Synthetic and natural small molecule TLR4 antagonists inhibit motoneuron death in cultures from ALS mouse model

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ABSTRACT: Increasing evidence indicates that inflammatory responses could play a critical role in the pathogenesis of motor neuron injury in Amyotrophic Lateral Sclerosis (ALS). Recent findings have underlined the role of Toll-like receptors (TLRs) and the involvement of both the innate and adaptive immune responses in ALS pathogenesis. In particular, abnormal TLR4 signaling in

pro-inflammatory microglia cells has been related to motoneuron degeneration leading to ALS. In this study the effect of small molecule TLR4 antagonists on in vitro ALS models has been investigated. Two different types of synthetic glycolipids and the phenol fraction extracted from commercial extra-virgin olive oil (EVOO) were selected since they efficiently inhibit TLR4 stimulus in HEK cells by interacting with the TLR4·MD-2 complex and CD14 co-receptor. Here, TLR4 antagonists efficiently protected motoneurons from LPS-induced lethality in spinal cord cultures, and inhibited the interleukine-1β production by LPS-stimulated microglia. In motoneurons/glia cocultures obtained from wild type or SOD1 G93A mice, motoneuron death induced by SOD1mut glia was counteracted by TLR4 antagonists. The release of nitric oxide by LPS treatment or SOD1mut glia was also inhibited by EVOO, suggesting that the action of this natural extract could be mainly related to the modulation of this inflammatory mediator.

KEYWORDS

TLR4; Amyotrophic Lateral Sclerosis (ALS); extra virgin olive oil; inflammation; motoneuron; microglia.

ABBREVIATIONS

ALS Amyotrophic Lateral Sclerosis; EVOO Extra Virgin Olive Oil; MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; SOD1 copper zinc superoxide dismutase 1; TLRs Toll-like Receptors.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a rare adult-onset neurodegenerative disease characterized by selective motoneuron death in the spinal cord, brain stem and motor cortex. The etiology is still elusive and at present ALS is regarded as a multifactorial disease in which several susceptibility genes act in concert in a complex interaction with environmental factors (1,2). Altered immune system responses and the consequent activation of the neuroinflammatory cascade have

been proposed amongst the pathological mechanisms (3-5). At a molecular level, a prominent role in mediating inflammation and innate immunity responses is played by Toll-like receptors (TLRs). TLR2, TLR4, and their co-receptor CD14, have been reportedly identified as the major molecular players in the neuroimmune dysregulations involved in ALS (6,7). It has been recently demonstrated that mutant SOD1 (copper zinc superoxide dismutase 1), which is responsible for 20% of familial ALS and causes selective motoneuron death in mouse models, exhibits its neurotoxic effect in motoneuron-microglia cocultures by activating proinflammatory microglia through interaction with CD14 in concert with TLR2 and TLR4 (8,9).

Expression of CD14 is increased in spinal cord of both mutant SOD1 mice and sporadic ALS patients (10,11). and the chronic stimulation of CD14/TLR pathway by LPS was found to exacerbate disease in ALS mice (12). TLR4 and one of its major endogenous ligands, high-mobility group box 1 (HMGB1) are specifically expressed by activated microglia and astrocytes and increase during disease progression in SOD1G93A mice (13,14). Moreover, deletion of TLR4 significantly extends survival and transiently improves hind-limb grip strength in this ALS disease model (14). Thus, inhibition of TLR4 pathway may provide beneficial effects and represent a new putative target for neuroprotective strategies. We previously demonstrated that cyanobacteria-derived TLR4 antagonists exert anti-inflammatory and neuroprotective effects in LPS-treated spinal cord cultures, by inhibiting microglia morphological activation and release of proinflammatory cytokines, eventually counteracting motoneuron death (15). Here we characterize the antinflammatory and neuroprotective effects of two synthetic small-molecule TLR4 antagonists developed by our group, (16-19) and of the phenol fraction of extra virgin olive oil (EVOO) in two related models. The first is the LPS-driven neurotoxicity model that has been extensively characterized by our group (15) and represents a useful and simple tool for investigations on TLR4 antagonists with promising neuroprotective properties. A second model was used to verify the neuroprotective potentiality of the tested compounds in a ALS-like in vitro setting, consisting in spinal cord primary cultures from

SOD1 mutated (SOD1 G93A) mice. In this model, glia-driven neurotoxicity has been extensively demonstrated and a role for TLR4 has been proposed.

