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Immunomodulatory influence of bone marrow-derived mesenchymal stem cells on neuroinflammation in astrocyte cultures

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ABSTRACT

The therapeutic benefits associated with mesenchymal stem cells (MSCs) largely result from their immunomodulatory and neurotrophic properties. In this study, we evaluated the effects of MSCs on astrocyte cultures exposed to lipopolysaccharide. In response to this inflammatory trigger, astrocytes showed an increased expression of pro-inflammatory genes (IL-1β, TNFα, IL-6), which was attenuated by pre-exposure to MSC conditioned medium. Furthermore, mediators released by MSCs increased cell proliferation and altered the regulation of intermediate filaments (GFAP, vimentin), pro-inflammatory enzymes (iNOS, COX-2) and receptors (TLR4, CD14, mGluR3, mGluR5). These data demonstrate that MSCs influence diverse cell types participating in the response to neuroinflammation.

1. Introduction

Extensive research on stem cells during the last decade has led to the identification of several types of stem cells isolated from different sources that could be used in cellular therapies for a variety of diseases. Thanks to their large availability, remarkable viability and proliferative capacity, mesenchymal stem cells (MSCs) derived from either bone marrow or adipose tissue have received considerable interest. While the potential benefit provided by these multipotent adult stem cells in several disease models has been largely documented, it is noteworthy that specific cell differentiation and functional integration into the host tissue are not the rule. However, there is accumulating evidence that MSCs grafted into lesioned tissues possess other beneficial capabilities that may promote tissue healing and repair or regeneration. In fact, MSCs were found to attenuate graft-versus-host disease (Le Blanc et al., 2008) and experimental autoimmune encephalomyelitis (EAE) (Gerdoni et al., 2007). Their immunomodulatory activity has also been documented in a plethora of other tissues and disease models of renal and cardiovascular disease (Salem and Thiemermann, 2010), spinal cord injury (Abrams et al., 2009), stroke (Li et al., 2005) and ALS, in the latter case prolonging survival and improving motor-behaviour (Boucherie et al., 2009). *In-vitro* studies have mainly focussed on peripheral immune cells, demonstrating that MSCs promote their survival and stimulate activation and proliferation of CD4+ T lymphocytes (Hoogduijn et al., 2010). In turn, MSCs can suppress the response of T cells to mitogenic and polyclonal stimuli and inhibit the proliferation of stimulated NK cells (Trento and Dazzi, 2010). Effects of MSCs on cells of the central nervous system (CNS) have only been described in a few studies, revealing modulated microglia responses to lipopolysaccharide (LPS) by reduced production of tumour necrosis factor α (TNFα) and nitric oxide (NO) (Zhou et al., 2009; Ooi et al., 2010). Furthermore, MSCs are able to produce and release a variety of growth factors, anti-inflammatory proteins and neurotrophic factors (Caplan and Dennis, 2006; Zhou et al., 2009).

For a long time microglia were thought to be the only cell-type participating in neuroinflammation, being recruited and activated within minutes or hours after an insult and constituting the first line of immune defence. It has since been shown that astrocytes play an essential role in mediating inflammatory processes in the brain and spinal cord (Dong and Benveniste, 2001; Moynagh, 2005). Astrocytes express receptors that recognise pathogenic molecules, such as toll-like receptors (TLR), and can directly respond to pro-inflammatory stimuli *in-vitro* (van Neerven et al., 2010a; Berger et al., 2012) or CNS-injuries *in-vivo*. Through constant interaction with microglial cells, astrocytes are involved in neuroinflammatory processes as they release both pro- and anti-inflammatory mediators. Indeed, activated astrocytes migrate, proliferate and release pro-inflammatory cytokines like TNFα, interleukin-1β (IL-1β) and IL-6. They show increased production of NO and prostaglandin E_2 (PGE_2), but also enhanced release of anti-inflammatory mediators like IL-10 or transforming growth factor β (TGFβ) as well as neurotrophic factors (Schmalenbach and Muller, 1993; Ledeboer et al., 2002).

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To our knowledge, the immunomodulatory effects of MSCs on astrocytes have not yet been explored. Therefore, we investigated the effects of conditioned medium from MSCs on primary cultures of astrocytes activated by LPS, a bacterial endotoxin that binds to CD14 and TLR4. Using this experimental paradigm, we demonstrate that the functional adaptation of astrocytes to inflammatory cues is considerably influenced by soluble mediators released by MSCs.

2. Materials and methods

All experiments were performed in strict accordance with the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC).

2.1. Materials

Culture media, foetal bovine serum, horse serum, penicillin–streptomycin, fungizone, proline, trypsin–EDTA and PCR primers were purchased from Invitrogen (Merelbeke, Belgium). Poly-L-lysine and 4′,6-diaminidino-2-phenylindole (DAPI) were obtained from Sigma (Bornem, Belgium) and ultrapure LPS (*Escherichia coli*) from InvivoGen (Toulouse, France). Percoll™/RediGrad™ reagent was acquired from GE Healthcare (Uppsala, Sweden). TriPure RNA Isolation Reagent was bought from Roche Diagnostic (Vilvoorde, Belgium) and iScript cDNA synthesis kit as well as IQ™ SYBR® Green supermix from BioRad Laboratories (Nazareth, Belgium). The rabbit antibody against glial fibrillary acidic protein (GFAP) was from DAKO (Biognost, Heule, Belgium), while mouse antibody against CD11b was from AbD Serotec (Oxford, United Kingdom). FITC-conjugated donkey anti-rabbit IgG and Cy-3-conjugated goat anti-mouse IgG were obtained from Jackson Immunoresearch Laboratory (DePinte, Belgium). Culture plastic ware was purchased from Greiner Bio-One (Wemmel, Belgium).

