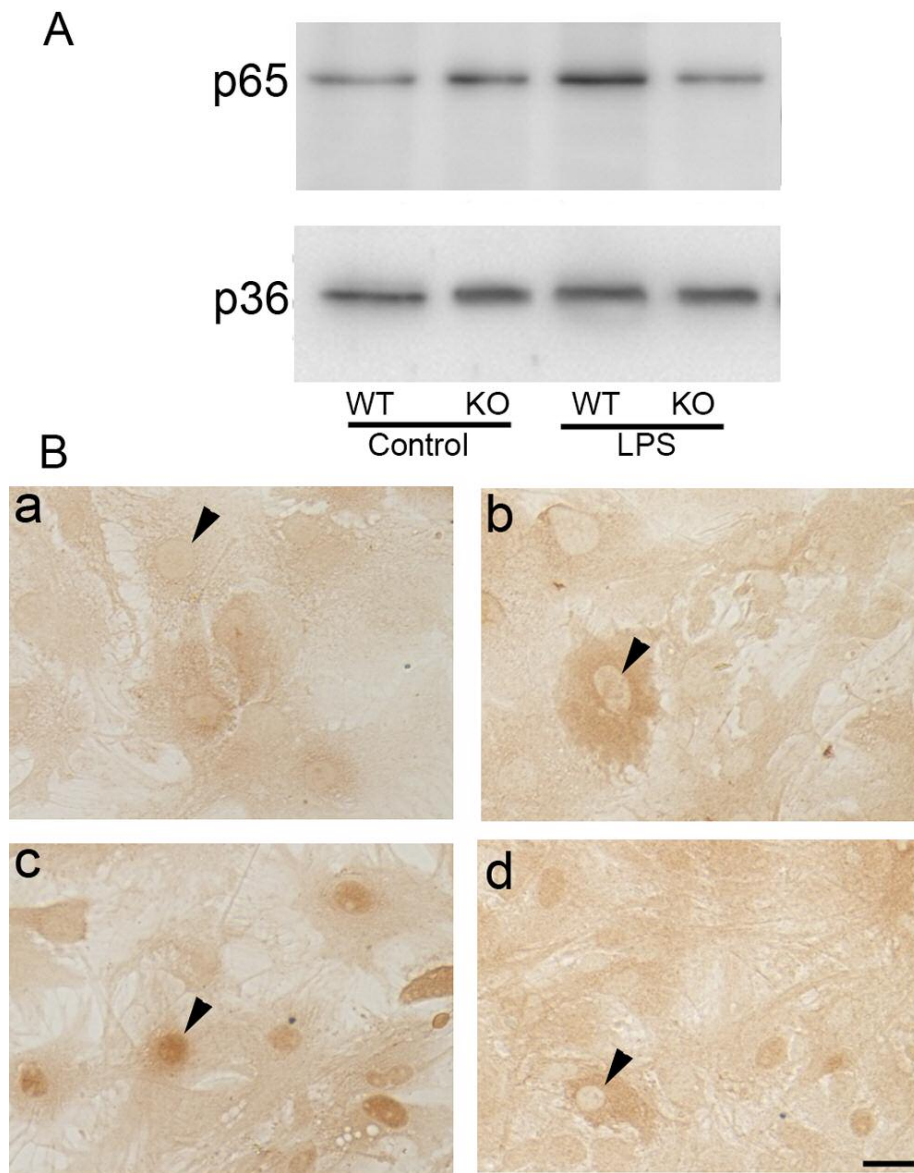
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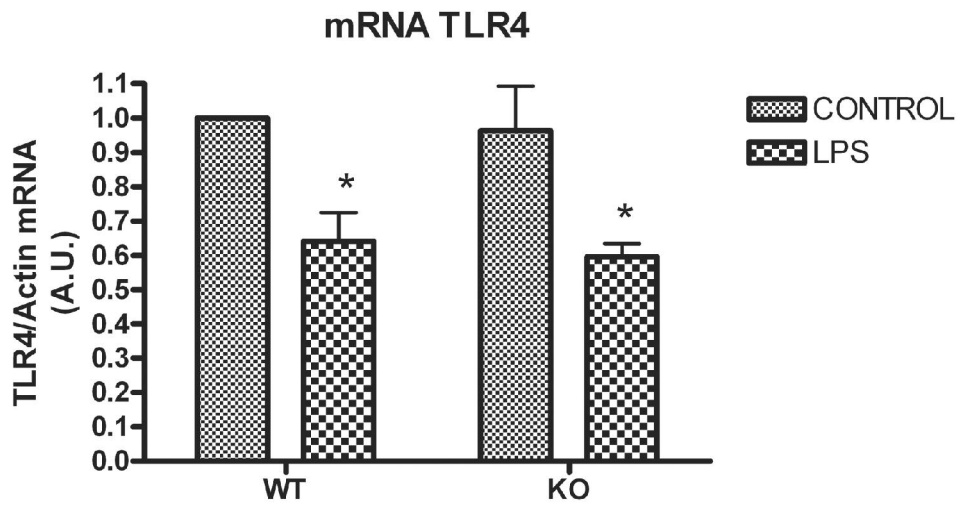
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