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C/EBPβ expression in activated microglia in amyotrophic lateral sclerosis

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Abstract.

Neuroinflammation is thought to play a pathogenic role in many neurodegenerative disorders including amyotrophic lateral sclerosis (ALS). In this study we demonstrate that the expression of NO synthase-2 (NOS2) and cyclooxygenase-2 (COX-2) induced by lipopolysaccharide (LPS) + interferon-γ is higher in microglial-enriched cultures from G93A-SOD1 mice, an ALS animal model, than from wild-type mice. The levels of CCAAT/enhancer binding protein β (C/EBPβ), a transcription factor that regulates proinflammatory gene expression, are also upregulated in activated G93A-SOD1 microglial cells. In vivo, systemic LPS also induces an exacerbated neuroinflammatory response in G93A-SOD1 mice vs wild-type mice, with increased expression of GFAP, CD11b, NOS2, COX-2, proinflammatory cytokines and C/EBPβ. Finally, we report that C/EBPβ is expressed by microglia in the spinal cord of ALS patients. This is the first demonstration to our knowledge of microglial C/EBPβ expression in human disease. Altogether these findings indicate that G93A-SOD1 expression results in an exacerbated pattern of neuroinflammation and suggest that C/EBPβ is a candidate to regulate the expression of potentially neurotoxic genes in microglial cells in ALS.

Keywords

Neuroinflammation; microglia; astrocyte; C/EBP beta; amyotrophic lateral sclerosis; NOS2; COX-2; IL-1 beta; IL-6; TNF alpha; lipopolysaccharide

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive form of motor neuron disease that invariably leads to skeletal muscle atrophy, paralysis and death. The incidence rate increases with age being maximal, 12/100,000 per year, in the 70-79 years range (for review, see Mitchell and Borasio, 2007). Most cases of ALS are sporadic but about 5-10% are familial. The most frequent and best characterized mutations associated with familial ALS are those in the gene encoding superoxide dismutase (SOD) (Rosen et al., 1993), with mutations in the DNA/RNA binding proteins TDP-43 (Gitcho et al., 2008; Sreedharan et al., 2008) and FUS/TLS (Kwiatkowski, Jr. et al., 2009), (Vance et al., 2009) being less frequent. Cytoplasmic ubiquitinated protein inclusions are characteristic of ALS. Interestingly, TDP-43 is a major component of such inclusions in most sporadic ALS cases (Arai et al., 2006, Neumann et al., 2006)

Transgenic mice with ubiquitous overexpression of the pathogenic G93A human SOD1 mutation (G93A-SOD1) show selective motor neuron degeneration and progressive paralysis that resemble human ALS (Hensley et al., 2006). The specific reduction of G93A-SOD1 expression in microglial cells slows disease progression and prolongs survival (Beers et al., 2006; Boillee et al., 2006) suggesting that microglial G93A-SOD1 is involved in disease progression in this ALS model. How this may occur has not yet been resolved. However, microglial cells with G93A-SOD1 expression show increased neurotoxicity and increased production of tumor necrosis factor-α (TNFα), NO, O_2^-, interleukin-6 (IL-6) and MCP-1 when activated in vitro (Beers et al., 2006;Liu et al., 2009; Sargsyan et al., 2009; Weydt et al., 2004; Xiao et al., 2007).

Accumulating evidence supports the hypothesis of a role of glial activation in the pathogenesis of most neurodegenerative disorders, including ALS (Frank-Cannon et al., 2009). Glial activation is the response of astrocytes and microglia to stimuli such as bacteria, viruses, damaged cells, cell debris, abnormal protein deposits, etc. Glial activation is not a unique process. The final phenotype of activated astrocytes and microglial cells can be strongly affected by factors such as the nature of the triggering stimulus, the duration of the stimulation, the participation of other cell types (lymphocytes, neutrophiles, dendritic cells, etc), the integrity of the blood-brain barrier or the CNS region where this response takes place. Although this response is primarily neuroprotective, some elements of glial activation, particularly of microglial activation, are potentially neurotoxic. Thus, the expression of the proinflammatory cytokines

TNFα, interleukin-1β (IL-1β) or IL-6 or the enzymes nitric oxide synthase 2 (NOS2), cyclooxygenase-2 (COX-2) or NADPH oxidase is increased in activated glial cells and these molecules or their products have the potential of damaging neurons (Glass et al., 2010). In the promoters of the genes of TNFα, IL-1β, IL-6, NOS2 and COX-2 functional binding sites for the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) are found. C/EBPβ is a member of the C/EBP family of b-zip transcription factors and we and others have shown that C/EBPβ levels are increased in activated glial cells (Cardinaux et al., 2000; Ejarque-Ortiz et al., 2007). Therefore, C/EBPβ can be a candidate to mediate neurotoxic effects of activated glial cells.

In this work we have investigated the proinflammatory profile of activated microglial cells from G93A-SOD1 mice in vivo and in vitro. Our results indicate that G93A-SOD1 microglial cells show an exacerbated response to LPS and suggest that C/EBPβ could mediate the expression of proinflammatory genes in this ALS model. Finally, we have observed the microglial expression of C/EBPβ in human ALS spinal cord sections. This is to our knowledge the first demonstration of C/EBPβ expression in human microglia

2. Methods

2.1. G93A-SOD1 mice

G93A-SOD1 mice (Gurney et al., 1994) were generously provided by Dr. Manuel Portero (Facultat de Medicina, Universitat de Lleida, Spain) and were kept at the Animal facilities of the University of Barcelona. 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 procedure was approved by the Ethics and Scientific Committees of the Hospital Clínic de Barcelona and registered at the “Departament d’Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya”.

2.2. In vivo treatments

G93A-SOD1 mice were used when symptoms of severe hindlimb motor deficits were observed. This occurred between 120 and 130 days and at this point the life expectancy of the animals in our colony was typically less than 7 days. Age-matched wild-type C57Bl/6 mice were used. Twenty-eight mice (4 groups, 7 mice per group) were used in the in vivo experiments. Mice were injected intraperitoneally with LPS (055:B5, Sigma-Aldrich) at a final dose of 200 μg per animal or vehicle (saline solution).

Mice were deeply anesthetized with isofluorane 6 hours after intraperitoneal injection, sacrificed and the cervical (for Western blot), thoracic and lumbar (for qRT-PCR) regions of the spinal cord were carefully removed, pooled and frozen in carbonic ice. For immunohistochemistry, 24 hours after LPS injection, the mice were deeply anesthetized, sacrificed and the cervical and thoracic regions of the spinal cord were carefully removed and frozen in carbonic ice. Thirty µm-thick cryostat sections were cut and stored at -20°C until immunostaining.

2.3. Cell Cultures and Treatment

Microglial cultures were prepared from 1- or 2-day-old neonatal G93A-SOD1 or wild-type (wt) mice both from C57BL/6 genetic background as described (Saura et al., 2003). Briefly, mice cerebral cortices were dissected, their meninges were totally removed and cortices were 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 µg/mL and amphotericin B 0.5 µg/mL) with 0.02% deoxyribonuclease I. The solution was pelleted (5 min, 200g), resuspended in culture medium and brought to a single cell suspension by repeated pipetting followed by passage through a 100 µm pore mesh. Glial cells were seeded at a density of $3.5x10^5$ cells/mL and cultured at 37°C in humidified 5% CO_2–95% air. The medium was replaced every 5–7 days. 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 Ca^{2+}. 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. For immunocytochemistry studies cells were seeded in 48-well plates. For the isolation of nuclear proteins from microglial cultures, cells were seeded in 6-well plates. Microglial cultures were treated 24h after their isolation. Cells were treated with LPS (100 ng/ml) + Interferon-γ (IFNγ, 0.1 ng/ml) in culture medium. Control cells were treated with culture medium.

2.4. Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 23–25°C. For immunocytochemistry using peroxidase labelling, cells were permeated and endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 10 min. Non-specific staining was blocked by incubating the cells with 10% normal goat or horse serum in PBS containing 1% BSA for 20 min at 23–25°C. Cells were then incubated overnight at 4°C with one of the

following primary antibodies: polyclonal rabbit anti-NOS2 (1:300, BD, USA); polyclonal rabbit anti-COX-2 (1:1000, Santa Cruz Biotechnologies, USA), polyclonal rabbit anti-C/EBPβ (1:2000, Santa Cruz Biotechnologies, USA), polyclonal rabbit anti-p65-NFκB (1:1000, Santa Cruz Biotechnologies, USA), monoclonal rat anti-CD11b (1:300, Serotec, Oxford, UK), monoclonal mouse anti-GFAP (1:2000, DAKO). After rinsing in PBS, cells were incubated for 1 h at room temperature with biotinylated horse anti-mouse. goat anti-rabbit or goat anti-rat secondary antibody (1:200, Vector). Then, after rinsing in PBS, cells were incubated for 1 h with ExtrAvidin-HRP (1:500, Sigma-Aldrich) and the immunostaining was developed with 0.1% diaminobenzidine (Sigma-Aldrich) and 0.3% H2O2. Microscopy images were obtained with an Olympus IX70 microscope (Olympus, Okoya, Japan) and a digital camera (CC-12, Soft Imaging System GmbH, Munich, Germany).