2.2. Generation of rat bone marrow-derived MSCs and collection of conditioned medium

MSCs were isolated from the bone marrow of tibias and femurs of 8-week old male Sprague–Dawley rats by plastic adhesion to non-coated tissue culture flasks. Cells were propagated for 15 passages and characterised as previously described (de Hemptinne et al., 2004; de Hemptinne et al., 2006). MSCs were harvested by trypsinisation and plated at a density of 1×10^5 cells per well in six well plates in 2 mL/well DMEM with high glucose, 10% FBS, 1% Penicillin–Streptomycin and 1% Fungizone. After 24 h the medium was changed and incubated for 48 h. Supernatant was collected and centrifuged at 274 ×*g* for 5 min to remove cell debris. Control medium was incubated in culture flasks for 48 h at 37 °C, then collected and centrifuged in the same manner. Conditioned and control media were frozen and stored at −20 °C until used.

2.3. Astroglial culture

Primary cultures were performed from one day old wild-type Wistar rats. Cerebral cortices were dissected and meninges removed in PBS, supplemented with 0.2% glucose. Tissues from up to 3 brains were combined in 2 mL of PBS/glucose and dissociated with the help of a Pasteur pipette. The cell solution was then centrifuged for 5 min at 274 ×*g* and the pellet resuspended in 1 mL of culture medium (DMEM with 10% FBS, 1% proline, 1% fungizone and 1% Penicillin/Streptomycin). In order to avoid contamination with microglial cells that would inevitably contribute to inflammatory responses (Saura, 2007), astrocytes were purified by a Percoll-density-gradient-centrifugation: a 100% Percoll solution was obtained by diluting Percoll 9:10 with 10× PBS, and further dilutions were made using 1× PBS/glucose. After placing 2 mL of 60% Percoll on the bottom of a new 15 mL tube, a layer of 2 mL 30% Percoll was carefully added on top. The cell homogenate was then slowly added and the tubes were centrifuged for 20 min at 550 ×*g*. Following centrifugation, a cell layer of purified astrocytes was present between the culture medium and the 30% Percoll. After cautiously collecting the cells and transferring them into a new 15 mL tube, the full volume was completed with PBS/glucose and the tube centrifuged for 20 min at 550 ×*g* to eliminate most of the Percoll. The pellet was resuspended with culture medium and the cells (approximately 1 flask per pup in a volume of 40 mL) were transferred into a 175 cm^2 flask and cultured for 14 days. The medium was changed after 7 days. On day 14, cells were trypsinised, counted and plated at a density of 3×10^4 cells/cm^2 for RNA extraction. For immunocytochemistry, cells were grown on poly-L-lysine coated coverslips in 24 well plates at a density of 1×10^4 cells/cm^2. This protocol yielded a purity of astrocytes amounting to 96.4 ± 0.4% as identified by immunostaining for GFAP with minimal contamination by CD11b positive microglial cells.

2.4. Cell activation and treatment protocol

All cells were treated 24 h after plating. Two millilitres of MSC conditioned medium or control medium was added to each well and cells were incubated for 24 h. The medium was then renewed and either supplemented with LPS (100 ng/mL) or left as control. After another 24 h of incubation, cell plates were transferred onto ice, washed with 1× PBS and 1 mL TriPure Isolation Reagent was added to each well. Until RNA extraction, the plates were stored at −20 °C.

2.5. RNA extraction, reverse transcription and real-time PCR

RNA was extracted according to the manufacturer's protocol and treated with DNAse at 37 °C for 30 min. Reverse transcription was carried out with the iScript cDNA synthesis kit, using 1 μg of RNA in a total reaction volume of 20 μL. Real-time qPCR was performed for

Table 1
Real-time qPCR primers (F: forward primer, R: reverse primer) and size of amplicon.

Gene	Primer sequence	Amplicon (bp)
CD14	F: 5′-GTGTGAGTGGTAGCCAGCAA-3′ R: 5′-TGCGCAGCGCTAAAACTTG-3′	132
COX2	F: 5′-GTGTGAGTGGTAGCCAGCAA-3′ R: 5′-CCCACAGGAGGATCTGAAAA-3′	146
GAPDH	F: 5′-CCCCCAATGTATCCGTTGTG-3′ R: 5′-TAGCCCAGGATGCCCTTTAGT-3′	115
GFAP	F: 5′-TGGCCACCAGTAACATGCAA-3′ R: 5′-CAGTTGGCGGCGATAGTCAT-3′	134
IL-1β	F: 5′-GGAAGGCAGTGTCACTCATTGTG-3′ R: 5′-GGTCCTCATCCTGGAAGCTCC-3′	84
IL-6	F: 5′-GGGACTGATGTTGTTGACAGCC-3′ R: 5′-CATATGTAATTAAGCCTCCGACTTGTG-3′	128
IL-10	F: 5′-GAAGCTGAAGACCCTCTGGATACA-3′ R: 5′-CCTTTGTCTTGGAGCTTATTAAAATCA-3′	117
iNOS	F: 5′-TGGTCCAACCTGCAGGTCTT-3′ R: 5′-CAGTAATGGCCGACCTGATGT-3′	121
mGluR3	F: 5′-CTGGTGATCCTATGCACTGT-3′ R: 5′-GAGGAATGCCAACCAGATGA-3′	120
mGluR5	F: 5′-ACCCTTCACCAATGACTCCTATG-3′ R: 5′-ATGATGACTGCAGCAAATCGC-3′	124
S100β	F: 5′-GATGTCTGAGCTGGAGAAG-3′ R: 5′-CTCCTGGAAGTCACACTCC-3′	220
TNFα	F: 5′-CCCCGACTATGTGCTCCTCAC-3′ R: 5′-AGGGCTCTTGATGGCGGA-3′	91
TGFβ	F: 5′-GGGGGTCAGCAGCCGGTTAC-3′ R: 5′-CCGCAACAACGCCATCTATGA-3′	223
TLR4	F: 5′-GATTGCTCAGACATGGCAGTTTC-3′ R: 5′-CACTCGAGGTAGGTGTTTCTGCTAA-3′	135
Vimentin	F: 5′-GAACGTAAAGTGGAATCCTTGC-3′ R: 5′-CATACTGCTGGCGGACATC-3′	169