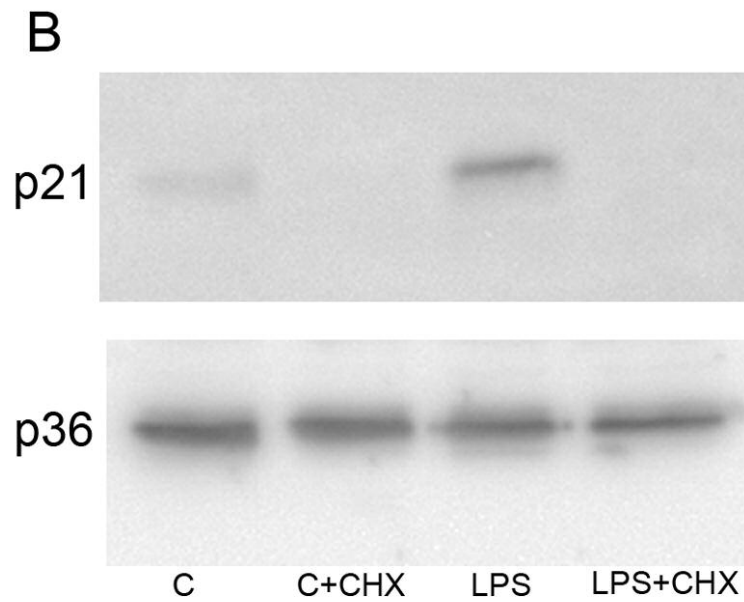
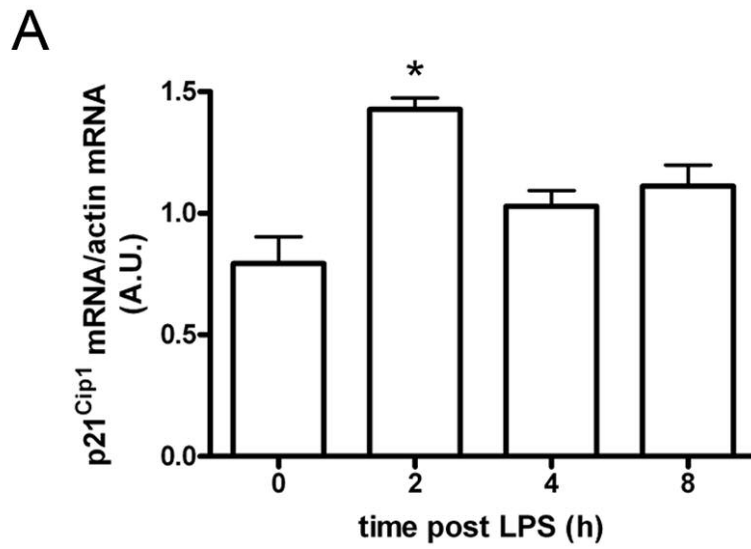
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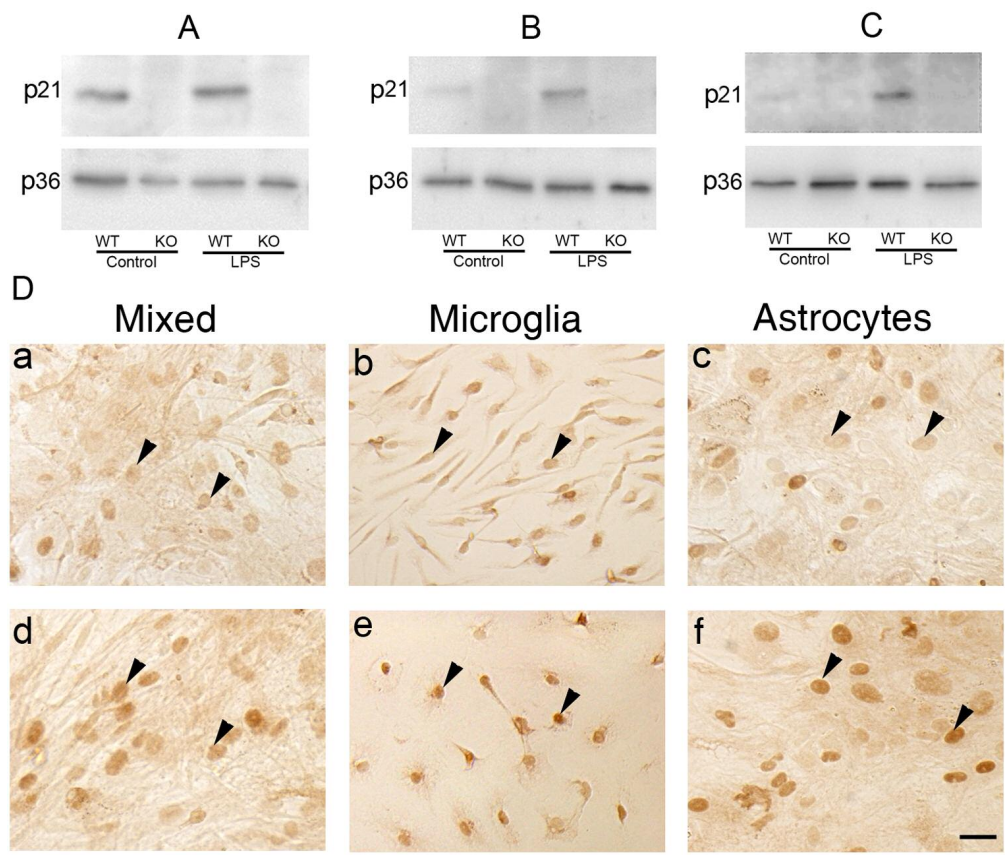