Both LPS and and SOD1 G93A models are characterized by a strong neuroinflammatory component combined with toxic effect on motoneurons, thus suggesting interesting parallelisms in new emerging findings from the combination of the two models (8,9).

IAXO102 and FP7 are two ionic, amphiphilic glycolipids (respectively, positively and negatively charged) first synthesized by our group and with potent activity as TLR4 antagonists. The cationic glycolipid IAXO102 (Fig.1) is a TLR4 antagonist in preclinical phase of development (17) that blocks LPS-induced TLR4 activation in TLR4-transfected HEK cells (HEK-TLR4) with an IC_{50} of about 5 μM by binding to CD14 and MD-2 co-receptors (20). The use of IAXO102 to target CNS syndromes has been investigated in the treatment of neuropathic pain that has been related to abnormal TLR4 activation in the spinal cord by endogenous factors (21). Although it is not yet clear if this compound can cross the spinal cord blood barrier, this observation make this compound attractive to be examined for its potential neuroprotective effect on motoneurons. The anionic glycolipid FP7 (Fig. 1) is a monosaccharide lipid A analogue that directly binds to the TLR4.MD-2 dimer thus inhibiting TLR4 activation with an IC_{50} of 0,4 μM (22).

Phenolic compounds present in EVOO have recognized anti-oxidant and anti-inflammatory properties. A recent study aimed at clarifying the mechanism of action of EVOO phenols showed that these compounds reduced LPS-induced oxidative stress and inflammatory responses through decreasing NO and radical oxygen species (ROS) generation (23). We show here that EVOO phenols are also active in inhibiting TLR4 activation in HEK-TLR4 cells, and thus we tested phenols, together with synthetic TLR4 antagonists, in the ALS-like in vitro models.

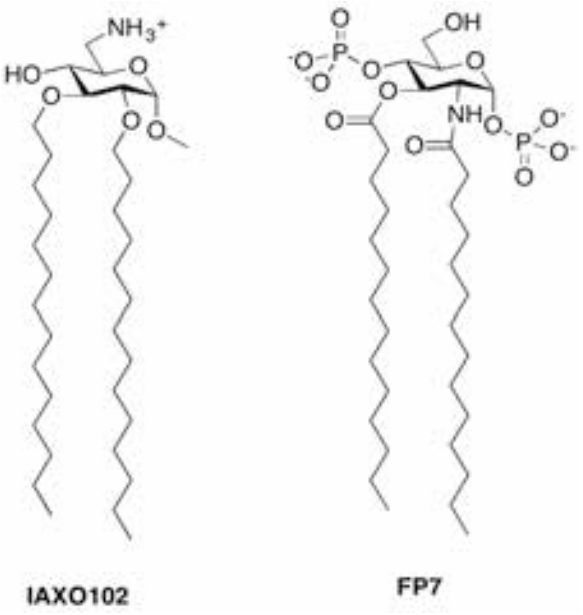

Fig. 1. Chemical structures of glucose-derived cationic amphiphile IAXO102 and anionic amphiphile FP7.

MATERIAL AND METHODS

Spinal cord cultures. Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS Istituto di Ricerche Farmacologiche "Mario Negri" in compliance with national (D.L. 116/92, 1992; Circolare 8/94, 1994) and international laws and policies (Directive 86/609/EEC, 1987; US National Research Council, 2011). Cultures were obtained from the spinal cord of 13-day-old C57 BL/6J mouse embryos as routinely used in our laboratory (15). Briefly, ventral horns were dissected from spinal cords, exposed to DNAse and trypsin (Sigma-Aldrich) and centrifuged through a bovine serum albumin (BSA) cushion. Cells obtained at this step were a mixed neuron/glia population and were centrifuged (800 g for 15 min) through a 6% iodixanol (OptiPrep™; Sigma-Aldrich) cushion for motoneuron enrichment. A sharp band (motoneuron–enriched fraction) on the top of the iodixanol cushion and a pellet (glial fraction) were obtained. The glial feeder layer was prepared by plating the glial fraction at a density of 25,000 cells/cm^2 into 12-well plates or into flasks, both previously precoated with poly-L-lysine (Sigma-Aldrich). To establish neuron/glia cocultures, the motoneuron–enriched fraction (ob-