quantification of the genetic expression of IL-1β, TNFα, IL-6, IL-10, TGFβ, iNOS, COX2, CD14, TLR4, mGluR3 and mGluR5 using the iCycler multicolour real-time PCR detection system (BioRad, Nazareth, Belgium). Each reaction was carried out in a total volume of 25 μL, containing 2 ng cDNA template, 350 nM of both specific forward and reverse primers (Table 1) and the IQ SYBR green super mix (BioRad). The protocol consisted of 50 amplification cycles, each conducted as follows: 15 s of denaturation at 95 °C, 45 s annealing at 60 °C and 15 s of elongation at 79 °C. For quantitative analysis, a standard curve was generated by amplification of serial dilutions of a cDNA template mix, consisting of all analysed samples. Each sample was normalised to the relative amplification of the housekeeping gene GAPDH. Raw data sets were analysed by the "post run data analysis" software provided by BioRad.

2.6. Immunocytochemistry

Before being fixed for immunostaining, the morphology of the cells was examined under an Olympus CKX41 transmission microscope, using 10× magnification and Pixellink software Capture SE Version 3.1. Afterwards, cells were fixed with 95% ethanol for 15 min at room temperature and washed 3 times with PBS. The cells were permeabilised with 0.5% Triton X-100 in PBS for 15 min at room temperature. After 3 more washes with PBS and a pre-incubation for 1 h with 3% milk in PBS at 37 °C, samples were incubated overnight at 4 °C with the primary antibodies diluted in a 3% milk/PBS solution (mouse anti-CD11b 1:500, rabbit anti-GFAP 1:1500). The next day started with another washing step after which cells were incubated for 1 h at room temperature with the secondary antibodies, diluted in 3% milk/PBS (FITC-conjugated

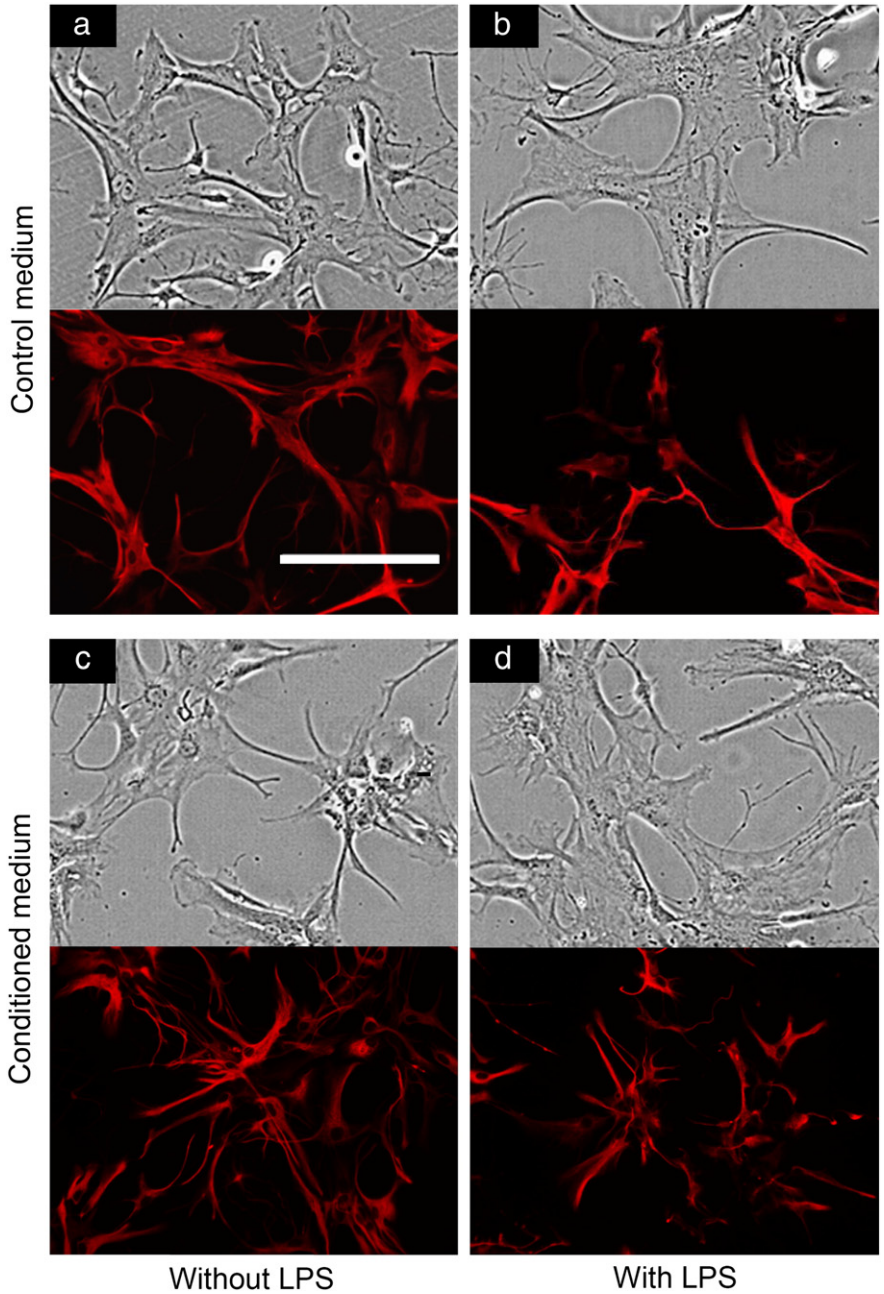

Fig. 1. Influence of MSC conditioned medium and LPS on astrocyte morphology. The morphology of cultured astrocytes was examined at single cell level by phase contrast microscopy or by fluorescence microscopy after GFAP immunostaining. When indicated, cultures were incubated for 48 h in medium conditioned or not with MSCs and LPS (100 ng/mL) was added during the last 24 h. Shown are representative images from 4 experiments. Scale bar 200 μm. Neither LPS treatment (b, c) nor exposure to MSC conditioned medium (c, d) provoked any morphological changes when examined at high magnification.

donkey anti-rabbit and Cy3-conjugated goat anti-mouse, both 1:500). After washing again, nuclei were stained by 5 min incubation with DAPI (1:5000 in PBS), followed by a final washing and then mounted with Fluoprep. Examination took place using a fluorescent inverted microscope (EVOS *fl*, AMG, Westburg, Leusden, Belgium), with the same parameters for all different conditions. Quantification of cell number (DAPI and GFAP-positive cells) as well as GFAP-staining intensity and area fraction was performed with the software ImageJ 1.44. Experiments were repeated 4 times in duplicates. For quantification, pictures of at least 5 different areas of each coverslip were taken for analysis.