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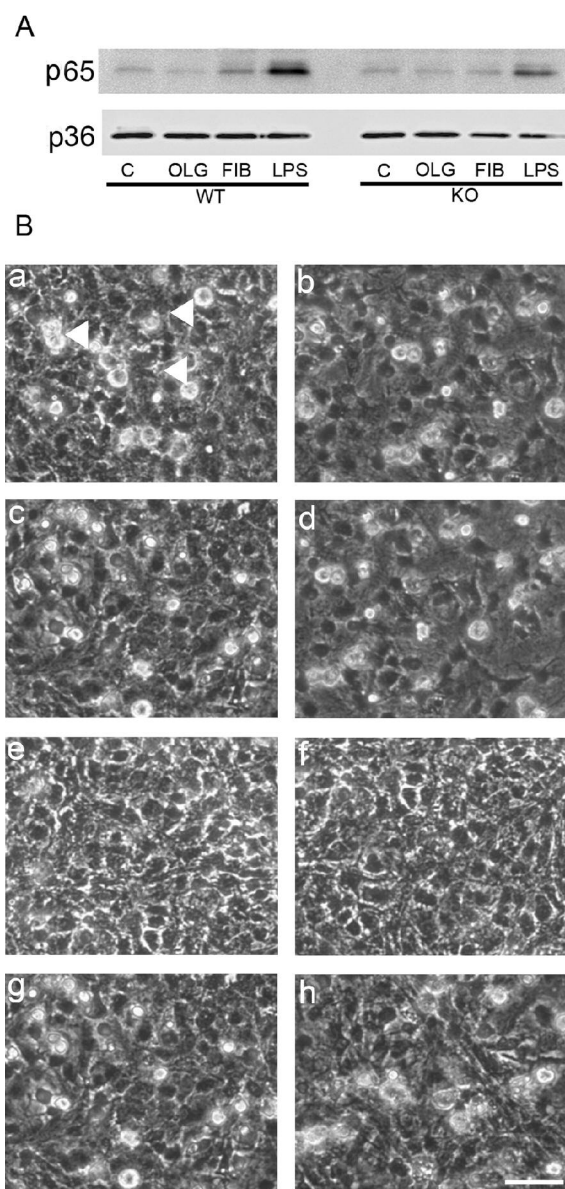
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