tained from the iodixanol-based separation) was seeded at a density of 10,000 cells/cm2 onto mature glial layers. In these cocultures, about 84 ± 5% of the neuronal cells were SMI32-positive cells with the typical motoneuron morphology (15). For combined wild-type/SOD1mut cocultures motoneurons were obtained from C57 BL/6J and glia from SOD1 G93A or NTg mouse embryos (C57/BL6, Harlan, Italy). Purified microglia were obtained from mixed glia as previously reported (15). Briefly, flasks containing confluent mixed glial cultures were shaken and the supernatants (containing microglial cells) were collected and seeded at a density of 20,000 cells/cm2 into 12-well plates.

Synthetic TLR4 antagonists. Pure compounds IAXO102 (17) and FP7 (22) (Fig.1) were obtained by multistep synthesis. The chemical identity and purity of compounds were assessed by NMR, mass and HPLC analysis.

Phenols extraction from EVOO. The phenolic extract was obtained from a commercial Italian EVOO (Sagra). The EVOO (10 g) was diluted with *n*-hexane (10 mL) and the polar phenolic fraction was extracted with a 8:2 solution of MeOH/H_2O (100 mL for 3 times). The polar fractions were collected and evaporated in vacuo at 25 °C to avoid phenols degradation. Phenolic extract was then dissolved and stocked in EtOH (10 mL). The total phenol content of this solution was evaluated using the Folin-Ciocalteu method (24) and resulted to be 2.8 mg/mL.

HEK-Blue™ cell culture. HEK-Blue™-4 cells were purchased from InvivoGen and cultured according to manufacturer's instructions. Briefly, cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 µg/ml streptomycin, 1x Normocin™ (InvivoGen). HEK-Blue™-4 cells were maintained with the addition of 1x HEK-Blue™ Selection (InvivoGen).

HEK-Blue™ cells assay. HEK-Blue-TLR4 cells (InvivoGen) were cultured as described before. Cells were detached by the use of a cell scraper and the cell concentration was estimated by using Try-

pan Blue (Sigma-Aldrich). The cells were diluted in DMEM high glucose medium supplemented as described before and seeded in multiwall plate at a density of 20,000 cells/well in 200 µL. After overnight incubation (37 °C, 5% CO_2, 95% humidity), supernatants were removed and cell monolayers were washed with warm PBS without Ca^{2+} and Mg^{2+} and treated with increasing concentrations of phenol extracts from EVOO of italian origin. Each concentration was tested in triplicate. After 30 min, the cells were stimulated with 100 ng/mL LPS from *E. coli* O55:B5 (Sigma- Aldrich) and incubated overnight at 37 °C, 5% CO_2 and 95% humidity. As a control, the cells were not treated with LPS. Then the supernatants were collected, and 50 µL of each sample was added to 100 µL PBS, pH 8, 0.84 mM paranitrophenylphosphate (pNPP) for a final concentration of 0.8 mM pNPP. Plates were incubated for 2-4 h in the dark at rt, and then the plate reading was assessed by using a spectrophotometer at 405 nm (LT 4000, Labtech). The results were normalized with positive control (LPS alone) and expressed as the mean of percentage ± SD of at least three independent experiments using GraphPad Prism v.5.01. As a negative control, the assay was performed by using HEK-293 cell line (InvivoGen) transfected with the same plasmids as HEK-Blue but without TLR4, MD-2 and CD14 genes.

MTT Cell Viability Assay. HEK-Blue-TLR4 cells were seeded in 100 µL of DMEM without Phenol Red at a density of 20,000 cells/well in 100µL. Each concentration was tested in triplicate. After overnight incubation, 10 µL of phenolic extract were added and the plates were incubated overnight at 37 °C, 5% CO_2, 95% humidity. DMSO and PBS were included as control. Then 10 µL of MTT solution (5 mg/mL in PBS) were added to each well. After 3 h incubation (37 °C, 5% CO_2, 95% humidity), HCl 0.1 N in 2-propanol was added (100 µL/well) to dissolve formazan crystals. Formazan concentration in the wells was determined by measuring the absorbance at 570 nm (LT 4000, Labtech). 10% DMSO was used as positive (toxic) control. The results were normalized with untreated sample (PBS) and expressed as the mean of percentage ± SD of three independent experiments using GraphPad Prism v.5.01.