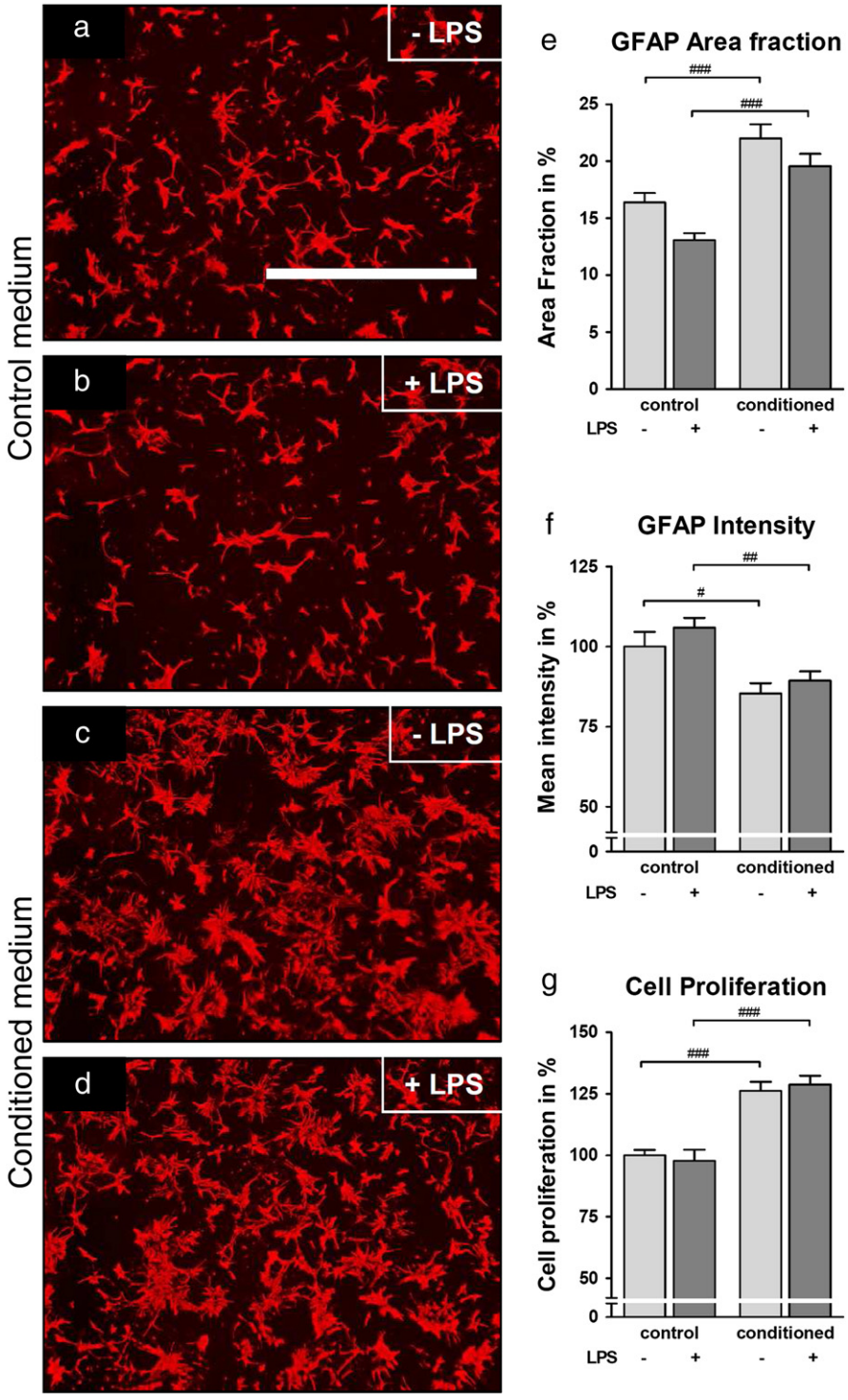

Fig. 2. Influence of MSC conditioned medium on the proliferation of astrocytes. (a–d) Astrocyte cultures where examined at low magnification by fluorescence microscopy after GFAP immunostaining. Where indicated, cultures were incubated for 48 h in medium conditioned or not with MSCs and LPS (100 ng/mL) was added during the last 24 h. Shown are representative images from 4 experiments. Scale bar 2 mm. (e) Quantification of the percentage of the image showing positive GFAP immunostaining (area fraction). (f) Quantification of the mean GFAP intensity of all positive astrocytes. (g) Numeration of DAPI stained nuclei. Data shown are mean values ± SEM from 4 independent experiments, analysed by one-way ANOVA followed by Tukey post-hoc test, #$p<0.05$, ##$p<0.01$, ###$p<0.001$.

2.7. Statistical analysis

All data are presented as means ± standard error of the mean (S.E.M.) and statistical analyses were performed with GraphPad Prism version 5.02 (GraphPad software, CA, USA). Differences between groups were compared by a one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test correcting for multiple comparisons. Statistical differences are indicated as */#$p<0.05$, **/##$p<0.01$ and ***/###$p<0.001$. * refers to the comparison between LPS-activated and non-activated treatment groups, whereas # refers to the comparison between control and MSC conditioned medium treatment groups.