2.5. Immunohistochemistry

Cervical/thoracic spinal cord sections were washed in PBS and fixed in 4% paraformaldehyde during 20 minutes. After rising in PBS, the sections were treated with 2% H_2O_2 in methanol during 10 minutes. The sections were rinsed in PBS-0.5% Triton, blocked with 10% of normal horse serum and incubated overnight with the monoclonal rat anti-CD11b antibody (1:500, Serotec, Oxford, UK) or polyclonal rabbit anti-GFAP (1:1000, DAKO). After that, sections were incubated with biotinylated goat anti-rabbit or goat anti-rat secondary antibody (1:200, Vector). Then, after rinsing in PBS, sections were incubated for 1 h with ExtrAvidin-HRP (1:500, Sigma-Aldrich) and the immunostaining was developed with 0.1% diaminobenzidine (Sigma-Aldrich) and 0.3% H_2O_2. After washed in PBS, the sections were dehydrated and mounted in DPX medium.

For double immunohistofluorescence, the sections were incubated overnight at 4°C with both polyclonal rabbit anti-C/EBPβ (1:2000, Santa Cruz Biotechnologies, USA) and monoclonal rat anti-CD11b (1:300, Serotec, Oxford, UK) or monoclonal mouse anti-GFAP (1:1000, DAKO) primary antibodies. After rinsing in PBS, sections were incubated 1 h at room temperature with goat anti-rabbit ALEXA 546 (1:1000) and/or goat anti-rat or anti-mouse ALEXA 488 (1:500) secondary antibodies (Molecular Probes, Eugene, OR, USA). Finally, after washed in PBS the sections were mounted in mowiol medium.

For triple immunofluorescence, sections were incubated overnight at 4°C with polyclonal rabbit anti-C/EBPβ (1:1000, Santa Cruz Biotechnologies, USA), monoclonal mouse anti-GFAP (1:1000, DAKO) and biotinylated tomato lectin (1:200, Sigma). After rinsing in PBS, sections were incubated 1 h at room temperature with goat anti-rabbit

ALEXA 546 (1:500), anti-mouse ALEXA 647 (1:500) and Streptavidin ALEXA 488 (1:500). Finally, the sections were counterstained with DAPI and analyzed using a Leica TCS 4D laser confocal fluorescence microscope with a x40 objective at the Serveis Científico-Tècnics, School of Medicine, University of Barcelona. For each animal (WT saline, n=3; WT LPS, n=4; SOD saline, n=3; SOD LPS, n=4) 12-15 sequential confocal images were obtained at 2 μm-z-intervals in two fields of the gray matter between reticulospinal and anterior spinothalamic tracts. Total C/EBPβ positive cells and double C/EBPβ-tomato lectin or C/EBPβ-GFAP positive cells were counted. Adjacent images were compared and cells that were present in more than one z-image were counted only once.

2.6. Quantitative real-time PCR (qRT-PCR)

mRNA expression was determined 6 h after in vivo treatment. Total RNA from thoracic/lumbar spinal cord pools was isolated with Trizol method (TriRReagent, Sigma-Aldrich), a modification of the method originally described by (Chomczynski and Sacchi, 1987). 1.5 μg of RNA for each condition was reverse transcribed with random primers using Transcriptor Reverse Transcriptase (Roche Diagnostics). cDNA was diluted 1/10 to perform real-time PCR. The primers (Roche Diagnostics) used to amplify mouse mRNA were: C/EBPα, 5'-TGGACAAGAACAGCAACGAGTAC-3' and 5'-TGCGCAGGCGGTCATT-3'; C/EBPβ, 5'-AAGCTGAGCGACGAGTACAAGA-3' and 5'-GTCAGCTCCAGCACCTTGTG-3'; NOS2, 5'-GGCAGCCTGTGAGACCTTTG-3' and 5'-GCATTGGAAGTGAAGCGTTTC-3'; COX-2, 5'-CATCCTGAGTGGGGTGATGAG-3' and 5'-GGCAATGCGGTTCTGATACTG-3'; IL-1β, 5'-TGGTGTGTGACGTTCCCATTA-3' and 5'-CAGCACGAGGCTTTTTTGTTG-3'; IL-6, 5'-CCAGTTTGGTAGCATCCATC-3' and 5'-CCGGAGAGGAGACTTCACAG-3', TNFα, 5'-TGATCCGCGACGTGGAA-3' and 5'-ACCGCCTGGAGTTCTGGAA-3'; GFAP 5'-AAGGTCCGCTTCCTGGAA-3' and 5'-GGCTCGAAGCTGGTTCAGTT-3'; CD11b, 5'-AGCTTGAAAGGACCCCAGTG-3' and 5'-AGCAGGAGGTGACCATGAGA-3'; (forward and reverse primers, respectively). For normalization of cycle threshold (Ct) values to an endogenous control, the following mouse actin and S18 mRNA primers were used: Actin, 5'-CAACGAGCGGTTCCGATG-3' and 5'-GCCACAGGATTCCATACCCA-3'; S18, 5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGTAGCG-3'. Real-time PCR was carried out with IQ SYBR Green SuperMix (Bio-Rad Laboratories) and iCycler MyIQ equipment (Bio-Rad Laboratories). Primer efficiency was estimated from standard curves generated by dilution of a cDNA pool. Samples were run for 50 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 15 s). Relative gene expression values were calculated with the comparative

Ct or ΔΔCt method (Livak and Schmittgen, 2001) using iQ5 2.0 software (Bio-Rad Laboratories). Ct values were corrected by the amplification efficiency of the respective primer pair which was estimated from standard curves generated by dilution of a cDNA pool.

2.7. Isolation of nuclear and total proteins

Nuclear protein and total protein extraction from cell cultures were performed as previously described (Ejarque-Ortiz et al., 2010). C/EBPβ and p65-NFκB levels were determined in nuclear protein extracts from microglial cell cultures 24 hours after treatments, using one or two wells from 6-well plates for each experimental condition. NOS2 and COX-2 levels were determined in total protein extracts from microglial cell cultures 24 h after treatments. Protein concentration was determined by the Lowry assay (Total Protein kit micro-Lowry, Sigma-Aldrich). For total protein extraction for in vivo Western blot spinal cord samples were first sonicated at 4º C in RIPA buffer containing protease inhibitor cocktail (1 ml of ice cold RIPA buffer per gram of tissue). After 30 minutes of incubation in ice, samples were centrifuged at 5000 rpm for 5 minutes at 4° C and the supernatants were collected. Protein concentration was determined by the Lowry assay as above.

2.8. Western blot

For in vitro western blots, 20–40 μg of protein from denatured (95°C for 5 min) total or nuclear cell culture extracts, were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 7% (NOS2 and COX-2) or 10% (p65-NFκB and C/EBPβ) polyacrylamide gel, together with a molecular weight marker (Fullrange Rainbow Molecular Weight Marker, Amersham, Buckinghamshire, UK), and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After washing in Tris-buffered saline (TBS: 20 mM Tris, 0.15 M NaCl, pH 7.5) for 5 min, dipping in methanol for 10 s and air dry, the membranes were incubated with primary antibodies overnight at 4°C: polyclonal rabbit anti-C/EBPβ (1:500, Santa Cruz Biotechnology), polyclonal rabbit anti-p65-NFκB (1:500, Santa Cruz Biotechnology), polyclonal rabbit anti-COX-2 (1 : 2000, Santa Cruz Biotechnology), polyclonal rabbit anti-NOS2 (1:200, Chemicon, Temecula, CA, USA), monoclonal mouse anti-β actin (1:40 000, Sigma-Aldrich) or polyclonal goat anti-lamin B (1:5000, Santa Cruz Biotechnology) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk). Then, the membranes were washed twice in 0.05% Tween-20 in TBS for 15 s and incubated in horseradish peroxidase (HRP)-labelled secondary antibodies for 1 h at room temperature: donkey anti-rabbit (1:5000, Amersham), goat anti-mouse (1:5000, Santa

Cruz Biotechnology) or mouse anti-goat/sheep (1:2000, Sigma-Aldrich). After extensive washes in 0.05% Tween-20 in TBS, they were incubated in ECL-Plus (Amersham) for 5 min. Membranes were then exposed to the camera of a VersaDoc System (Bio-Rad Laboratories, Hercules, CA, USA), and pixel intensities of the immunoreactive bands were quantified using the % adjusted volume feature of Quantity One 5.4.1 software (Bio-Rad Laboratories). Data are expressed as the ratio between the intensity of the protein of interest band and the loading control protein band (lamin B for nuclear extracts or β-actin for total extracts). For in vivo western blots, 40-60 µg of protein from denatured (95°C for 5 min) total extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and processed for Western blot as described above. The membranes were incubated with polyclonal rabbit anti-GFAP (1:150.000, DAKO) and monoclonal mouse anti-β actin (1:300.000, Sigma).