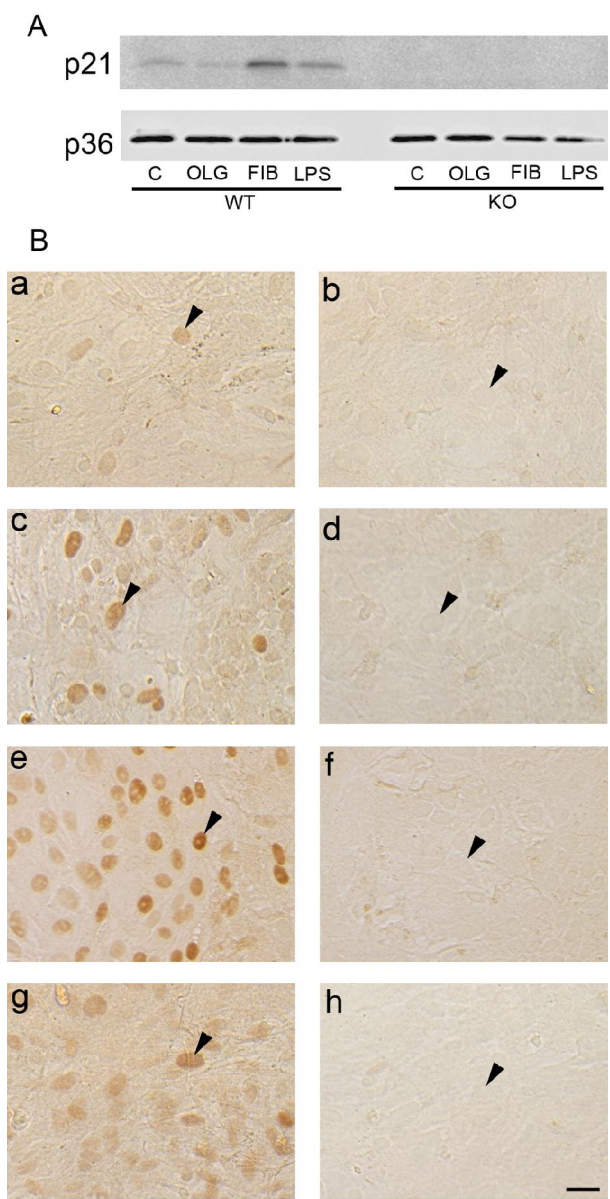
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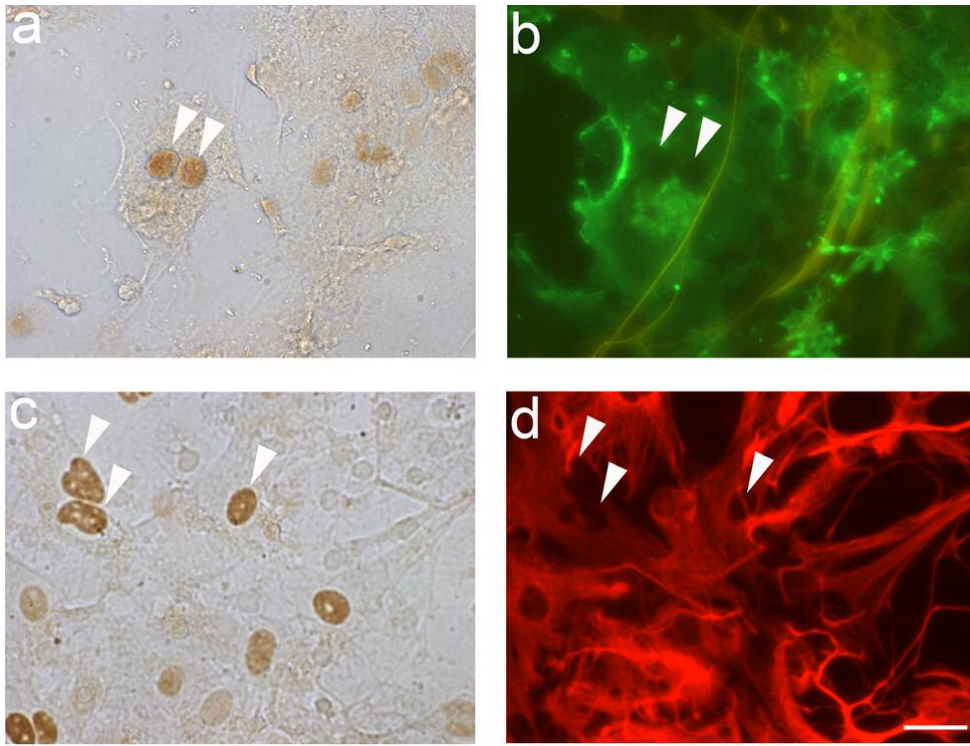
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