3. Results

3.1. LPS-mediated alteration of astrocyte morphology and proliferation

In-vivo, astrogliosis involves the proliferation of astrocytes and is associated with increased expression of GFAP and a change of cell morphology into a hypertrophic state. In our experimental paradigm, neither adding LPS to the culture medium (24 h) nor exposure to the MSC conditioned medium (48 h) triggered noticeable morphological changes when examined at high magnification (single cell level, Fig. 1). Also, immunodetection of GFAP did not reveal any differences between the culture conditions tested. Nevertheless, examination at a lower magnification revealed a clear-cut difference in the density of GFAP immunopositive cells (Fig. 2a–d). This observation was confirmed by quantifying the area fraction of GFAP staining (percentage of positive immunostaining within a defined area). Thus, while exposure to LPS only modestly affected the intensity of GFAP staining, the maintenance of astrocytes in MSC conditioned medium considerably increased the area fraction of GFAP staining (by 34% for control cells and by 50% for LPS-activated cells, Fig. 2e). Of note, while it increased the GFAP area fraction, this treatment with MSC conditioned medium also decreased the average GFAP intensity in individual cells (Fig. 2f). The influence of LPS and MSC conditioned medium on astrocyte proliferation was examined by quantifying the cell density (counting DAPI stained nuclei, Fig. 2g). The 24 h LPS treatment had no significant influence on the cell density whereas the MSC conditioned medium was responsible for a significant increase in the number of astrocytes (26% increase in control conditions and 32% in LPS-activated cultures).

Upregulation of the intermediate filaments GFAP and vimentin as well as of the calcium-sensor protein S100β is a typical feature of astrogliosis. The mRNA levels for these astrocyte components were therefore examined by RT-qPCR, revealing that the activation of the cultured astrocytes with LPS significantly decreased their expression (Fig. 3). This effect of LPS was also observed in cells that had been maintained in MSC conditioned medium, even though the constitutive levels of GFAP and vimentin mRNA were lower in this set of cultures.

3.2. MSC conditioned medium diminishes cytokine response to LPS

In order to examine whether the MSC conditioned medium influenced the pro- and/or anti-inflammatory responses triggered by LPS in astrocytes, we quantified the mRNA expression of key pro-inflammatory (TNFα, IL-1β and IL-6) and anti-inflammatory cytokines (IL-10 and TGFβ). As more commonly documented in microglia, exposure of astrocyte cultures to LPS for 24 h increased the expression of all tested targets. In cells that had been exposed to MSC conditioned medium, the constitutive expression of TNFα, IL-1β and IL-10 mRNA appeared slightly (but not significantly) decreased. However, with the exception of TGFβ, the increased expression of all cytokines induced by LPS was significantly attenuated (Fig. 4), indicating that soluble factors released by MSCs influence the inflammatory response in astrocytes.

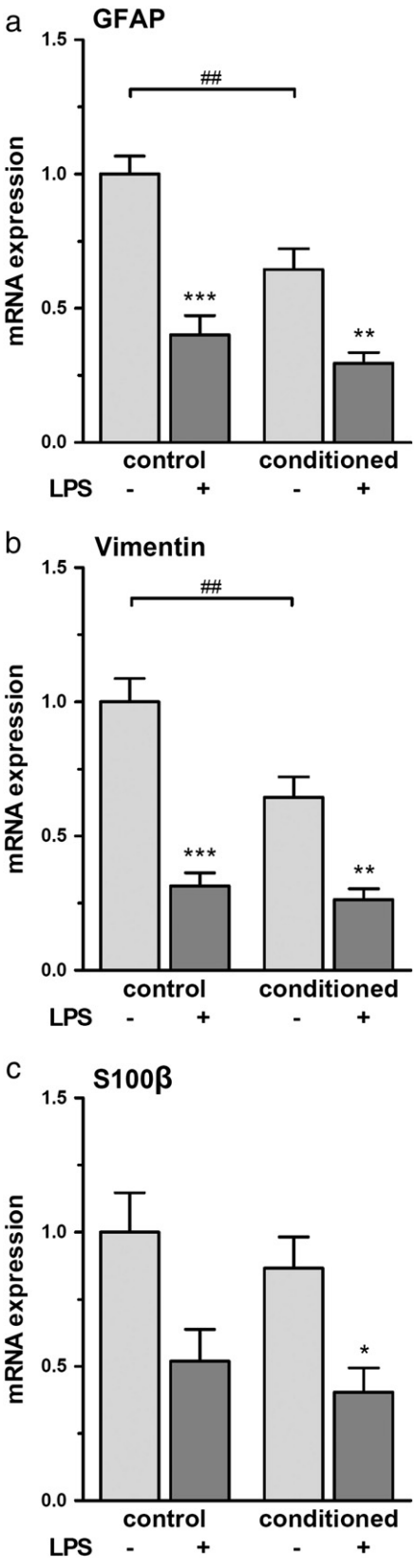

Fig. 3. Modulation of the gene expression of astrogliosis markers. The relative mRNA expression of GFAP (a), vimentin (b) and S100β (c) normalised to GAPDH was examined in astrocyte cultures incubated for 48 h in medium conditioned or not with MSCs and with or without LPS (100 ng/mL) during the last 24 h. Data shown are mean values ± SEM from 4 independent experiments, analysed by one-way ANOVA followed by Tukey post-hoc test, */#$p<0.05$, **/##$p<0.01$ and ***/###$p<0.001$. "*" symbols denote significant differences between LPS-activated and non-activated treatment groups and "#" symbols denote significant differences between control and conditioned medium treatment groups.