2.9. Human spinal cord samples

Post-mortem human spinal cord sections used in this study were supplied by the human neurological tissue bank at the Hospital Clínic (Barcelona, Spain) in accordance with the Helsinki Declaration, Convention of the Council of Europe on Human Rights and Biomedicine and Ethical Committee of Barcelona University. The clinical diagnosis of ALS was confirmed neuropathologically. Post-mortem histological spinal cord samples were obtained from control (n=5; 3 ♀, 2 ♂; age range 66-81; post-mortem delay range 3.5-23.5 h) and sporadic ALS (n=6; 1 ♀, 5 ♂; age range 58-79; post-mortem delay 7-19 h) patients. Tissues were fixed in 4% paraformaldehyde solution, processed for paraffin embedded blocks and sections of 5 µm were obtained.

2.10. Human immunohistochemistry

Sections were deparaffinised, hydrated, and washed in PBS containing 0.1% Tween-20 (PBS-TW). Antigen retrieval was performed incubated the sections in citrate buffer (pH = 6.0) at 95 ºC during 1 hour. Then, sections were rinsed in citrate buffer at room temperature and treated with 2 N HCl at 37 ºC during 30 minutes. After rinsing in PBS, the sections were treated with 2% H_2O_2 in methanol during 10 minutes. rinsed in PBS-TW, blocked with 10% of foetal bovine serum and incubated overnight with the monoclonal mouse anti-CR3 antibody (1:500, DAKO) or monoclonal mouse anti-GFAP (1:10000, DAKO). Sections were then incubated with alkaline phosphatase goat anti-mouse antibody (1:500, Sigma), and developed with the substrates for alkaline phosphatase, 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt and Nitro-Blue Tetrazolium Chloride, until a specific blue colour was observed in microglial or

astroglial cells, respectively. After alkaline phosphatase development, the sections were rinsed for 1 hour in PBS-TW and incubated with the polyclonal anti-C/EBPβ antibody (1:300, Santa Cruz Biotechnology). Next, the sections were washed in PBS-TW, incubated for one hour at room temperature with biotinylated goat anti-rabbit antibody (1:500, Sigma) and then with ExtrAvidin-HRP (1:500, Sigma-Aldrich). Finally, the sections were developed with 0.05% diaminobenzidine-0.01% H_2O_2 (brown colour stain), washed in PBS-TW and mounted in mowiol medium. For each human sample (control, n=5; sporadic ALS, n=6), 3 spinal cord sections were used for cell counts. First, the tractus corticospinalis anterior and tractus corticospinalis lateralis were defined in an adjacent section by hematoxylin-eosin staining. Then double CR3-C/EBPβ positive cells in both the tractus corticospinalis anterior and lateralis were counted under the microscope with the x40 objective. Data are expressed as the number of double CR3-C/EBPβ positive cells per mm^2.

2.11. Data presentation and statistical analysis

All results are presented as mean ± SD. Statistical analyses were performed using two-way ANOVA followed by Bonferroni's multiple comparisons test, except for human results where statistical analyses were obtained by one-way ANOVA followed by Newman–Keuls post-hoc test when three or more experimental groups were compared. Values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism software.

3. Results

3.1. Exacerbated activation of G93A-SOD1 microglial cells in vitro

To confirm and extend previous reports of an exacerbated activation of microglia from G93A-SOD1 mice, highly-enriched microglial cultures from wt and G93A-SOD1 mice were treated with LPS+IFNγ and NOS2 and COX-2 protein levels were analyzed. The combination of the TLR4 agonist LPS and the host cytokine IFNγ induces a synergistic activation in primary murine microglial cultures (Kim et al., 1998). As expected, LPS+IFNγ induced the expression of NOS2 and COX-2 in wt microglia (Fig 1). Interestingly, NOS2 and COX-2 levels were higher in LPS-treated G93A-SOD1 microglia than in LPS-treated wt microglia by 1.8-fold ($p<0.01$) and 3.2-fold ($p<0.01$), respectively (Fig1 A-C). These findings were confirmed by immunocytochemistry (Fig 1 D,E) and indicate that the presence of G93A-SOD1 in microglial cells somehow enhances the activation response of these cells.

C/EBPβ and nuclear factor-κB (NFκB) are transcription factors that are able to regulate the expression of NOS2 and COX-2. We were interested to analyze whether the increased protein levels of NOS2 and COX-2 in LPS+IFNγ-treated G93A-SOD1 microglia could be attributed to enhanced nuclear levels of these transcription factors. LPS+IFNγ treatment did not induce a significant increase in the nuclear levels of C/EBPβ (Fig 2A) or the p65 isoform of NFκB (Fig 2B) in wt microglial cells, although a non-significant trend for an increase was observed. Note that at earlier time-points (4-8h) LPS+IFNγ induce significant increases in nuclear levels of C/EBPβ and p65-NFκB in wt microglial cultures (data not shown). In contrast, LPS+IFNγ induced significant increases in the nuclear levels of C/EBPβ (Fig 2A) and p65-NFκB (Fig 2B) in G93A-SOD1 microglial cultures. These findings were confirmed by immunocytochemistry (Figs 2 D, E).

3.2. Glial activation induced by LPS in G93A-SOD1 mice in vivo

We were next interested to study whether the exacerbated response of G93A-SOD1 microglia could also be observed in vivo. To this end mice were treated i.p. with 200 μg of LPS. Peripheral stimulation of the innate immune system with LPS induces a strong neuroinflammatory response. First, the expression of the microglial marker CD11b (Fig 3) and the astroglial marker GFAP (Fig 4) were analyzed. CD11b mRNA and GFAP mRNA and protein spinal cord levels were not altered in vehicle-treated G93A-SOD1 mice or in wt mice treated with LPS for 6 hours (Fig 3A, 4A-B) whereas both markers were moderately increased at the protein level as observed by immunohistochemistry in spinal cord sections 24 hours after treatments (Figs 3B, 4C). Interestingly, LPS i.p. markedly upregulated CD11b and GFAP mRNA levels in G93A-SOD1 mice, as assessed by qRT-PCR (Fig 3A, 4A) and CD11b and GFAP protein levels, as assessed by Western blot (Fig 4B) and immunohistochemistry (Fig 3B, 4C).

3.3. Exacerbated expression of proinflammatory genes and C/EBPβ is induced by LPS in G93A-SOD1 mice in vivo

We then analyzed the spinal cord mRNA levels of five key proinflammatory genes, namely NOS2, COX-2, IL-1β, IL-6 and TNFα by qRT-PCR. These genes are expressed by activated microglia and/or astrocytes and have been implicated in the neurotoxic effects of glial activation. A similar pattern of changes was observed for all five genes (Fig 5). The spinal cord mRNA levels were not affected by genotype alone, since the mRNA concentrations did not differ between vehicle-treated wt and G93A-SOD1 mice. Peripheral LPS injection induced a significant increase in all five proinflammatory mRNA levels in wt mice. Interestingly, in all five proinflammatory genes studied, the

mRNA levels in LPS-treated G93A-SOD1 mice were significantly higher than in LPS-treated wt mice. This exacerbation ranged from the 2-fold increase in COX-2 mRNA levels to the 6-fold increase in TNFα or IL-1β mRNA levels.