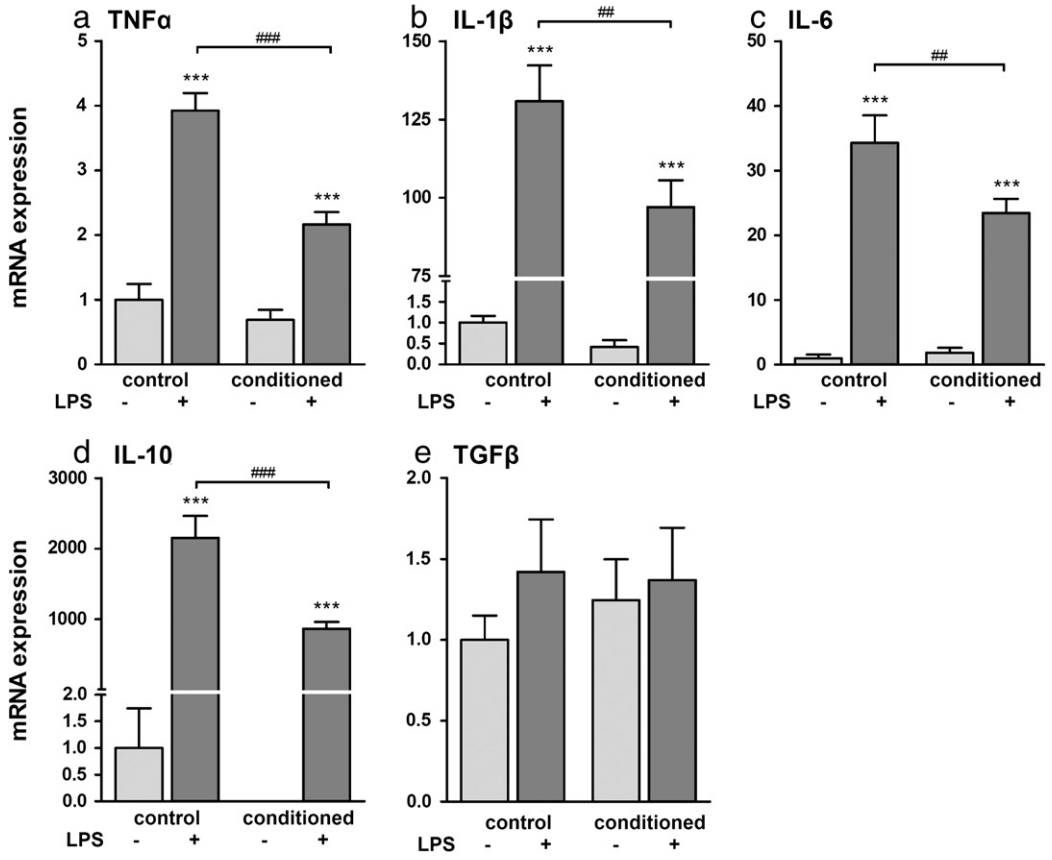

Fig. 4. Modulation of LPS-induced expression of inflammatory cytokines by astrocytes. The relative mRNA expression (normalised to GAPDH) of the pro-inflammatory cytokines TNFα, IL-1β and IL-6 (a–c) as well as of the anti-inflammatory cytokines IL-10 and TGFβ (d, e) was examined in astrocyte cultures incubated for 48 h in medium conditioned or not with MSCs and with or without LPS (100 ng/mL) during the last 24 h. Data shown are mean values ± SEM from 4 independent experiments, analysed by one-way ANOVA followed by Tukey post-hoc test, */#$p<0.05$, **/##$p<0.01$ and ***/###$p<0.001$. "*" symbols denote significant differences between LPS-activated and non-activated treatment groups and "#" symbols denote significant differences between control and conditioned medium treatment groups.

3.3. MSC conditioned medium influences astrocyte functions

Using the LPS-mediated activation of astrocytes as an *in-vitro* model of neuroinflammation, we wondered if other functional adaptations to inflammation were influenced by MSC conditioned medium. The enzymes COX2 and iNOS are typically upregulated under inflammatory conditions and participate in a pro-inflammatory immune response. As shown in Fig. 5, COX2 and iNOS mRNA levels in cultured astrocytes were strongly upregulated after exposure to LPS for 24 h. As observed for cytokines, the exposure of astrocytes to MSC conditioned medium substantially decreased the upregulation of both COX2 and iNOS triggered by LPS. Similarly, we investigated the influence of MSC conditioned medium on the regulation of TLR4. As shown in Fig. 5c, TLR4 gene expression was hardly modified after challenging the astrocytes with LPS in control medium. However, the same treatment was found to cause a downregulation of TLR4 gene expression in cells maintained in MSC conditioned medium. At variance, CD14, the TLR4 co-receptor for LPS was highly upregulated after exposure of the cells to LPS, but this response was not influenced by the MSC conditioned medium (Fig. 5d).

Types 3 and 5 are the most abundant metabotropic glutamate receptors (mGluR) expressed by astrocytes, modulating essential astrocyte functions such as glutamate uptake, cell proliferation and inflammatory responses. These receptors are differentially regulated in response to stimulation with cytokines. As shown in Fig. 5e,f, exposure of cultured astrocytes to LPS increased mGluR3 gene expression (by up to 280%) and decreased mGluR5 gene expression (by 80%). In cells pre-treated with MSC conditioned medium, the constitutive expression of both receptors was substantially decreased. In these cells, the upregulation of mGluR3 induced by LPS was preserved whereas the down-regulation of mGluR5 was almost totally suppressed (Fig. 5f).

4. Discussion

This study provides the first experimental evidence for an influence of MSCs on the adaptation of astrocytes upon *in-vitro* activation. Using a simplified experimental paradigm which consisted of the pre-incubation of cultured astrocytes with medium conditioned by bone marrow-derived MSCs before activation with LPS, we observed an altered response to this inflammatory stress. Thus, soluble factors released by MSCs were found to increase the proliferation of astrocytes and to inhibit the LPS-mediated induction of inflammatory markers.