C/EBPβ mRNA levels showed the same pattern of changes seen for the proinflammatory genes, e.g. no significant changes in vehicle-treated G93A-SOD1 mice, a significant increase induced by LPS in wt mice (4-fold, $p<0.01$) and a significantly stronger increase in LPS-treated G93A-SOD1 mice (2-fold increase vs LPS-treated wt mice, $p<0.001$) (Fig 6A). In contrast, the mRNA levels of C/EBPα, another member of the C/EBP family of transcription factors, were not altered in any of the experimental groups (Fig 6A). In agreement with the mRNA data, C/EBPβ immunoreactivity was low in the spinal cord of both vehicle-treated wt and G93A-SOD1 mice (Fig 6B). Peripheral LPS induced the appearance of C/EBPβ positive nuclei in spinal cord sections of wt mice and the number and intensity of these C/EBPβ positive nuclei was markedly higher in LPS-treated G93A-SOD1 mice (Fig 6B). Double labeling immunofluorescence experiments showed the presence of C/EBPβ in activated microglia and astrocytes, identified by CD11b and GFAP immunoreactivity respectively (Fig 6C). Analysis of confocal microscope images of triple labeling immunofluorescence experiments (Fig 6D) revealed that in LPS-treated G93A-SOD1 mice 26.2 $\pm$ 5.5 % of C/EBPβ positive nuclei corresponded to microglial cells and 21.2 $\pm$ 6.0 % corresponded to astrocytes.

3.4. C/EBPβ is expressed by microglial cells in ALS

The presence of C/EBPβ in human glial cells has not yet been demonstrated. To this end we analyzed by immunohistochemistry the presence of C/EBPβ in spinal cord sections of ALS patients and non-neurological controls. In control cases C/EBPβ positive nuclei were rarely observed (Fig 7Aa). In contrast, in ALS cases the number of C/EBPβ positive nuclei increased markedly (Fig 7Ab). These nuclei were particularly abundant in several white matter tracts: tractus corticospinalis lateralis and anterior, and tractus spinothalamicus lateralis. Double immunohistochemistry revealed that C/EBPβ positive nuclei often co-localized with the microglial marker CR3 (Fig 7Ac-d). Double C/EBPβ-CR3 positive cells were mainly found in the areas affected by motoneuron degeneration (tractus corticospinalis) demonstrating for the first time to our knowledge the presence of C/EBPβ in activated microglial cells in human disease. Quantification of double CR3-C/EBPβ positive cells revealed that the number of C/EBPβ positive microglial cells was higher in ALS than in control cases by 4.2 fold in

the tractus corticospinalis anterior ($p<0.001$) and by 7.3 fold in the tractus corticospinalis lateralis ($p<0.05$) (Fig 7B).

Discussion

To elucidate how the endogenous expression of the pathogenic G93A-SOD1 protein affects the pattern of microglial activation we have analyzed the expression of key proinflammatory genes in activated G93A-SOD1 microglial cells in vitro and in vivo. We have observed an exacerbated pattern of activation in G93A-SOD1 in both models which was accompanied by the increased expression of the transcription factor C/EBPβ. In order to determine the relevance of the increased microglial expression of C/EBPβ to human disease we have then analyzed the presence of C/EBPβ in ALS tissue and have observed the increased expression of C/EBPβ in microglial cells in ALS spinal cord.

Previous studies have shown that toll-like receptor (TLR) agonists induce an exacerbated response in microglial cells expressing G93A-SOD1, with increased expression of TNFα, NOS2 and MCP-1 (Beers et al., 2006; Sargsyan et al., 2009; Weydt et al., 2004; Xiao et al., 2007) and increased neurotoxicity associated to microglial activation (Beers et al., 2006; Xiao et al., 2007). In this report we confirm that NOS2 expression is exacerbated in LPS+IFNγ-treated G93A-SOD1 microglia and we extend this finding also to COX-2 expression. The upregulation of COX-2 in activated G93A-SOD1 microglia is relevant since COX-2 is markedly upregulated in sporadic ALS spinal cord (Almer et al., 2001; Maihofner et al., 2003) where it is expressed by activated microglia (Yiangou et al., 2006). On the other hand, COX-2 inhibitors are protective in ALS animal models (Drachman et al., 2002; Pompl et al., 2003), altogether suggesting the involvement of microglial COX-2 upregulation in ALS pathogenesis. It is not clearly established how the expression of mutant SOD1 in microglia leads to an exacerbated activation profile. A first unanswered question is whether this effect is triggered by intracellular or extracellular mutant SOD1 (Henkel et al., 2009). The observations that exogenously applied mutant SOD1 induce microglial activation and microglial neurotoxicity (Kang and Rivest, 2007; Urushitani et al., 2006; Zhao et al., 2010) favor the hypothesis of extracellular, probably misfolded, mutant G93A-SOD1 priming microglia for an exacerbated response to TLR agonists.

In this study we have extended these in vitro findings to show for the first time that the neuroinflammation induced by TLR agonists in vivo is also exacerbated when mutant SOD1 is expressed. In the absence of LPS treatment we did not observe the upregulation of glial markers or proinflammatory genes in G93A-SOD1 spinal cord. This is somewhat unexpected since in these animals a neuroinflammatory response has been demonstrated (Beers et al., 2011; Yang et al., 2011). It is interesting to note that the pattern of neuroinflammation is dependent on the spinal cord region and the animal age (Beers et al., 2011; Yang et al., 2011). The neuroinflammatory response is stronger in lumbar than in cervical spinal cord (Beers et al., 2011) and increases over time although for markers such as NOS2 (Yang et al., 2011) or IL-6 (Beers et al., 2011) expression levels return to normal values at the late-stage of the disease. Since in this study we have used late-stage animals, this could explain the absence of differences between wild-type and G93A-SOD1 in NOS2 and IL-6. Differences in the criteria used to define late-stage in G93A-SOD1 mice could underlie the absence of changes in markers such as GFAP, IL-1β or TNFα in these mice which are at odds with previous reports (Beers et al., 2011; Yang et al., 2011). On the other hand, the expression of the microglial marker CD11b, the astroglial marker GFAP and the proinflammatory genes NOS2, COX-2, TNFα, IL-1β and IL-6 induced by systemic LPS was markedly exacerbated in G93A-SOD1 mice. The proinflammatory genes included in this study have all been implicated in neurotoxic effects of neuroinflammation and are all expressed by activated microglia in neurodegenerative processes (Glass et al., 2010). Interestingly, chronic infusion of LPS, which is non toxic per se, exacerbates disease progression and motor axon degeneration in G37R-SOD1 mice (Nguyen et al., 2004). Our findings suggest that the systemic LPS-induced exacerbated expression of proinflammatory mediators such as TNFα, prostaglandins or NO in high concentrations could mediate the accelerated neurodegeneration reported in the ALS model (Nguyen et al., 2004). An exacerbating effect of systemic LPS on neuroinflammation and neurodegeneration has also been reported in animal models of Alzheimer's disease (Kitazawa et al., 2005; Sly et al., 2001) and in prion disease (Cunningham et al., 2009). Collectively these data support the hypothesis that infections, even subclinically, could trigger or accelerate disease in sporadic cases of neurodegeneration (Cunningham et al., 2009). In this respect, it is interesting to note that LPS plasma levels are elevated in ALS patients and correlate positively with clinical disease status and monocyte/macrophage activation (Zhang et al., 2009).

In parallel with the exacerbated expression of proinflammatory genes such as NOS2 or COX-2 we have also observed an exacerbated increase in the levels of the transcription factor C/EBPβ, both in LPS+IFNγ-treated G93A-SOD1 microglia in culture and in microglia and astrocytes of LPS-treated G93A-SOD1 mice in vivo. C/EBPβ activity is mainly regulated at the level of transcription (Bradley et al., 2003), therefore increased protein/mRNA levels of C/EBPβ are a good indicator of increased C/EBPβ activity as transcription factor. C/EBPβ expression in activated microglial cells has been reported in cultured microglia treated with TLR agonists (Chang et al., 2008; Ejarque-Ortiz et al., 2007; Samuelsson et al., 2008), proinflammatory cytokines (Jana et al., 2001; Jana et al., 2002; Jana et al., 2003) or activated T lymphocytes (Dasgupta et al., 2003). Increased C/EBPβ levels have also been reported in vivo in situations in which neuroinflammation occurs such as systemic LPS injection (Alam et al., 1992; Ejarque-Ortiz et al., 2007; Saito et al., 1999; Damm et al., 2011), cerebral ischemia (Kapadia et al., 2006), excitotoxic insult (Cortes-Canteli et al., 2008) or aging (Akar and Feinstein, 2009). To date, the presence of C/EBPβ in activated glial cells in vivo has only been reported in microglia or astrocytes after systemic LPS or kainic acid injection (Ejarque-Ortiz et al., 2007; Cortes-Canteli et al., 2008; Damm et al., 2011). The demonstration of the presence of C/EBPβ in activated microglial cells and astrocytes in the rodent CNS in vivo supports the hypothesis of a role of C/EBPβ in the regulation of proinflammatory gene expression in glial activation. In this respect, our finding of C/EBPβ expression in microglial cells in human ALS samples is particularly relevant. This is the first demonstration of the expression of C/EBPβ in human microglia in health or disease.