Activated astrocytes *in-vivo* generally exhibit changes in morphology associated with increased expression of the intermediate filaments GFAP and vimentin. In the present study, somewhat paradoxically, the gene expression of these markers of astrogliosis was strongly decreased upon LPS activation of the cell cultures. Nevertheless, similar observations have been reported with astrocyte cultures challenged with LPS, TNFα or conditioned medium from activated microglia (Selmaj et al., 1991; Letournel-Boulland et al., 1994; Murphy et al., 1995; Frank et al., 2007; Rohl et al., 2007). Indeed, we have previously noticed that such a decrease in GFAP and vimentin gene expression after exposure to activating triggers is transient as the initial decrease is followed by a progressive recovery within 72 h (Tilleux et al., 2007). It is noteworthy that the loss of GFAP mRNA induced by LPS was not reflected when examining GFAP by immunocytochemistry, although mRNA and protein are likely to

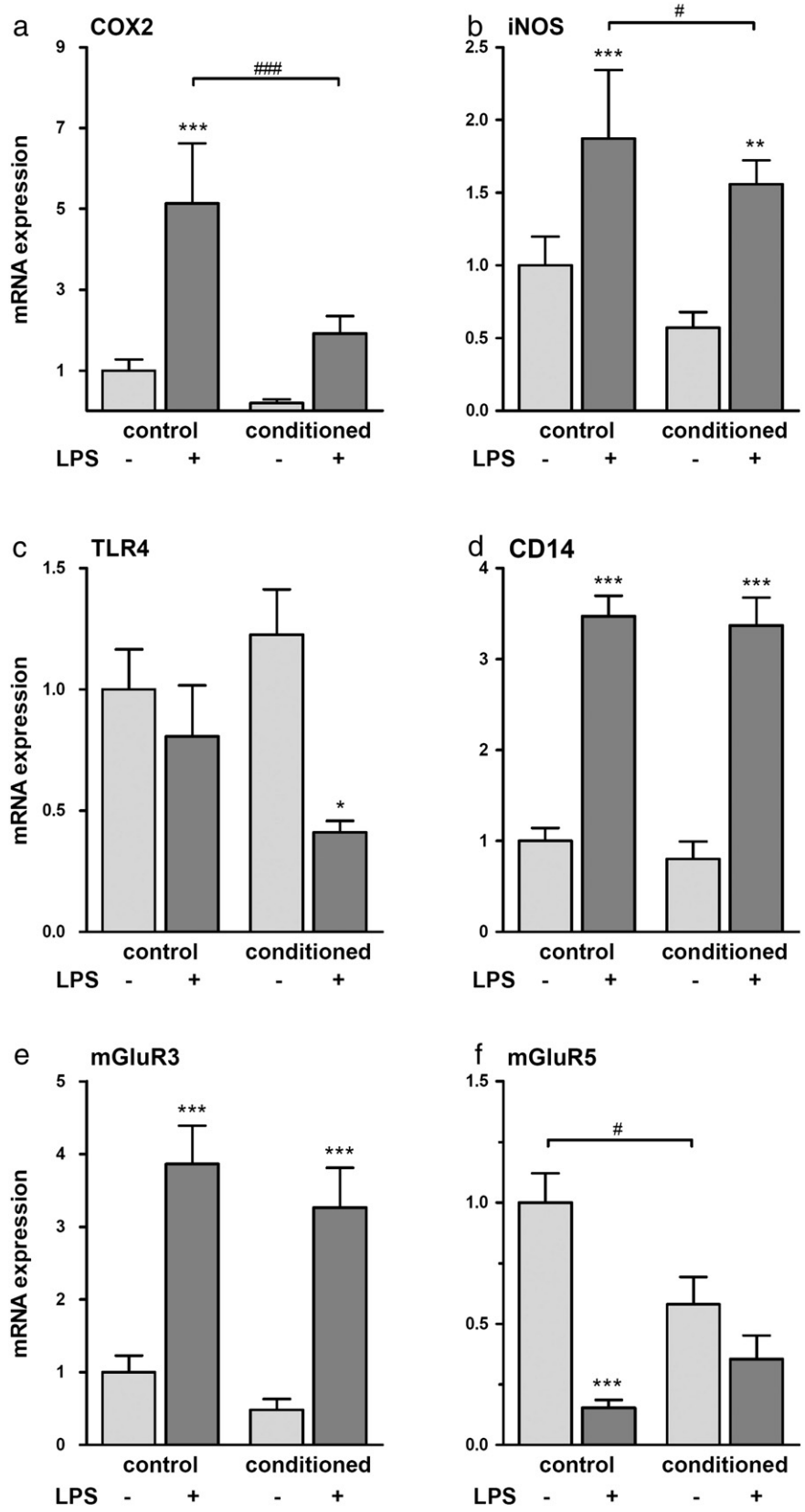

Fig. 5. Influence of MSC conditioned medium on essential genes for astrocyte functions. The relative mRNA expression (normalised to GAPDH) of the enzymes COX2 and iNOS (a, b), the LPS-receptor TLR4 and its co-receptor CD14 (c, d) and the metabotropic glutamate receptors mGluR3 and mGluR5 (e, f) was examined in astrocyte cultures incubated for 48 h in medium conditioned or not with MSCs and with or without LPS (100 ng/mL) during the last 24 h. Data shown are mean values ± SEM from 4 independent experiments, analysed by one-way ANOVA followed by Tukey post-hoc test, $^{*}/\#p<0.05$, $^{**}/\#\#p<0.01$ and $^{***}/\#\#\#p<0.001$. "*" symbols denote significant differences between LPS-activated and non-activated treatment groups and "#" symbols denote significant differences between control and conditioned medium treatment groups.

show different regulation time-courses. Furthermore, immunodetection of cytosolic proteins expressed all over the cells is profoundly biased by changes in cell morphology and proliferation which are in turn influenced by the exposure to LPS. Indeed, convincing preliminary evidence for the influence of soluble mediators released from MSCs on astrocytes is the increased proliferation observed in cultures exposed to MSC conditioned medium. When examined at low magnification, treated astrocyte cultures also showed increased total GFAP immunostaining which coincides with the higher cell density, but also with the increased size of the cells. Of note, individual cells in treated cultures showed lower GFAP staining intensity, which is consistent with a decreased GFAP gene expression.