C/EBPβ in human microglia may regulate the expression of genes with a strong potential for neuroinflammatory collateral damage such as NOS2, COX-2, TNFα, IL-1β or IL-6 since these genes contain functional C/EBP sites in their promoters (Gorgoni et al., 2001; Kolyada and Madias, 2001; Ray and Ray, 1995; Wedel et al., 1996; Yang et al., 2000). The role of C/EBPβ in activated microglia is probably similar to the key role C/EBPβ plays in specifying cell-specific patterns of TLR inducible gene expression in macrophages (Medzhitov and Horng, 2009). In the regulation of proinflammatory genes in microglia/macrophages C/EBPβ interacts in a cell-specific and promoter-specific manner with transcription factors such as C/EBPα (Ray and Ray, 1995; Wedel et al., 1996), C/EBPδ (Caivano et al., 2001; Kolyada and Madias, 2001), NFκB (Caivano et al., 2001; Hu et al., 2000; Kolyada and Madias, 2001; Ray and Ray, 1995), PU.1 (Yang et al., 2000), CREB (Caivano et al., 2001) or AP-1 (Pope et al., 2000; Zagariya et al., 1998). In most cases, these physical interactions determine functional synergies. The most likely situation in the promoters of tightly regulated genes such as those is that

multimolecular complexes are formed in which several transcription factors and coactivators interact. The role played by each individual transcription factor will be different in different cell/promoter scenarios. Our hypothesis is that C/EBPβ is necessary to obtain maximal transcription of proinflammatory genes such as NOS2, COX-2, IL-1β, IL-6 or TNFα in microglia. In consequence, C/EBPβ inhibition could result in an attenuated, less neurotoxic neuroinflammatory response. This hypothesis is supported by the observations of neuroprotection and reduced neuroinflammation in C/EBPβ deficient mice after ischemic (Kapadia et al., 2006) and excitotoxic insults (Cortes-Canteli et al., 2008). Since C/EBPβ-deficient mice show physiological alterations unrelated to their anti-inflammatory phenotype (Lekstrom-Himes and Xanthopoulos, 1998) experiments with mice with microglial- or astroglial-specific C/EBPβ deficiency would be useful to confirm the potential of glial C/EBPβ as a therapeutic target to attenuate the deleterious effects of neuroinflammation in neurodegenerative disorders such as ALS.

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Disclosure statement

None of the authors have a financial interest in publication of the manuscript.

Figure legends

Figure 1. Highly-enriched primary microglial cultures from wt or G93A-SOD1 neonates were treated with LPS (100 ng/ml) + IFNγ (0.1 ng/ml) or vehicle (Control) for 24 hours. NOS2 and COX-2 content in total protein homogenates was analyzed by Western blot. A and B show the quantification of 4 independent experiments with 2 replicates per condition and experiment. Data is expressed as mean + SD. **: $p<0.01$, ***: $p<0.001$. C shows a representative Western blot image. D and E show images of microglial cultures treated with LPS + IFNγ or vehicle for 24 hours and processed for NOS2 (D) and COX-2 (E) immunocytochemistry. Images shown are representative of 4 independent experiments. Magnification bars: 10 μm. Both Western blot and immunocytochemistry show that the increase in NOS2 and COX-2 protein induced by LPS + IFNγ is higher in G93A-SOD1 than in wt microglia.

Figure 2. Highly-enriched primary microglial cultures from wt or G93A-SOD1 neonates were treated with LPS (100 ng/ml) + IFNγ (0.1 ng/ml) (L+I) or vehicle (Control) for 24 hours. C/EBPβ and p65-NFκB contents in nuclear protein homogenates were analyzed by Western blot. A and B show the quantification of 4 independent experiments with 2 replicates per condition and experiment. Data is expressed as mean + SD. *: $p<0.05$, **: $p<0.01$. C shows a representative Western blot image. D and E show images of microglial cultures treated with LPS + IFNγ or vehicle for 24 hours and processed for C/EBPβ (D) and p65-NFκB (E) immunocytochemistry. Images shown are representative of 4 independent experiments. Magnification bars: 20 μm. Western blots and immunocytochemistry show that the LPS + IFNγ-induced increases in the nuclear levels of C/EBPβ and p65-NFκB are more marked in G93A-SOD1 than in wt microglia.

Figure 3. Adult wt or G93A-SOD1 mice were treated with 200 μg of LPS or vehicle (Control). A) The spinal cord mRNA levels of the microglial marker CD11b 6 hours after treatment were analyzed by qRT-PCR with triplicates for each animal. The graph shows mean + SD of 4 animals per group. **: $p<0.01$. B) CD11b immunoreactivity in spinal cord sections of mice treated with 200 μg of LPS or vehicle (Control) and sacrificed 24 hours after the treatment. Arrows point to individual CD11b-positive microglial cells. The images shown are representative of 3 animals per condition. Magnification bar: 10 μm.

Figure 4. Adult wt or G93A-SOD1 mice were treated with 200 μg of LPS or vehicle (Control). A) The spinal cord mRNA levels of the astroglial marker GFAP 6 hours after

treatment were analyzed by qRT-PCR with triplicates for each animal. The graph shows mean + SD of 4 animals per group. ***: p<0.001. B) Spinal cord protein GFAP levels were analyzed 6 hours after treatment by Western blot. The graph shows mean + SD of 4 animals per group. *: p<0.05. An image from a representative experiment is also shown. C) GFAP immunoreactivity in spinal cord sections of mice treated with 200 µg of LPS or vehicle (Control) and sacrificed 24 hours after the treatment. Arrows point to individual GFAP-positive astrocytes. The images shown are representative of 3 animals per condition. Magnification bar: 10 µm.

Figure 5. Adult wt or G93A-SOD1 mice were treated with 200 µg of LPS or vehicle (Control). The spinal cord mRNA levels of the proinflammatory genes NOS2 (A), COX-2 (B), IL-1β (C), IL-6 (D) and TNFα (E) were analyzed by qRT-PCR 6 hours after treatment with triplicates for each animal. The graphs show mean + SD of 4 animals per group. The LPS-induced expression of all 5 proinflammatory genes is significantly higher in G93A-SOD1 mice than in wt mice. *: p<0.05; **: p<0.01, ***: p<0.001.

Figure 6. Adult wt or G93A-SOD1 mice were treated with 200 µg of LPS or vehicle (Control). A) The spinal cord mRNA levels of the transcription factors C/EBPβ and C/EBPα were analyzed by qRT-PCR 6 hours after treatment with triplicates for each animal. LPS treatment increases C/EBPβ mRNA levels in wt mice and this effect is even higher in G93A-SOD1 mice. In contrast, C/EBPα spinal cord mRNA levels are not affected by LPS or genotype. The graphs show mean + SD of 4 animals per group. **: p<0.01, ***: p<0.001. B) The C/EBPβ protein distribution and abundance in the spinal cord was analyzed by immunohistochemistry 24 hours after treatment. The appearance of C/EBPβ positive nuclei was induced by LPS in wt mice and the number of C/EBPβ positive nuclei was markedly higher in LPS-treated G93A-SOD1 mice. Arrows point to individual C/EBPβ positive nuclei. The images shown are representative of 3 animals per condition. Magnification bar: 10 µm. C) Spinal cord sections of G93A-SOD1 mice treated with LPS (24 hours) were processed for double immunofluorescence GFAP+C/EBPβ (upper row) or CD11b+C/EBPβ (lower row). The arrow in the upper row points to a C/EBPβ positive astrocyte and the arrows in the lower row point to two C/EBPβ positive microglial cells. The images shown are representative of 3 animals per condition. Magnification bar: 10 µm. In the right column in C two high magnification images showing the typical morphology of an activated astrocyte (upper row) and an activated microglial cell (lower row) are shown. Magnification bar in this column is 5 µm. D) Confocal microscope images from the spinal cord of a G93A-SOD1 mouse treated with LPS for 24 hours. C/EBPβ is shown in red, the astroglial marker GFAP in

yellow, the microglial marker Tomato lectin (TL) in green and the nuclear marker DAPI in blue. Various combinations of double, triple or quadruple labeling are shown. C/EBPβ immunoreactivity is seen in microglial cells (arrows), astrocytes (arrowheads) and also in some non-astroglial/non-microglial cells. Magnification bar 5 μm

Figure 7. A) C/EBPβ immunohistochemistry in tissue sections of a control (a) and an ALS case (b). Note the increased number of C/EBPβ positive nuclei in ALS. c, d) Double immunohistochemical staining of C/EBPβ (brown) and the microglial marker CR3 (blue) in ALS spinal cord sections. C/EBPβ and CR3 often co-localize (arrows) but C/EBPβ is also seen in nuclei of CR3-negative cells (arrowheads). Magnification bar: 50 μm. B) Counts of double C/EBPβ-CR3 positive cells in the tractus corticospinalis anterior (TCsA) and tractus corticospinalis lateralis (TCsL) of control (n=5) and ALS cases (n=6). Data are expressed as mean + SD. *: $p<0.05$, ***: $p<0.001$. Note the marked increase of C/EBPβ expressing microglial cells in both spinal cord regions in ALS. The anatomical areas selected for cell counting are depicted in C.

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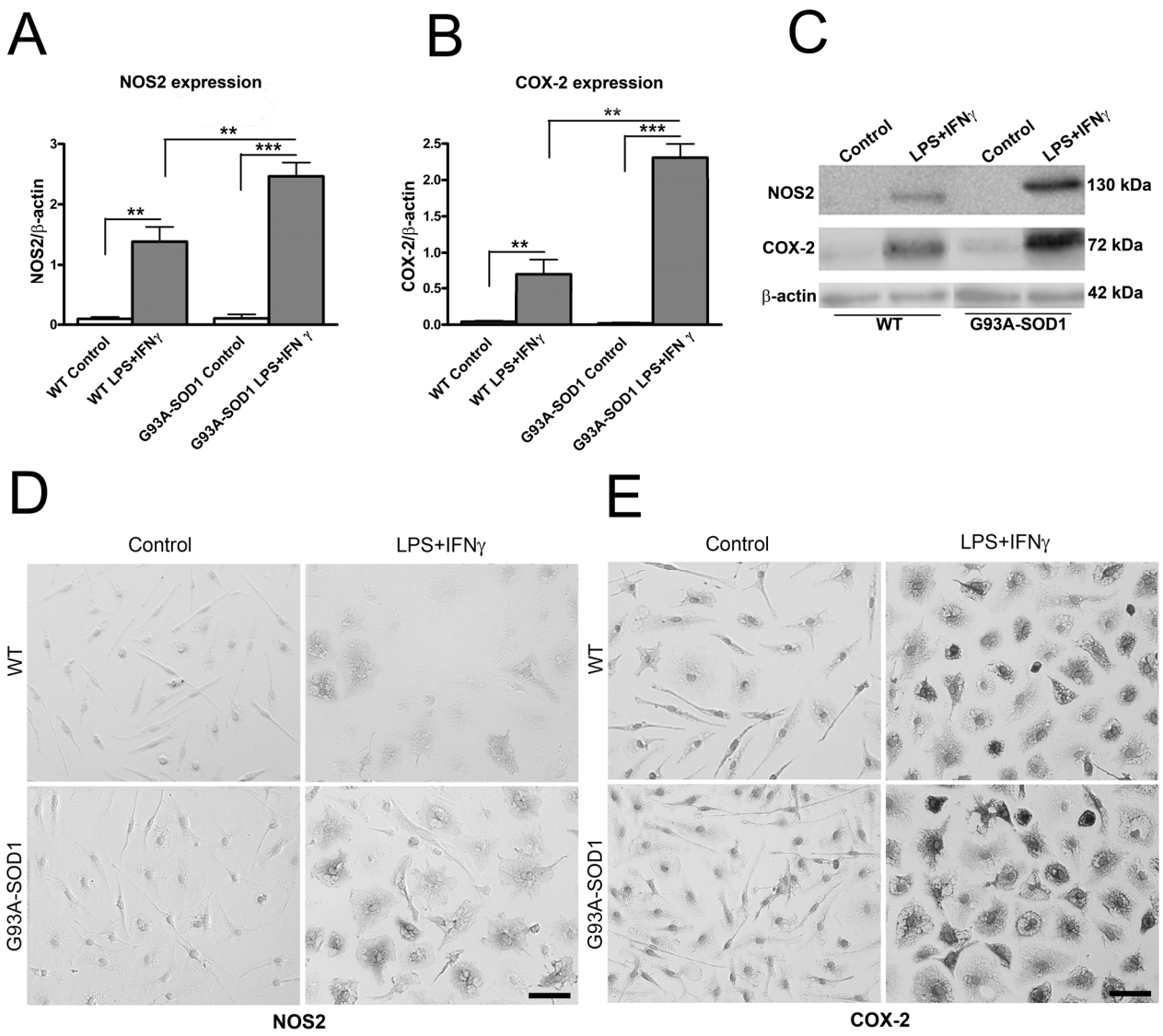
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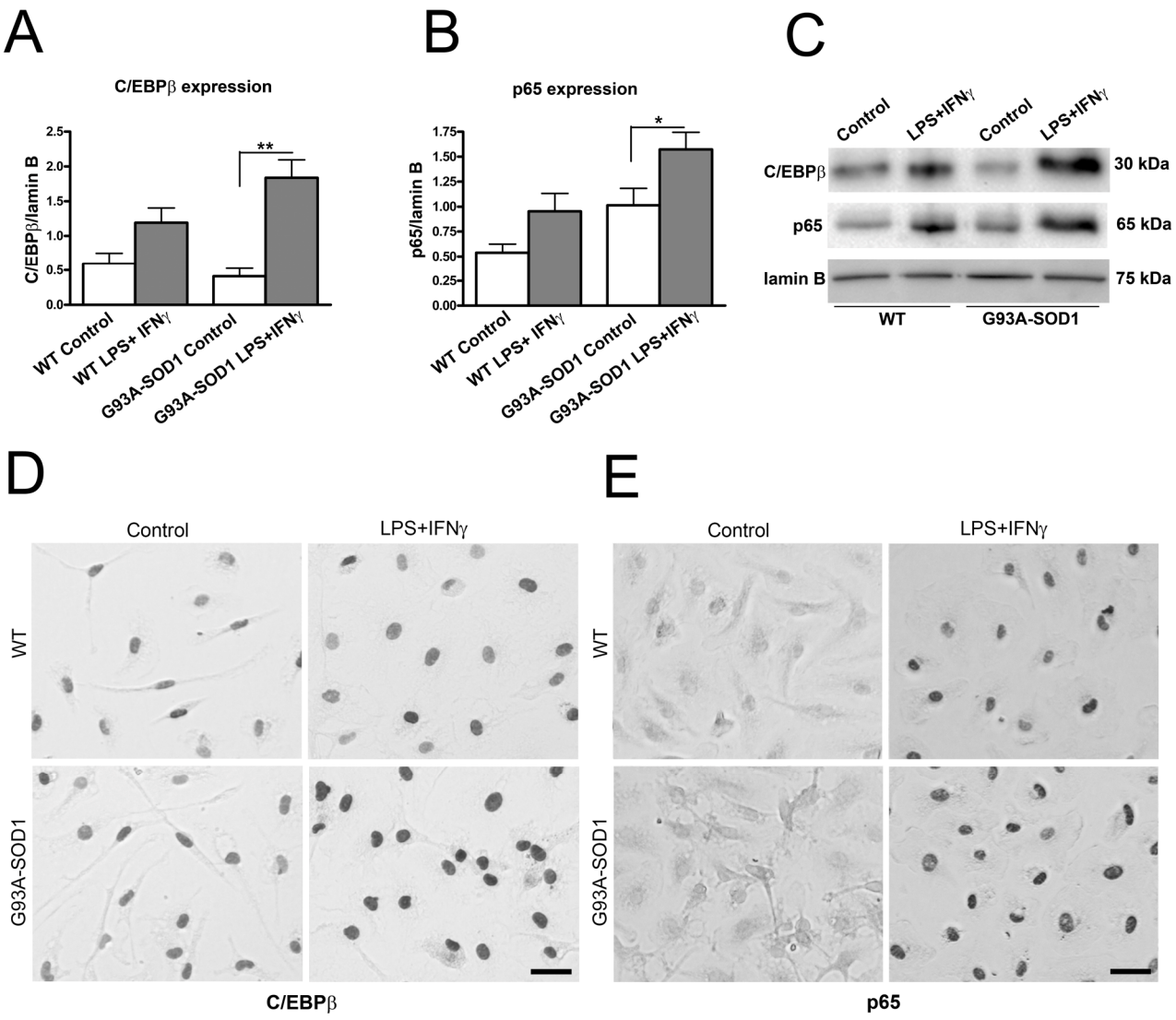
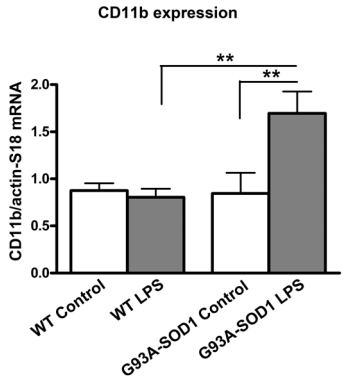
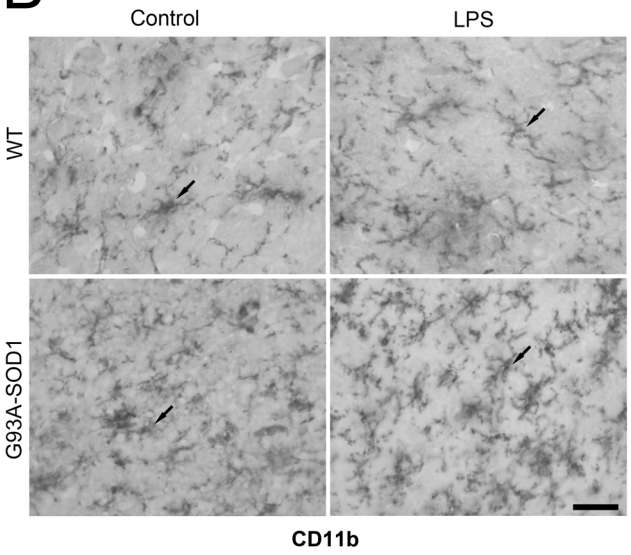
NOS2 expression
NOS2/β-actin
WT Control
WT LPS+IFNγ
G93A-SOD1 Control
G93A-SOD1 LPS+IFN γ
B
COX-2 expression
COX-2/β-actin
C
Control
LPS+IFNγ
NOS2
COX-2
β-actin
130 kDa
72 kDa
42 kDa
WT
G93A-SOD1
D
E
WT
G93A-SOD1
NOS2
COX-2