Activation of astrocytes promotes the release of a variety of pro- and anti-inflammatory cytokines and chemokines (Farina et al., 2007; van Neerven et al., 2010a, 2010b). Their balanced production following nervous insults supports either beneficial or harmful outcomes, respectively leading to neuroprotection or cell death and impeded regeneration. Hence, the pharmacological manipulation of the glial response is seen as a promising objective for the treatment of nervous lesions or neurodegenerative disorders (Sofroniew, 2009; Buffo et al., 2010; Fellner et al., 2011). The present study demonstrates that soluble factors released by MSCs can inhibit the production of cytokines in LPS-activated astrocyte cultures. Not only was the induction of pro-inflammatory cytokines attenuated, but also the expression of the anti-inflammatory IL-10, known to inhibit the production of TNFα, IL-1β and IL-6 (Owens et al., 2001). This supports the concept that MSCs are not strictly endowed with anti-inflammatory properties, but rather exhibit an immunosuppressive influence. It is however noteworthy that MSC conditioned medium had no influence on TGFβ. While the molecular mechanisms involved in the regulation of this anti-inflammatory growth factor remain largely unknown, our data indicate that MSCs might only influence certain signalling pathways in astrocytes.

Besides having an influence on the expression of cytokines, the soluble mediators constitutively released by MSCs were also found to change astrocyte functions. Thus, consistent with a modulation of immune response, the LPS-induced COX2 and iNOS expression was significantly attenuated when the cells were pre-exposed to MSC conditioned medium. Identical observations were reported by Zhou et al. (2009) on a model of cultured microglia, indicating that MSCs have similar effects on both types of cells. Using the same model of undifferentiated MSCs transplanted in the cerebral fluid of adult rodents, we recently evidenced a decrease in COX2 and NADPH oxidase (NOX-2) expression in the lumbar spinal cord of transgenic rats developing amyotrophic lateral sclerosis (Boucherie et al., 2009). Such a modulation of inflammatory markers and enzymes certainly explains the immunodepressive outcome described in several *in-vivo* studies where MSCs have been used as a therapeutic tool (Li et al., 2005; Abrams et al., 2009; Salem and Thiemermann, 2010).

The activation of glial cells by LPS requires the presence of the cognate receptor TLR4 as well as its co-receptor CD14, which facilitates recognition by the former. In the present study, exposure to LPS caused an increased expression of CD14 mRNA whereas TLR4 mRNA was unchanged. A similar upregulation of CD14 was shown in brain parenchyma after intraperitoneal administration of LPS in rats (Nadeau and Rivest, 2000) and also in mixed glia cultures treated with 500 ng/mL of LPS for 5 h (Lehnardt et al., 2002) while unchanged TLR4 mRNA levels have been reported in astrocyte cultures (Bellini et al., 2011; Santos-Galindo et al., 2011) and mixed glia cultures (Lehnardt et al., 2002) after LPS treatment investigating different time points. Surprisingly, maintaining astrocytes in MSC conditioned medium did not influence CD14 expression but significantly decreased TLR4 expression when simultaneously exposed to LPS. This loss of TLR4 expression likely reduces the response to LPS and may contribute to the inhibitory effects of MSC conditioned medium on the activation of astrocytes. Down-regulation of TLR4 was also observed when examining the impact of the insulin-like growth factor 1 (IGF1) on cultured astrocytes activated by LPS (Bellini et al., 2011). It is tempting to propose that IGF1 participates in the effects of MSCs, as recent studies have evidenced the production of this growth factor by cultured MSCs (Zhou et al., 2009; Nicaise et al., 2011). Indeed, IGF1 is also known to enhance the proliferation of cultured astrocytes (McMorris and Dubois-Dalcq, 1988; Tranque et al., 1992).

In order to examine how the neuroprotective functions of astrocytes under inflammatory conditions can be influenced by MSCs, we also studied the regulation of mGluR3 and mGluR5, the predominant glutamate receptors in cultured astrocytes. We recently reported on the opposite regulation of these two receptors in astrocytes exposed to the inflammatory cytokines IL-1β or TNFα (Berger et al., 2012). Considering the distinct signalling pathways associated with these receptors, we hypothesised that the concomitant upregulation of mGluR3 and downregulation of mGluR5 could reinforce the neuroprotective activity of glial cells under inflammatory conditions. Like cytokines, LPS was found to trigger the same opposite regulations of mGluR3 and mGluR5 in cultured astrocytes. The pre-treatment of astrocytes with MSC conditioned medium did not affect the upregulation of mGluR3 preserving the neuroprotective function ascribed for this receptor. At variance, the loss of mGluR5 induced by LPS was considerably attenuated in cells pre-treated with MSC conditioned medium. The role of this receptor subtype in astrocytes remains largely debated as authors have reported on both beneficial and harmful consequences of its activation on glutamate handling (Aronica et al., 2003; Byrnes et al., 2009; D'Antoni et al., 2011). Nevertheless, the possibility of differentially modulating mGluR3 and mGuR5 suggests that the regulation of these receptors operates through distinct molecular signalling mechanisms.

In conclusion, the present data indicate for the first time that MSCs have an influence on astrocytes under pro-inflammatory conditions *in-vitro*. While such modulation may contribute to the numerous benefits of MSCs observed in *in-vivo* studies, it is important to mention that no activation/stimulation or differentiation of MSCs appeared necessary to achieve the immunomodulatory effect, as previously stated (Hoogduijn et al., 2010). As demonstrated in peripheral tissues, the influence of MSCs in the central nervous system is thus not restricted to immune-specialised cells, but reaches other cell types that actively contribute to the neuroinflammatory microenvironment. This should be taken into account when examining the consequences of grafting MSCs in models of neurological disorders.

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