A
C/EBPβ expression
C/EBPβ/lamin B
2.5
2.0
1.5
1.0
0.5
0.0
**
WT Control
WT LPS+ IFNγ
G93A-SOD1 Control
G93A-SOD1 LPS+IFNγ
B
p65 expression
p65/lamin B
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
WT Control
WT LPS+ IFNγ
G93A-SOD1 Control
G93A-SOD1 LPS+IFNγ
C
Control
LPS+IFNγ
Control
LPS+IFNγ
C/EBPβ
30 kDa
p65
65 kDa
lamin B
75 kDa
WT
G93A-SOD1
D
Control
LPS+IFNγ
WT
G93A-SOD1
C/EBPβ
E
Control
LPS+IFNγ
WT
G93A-SOD1
p65

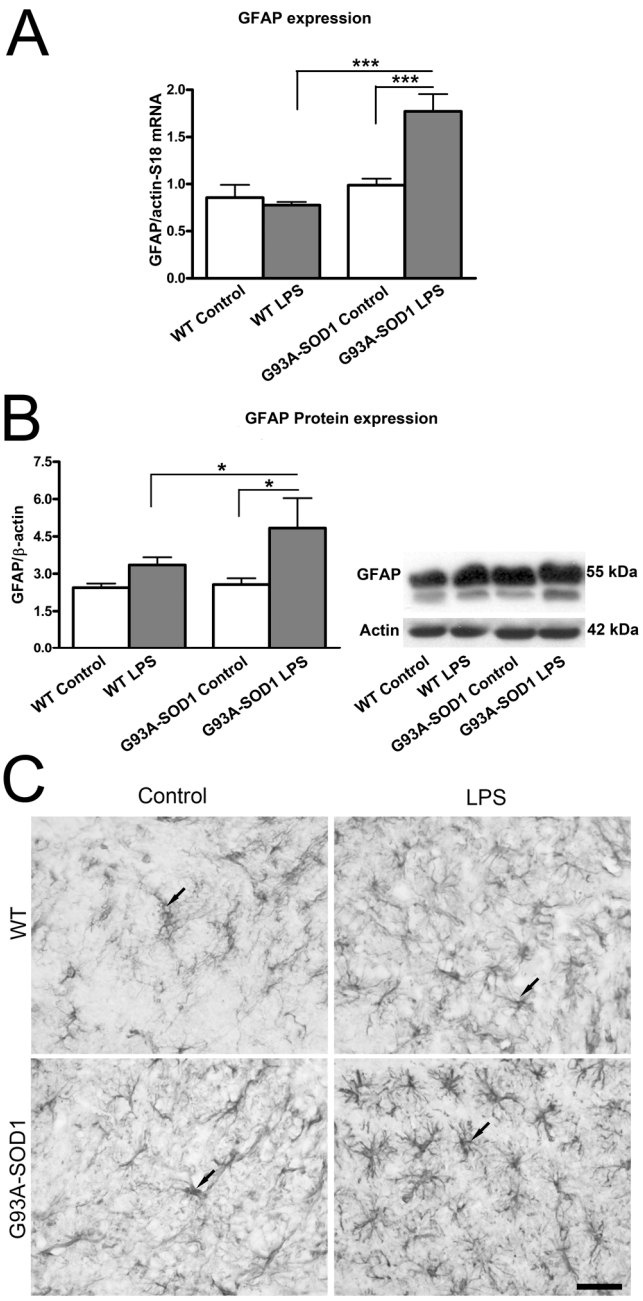
A

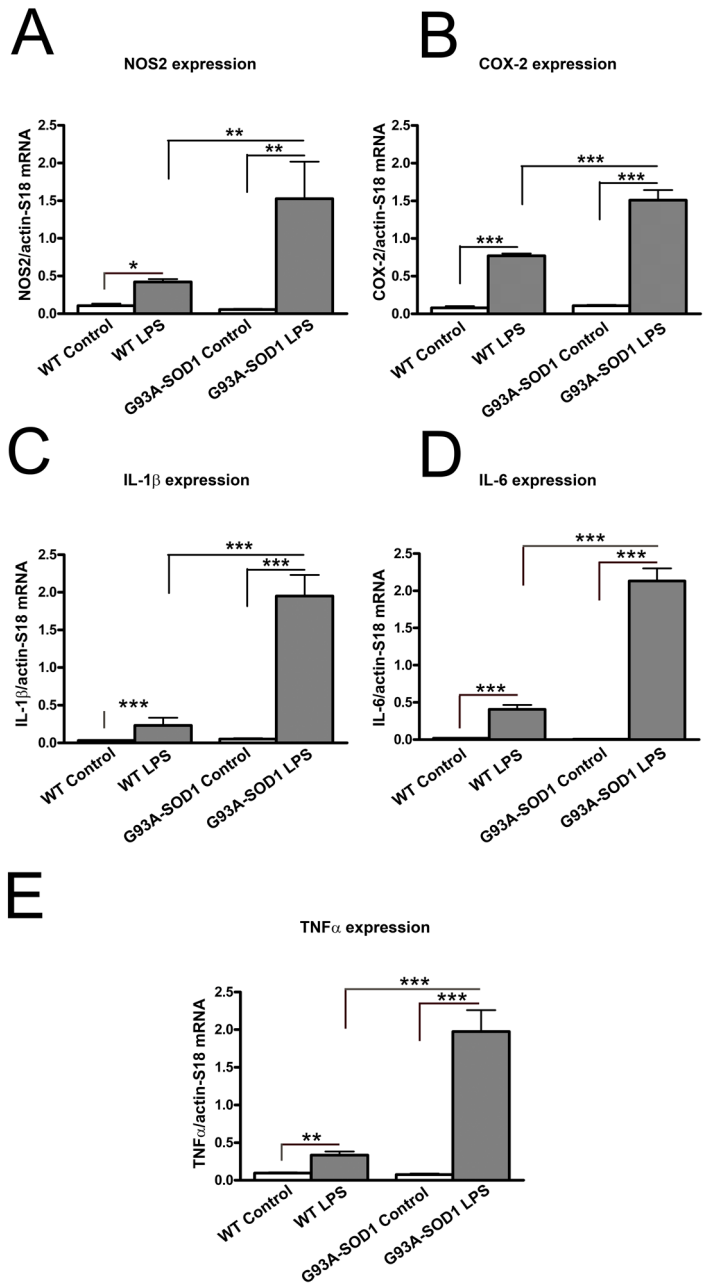

CD11b expression
**
**
CD11b/actin-S18 mRNA
2.0
1.5
1.0
0.5
0.0
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS

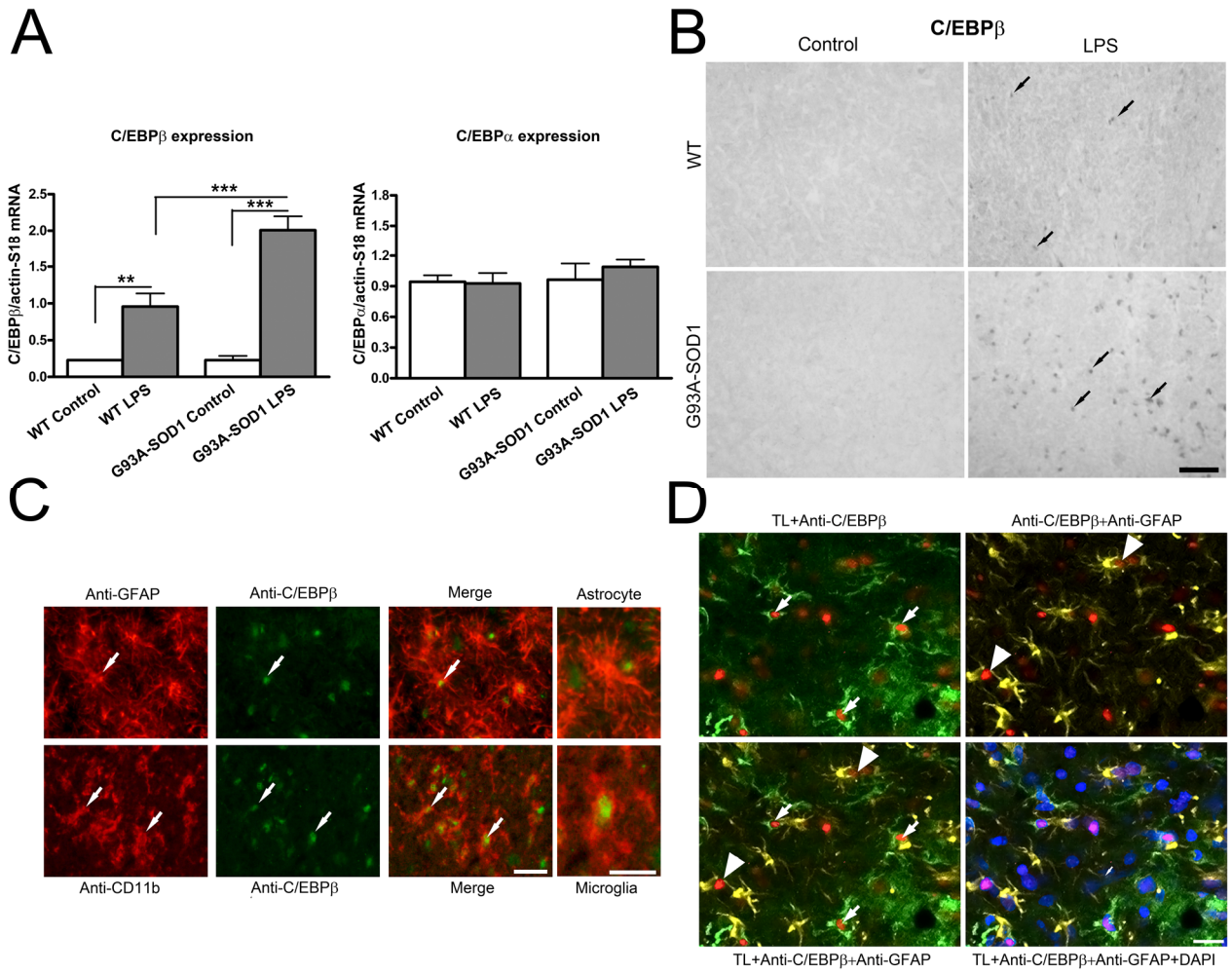
B

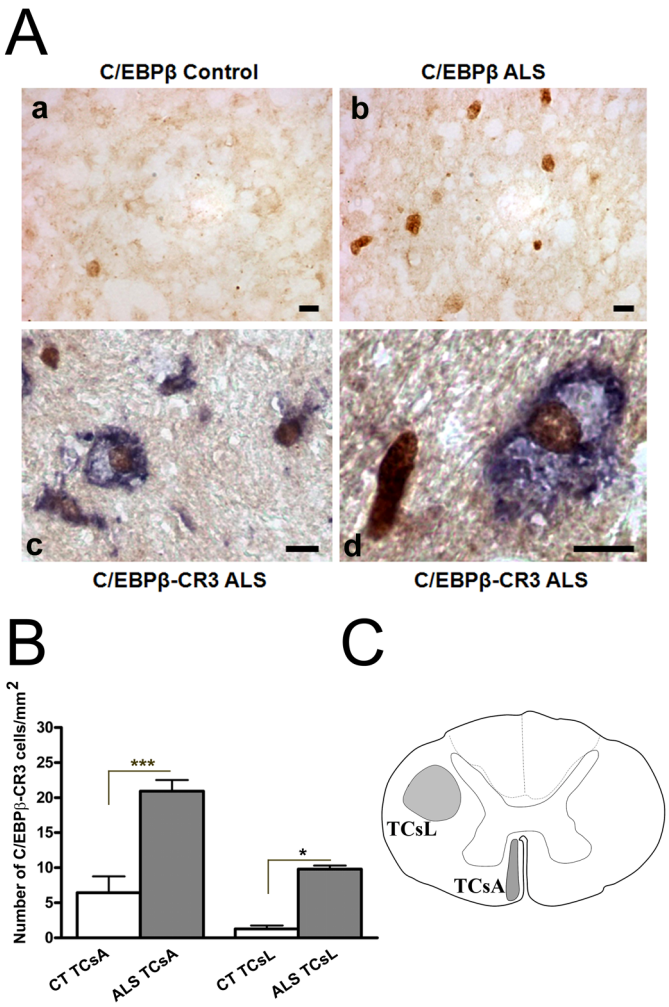

Control
LPS
WT
G93A-SOD1

CD11b


A
GFAP expression
***
***
GFAP/actin-S18 mRNA
2.0
1.5
1.0
0.5
0.0
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS
B
GFAP Protein expression
*
*
GFAP/β-actin
7.5
6.0
4.5
3.0
1.5
0.0
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS
GFAP
55 kDa
Actin
42 kDa
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS
C
Control
LPS
WT
G93A-SOD1


A
NOS2 expression
NOS2/actin-S18 mRNA
B
COX-2 expression
COX-2/actin-S18 mRNA
C
IL-1β expression
IL-1β/actin-S18 mRNA
D
IL-6 expression
IL-6/actin-S18 mRNA
E
TNFα expression
TNFα/actin-S18 mRNA
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS


A
C/EBPβ expression
C/EBPβ/actin-S18 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS
C/EBPα expression
C/EBPα/actin-S18 mRNA
1.8
1.5
1.2
0.9
0.6
0.3
0.0
WT Control
WT LPS
G93A-SOD1 Control
G93A-SOD1 LPS
B
C/EBPβ
Control
LPS
WT
G93A-SOD1
C
Anti-GFAP
Anti-C/EBPβ
Merge
Astrocyte
Anti-CD11b
Anti-C/EBPβ
Merge
Microglia
D
TL+Anti-C/EBPβ
Anti-C/EBPβ+Anti-GFAP
TL+Anti-C/EBPβ+Anti-GFAP
TL+Anti-C/EBPβ+Anti-GFAP+DAPI


A
C/EBPβ Control
C/EBPβ ALS
a
b
c
d
C/EBPβ-CR3 ALS
C/EBPβ-CR3 ALS
B
C
Number of C/EBPβ-CR3 cells/mm2
30
25
20
15
10
5
0
***
*
CT TCsA
ALS TCsA
CT TCsL
ALS TCsL
TCsL
TCsA