Spinal cord culture treatments and viability assessment. Cultures were treated on the sixth day *in vitro* (DIV). For the LPS-induced neurotoxic model, cultures were exposed to 1 μg/mL LPS (from Escherichia coli 0111:B4) for 24 hours. To assess the effect of the TLR4 antagonists, a pre-treatment with different concentrations of IAXO102, FP7 or phenolic extract of EVOO was performed for 2 hours; then, co-treatment with LPS was prolonged for further 24 hours. For the SOD1mut glia model, cultures were maintained with normal culture medium for 24 hours. Then, medium was replaced by normal medium (for control conditions) or TLR4 antagonists-containing medium and for the following 6 days. The viability of motoneurons was assessed by counting the SMI32-positive cells (see below for immunostaining) with typical morphology and large cell bodies (>20 μm) in the different treatment conditions. The number of viable motoneurons was normalized to the mean of SMI32-positive cells counted in the appropriate control wells (untreated cultures for tests in wild type cocultures; cocultures with NTg derived glia for studies on SOD1mut glia effects). In a typical experiment with cocultures, the number of counted SMI32-positive cells in control wells was 70 ± 11 (n = 12; not shown).

Immunocytochemistry. After treatments, cells were fixed with 4% paraformaldehyde and permeabilized by 0.2% Triton X-100 (Sigma Aldrich). Staining was carried out by overnight incubation with the primary antibody SMI32 (antinonphosphorylated neurofilament H antibody, mouse, 1:6,000; Covance), followed by incubation with a biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories). The biotinylated antibody signal was amplified with avidin and a biotinylated horseradish peroxidase macromolecular complex, finally revealed with diaminobenzidine (DAB, 0.5 mg/ml) and H_2O_2 (6μl/10ml).

Quantitative Enzyme-Linked Immunosorbent Assays. TNF-α and interleukin (IL)-1β concentrations in cell culture supernatants were quantified by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) (eBioscience). Samples from each experiment were tested in tripli-

cate, according to the manufacturer's instructions. The sensitivity of the kits was 8 pg/mL for both the cytokines.

Nitrites Measurement. Production of nitric oxide (NO) was determined by measuring the levels of accumulated nitrite, a metabolite of NO, in the culture supernatant using Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamide in 5% phosphoric acid; Promega Italia Srl). Briefly, after treatments, the culture supernatants were separately collected and added by an equal volume of Griess reagent, thenincubated at room temperature for 10 min. The absorbance was afterwards measured at 540nm (OD540).

Statistics. Treatment effects were compared by one-way analysis of variance (ANOVA) and Tukey or Dunnett test, or two-way ANOVA and Tukey or Bonferroni post-test, using GraphPad v6 (GraphPad Software).

RESULTS

TLR4 activity of olive oil phenolic fraction in TLR4-transfected HEK cells.

The TLR4 activity of phenolic fraction of EVOO was assessed in HEKBlue-4™ cells, which are HEK 293 cells stably transfected with human TLR4, MD-2 and CD14 genes. HEK-Blue-4™ cells stably express an optimized alkaline phosphatase gene engineered to be secreted (SEAP), placed under the control of an IL-12 p40 minimal promoter fused to five NF-κB and AP-1-binding sites. Stimulation with a TLR4 agonist (LPS 100 ng/mL from E. coli O55:B5) activates NF-κB and AP-1, which induce the production of SEAP.

Levels of SEAP can be easily determined by adding the chromogenic SEAP substrate *p*-nitrophenyl phosphate (pNPP), which is hydrolyzed to the yellow compound 4-nitrophenolate detectable by reading the absorbance (405 nm). EVOO phenols decreased the LPS-induced TLR4 activation in a concentration-dependent way (Fig.2) with an IC_{50} of about 20 μg/mL. Higher concen-

trations of EVOO phenols induced a complete inhibition of TLR4 activation. MTT assay (Fig.2) showed that phenol extract was not toxic in the concentration range used to determine TLR4 activity.

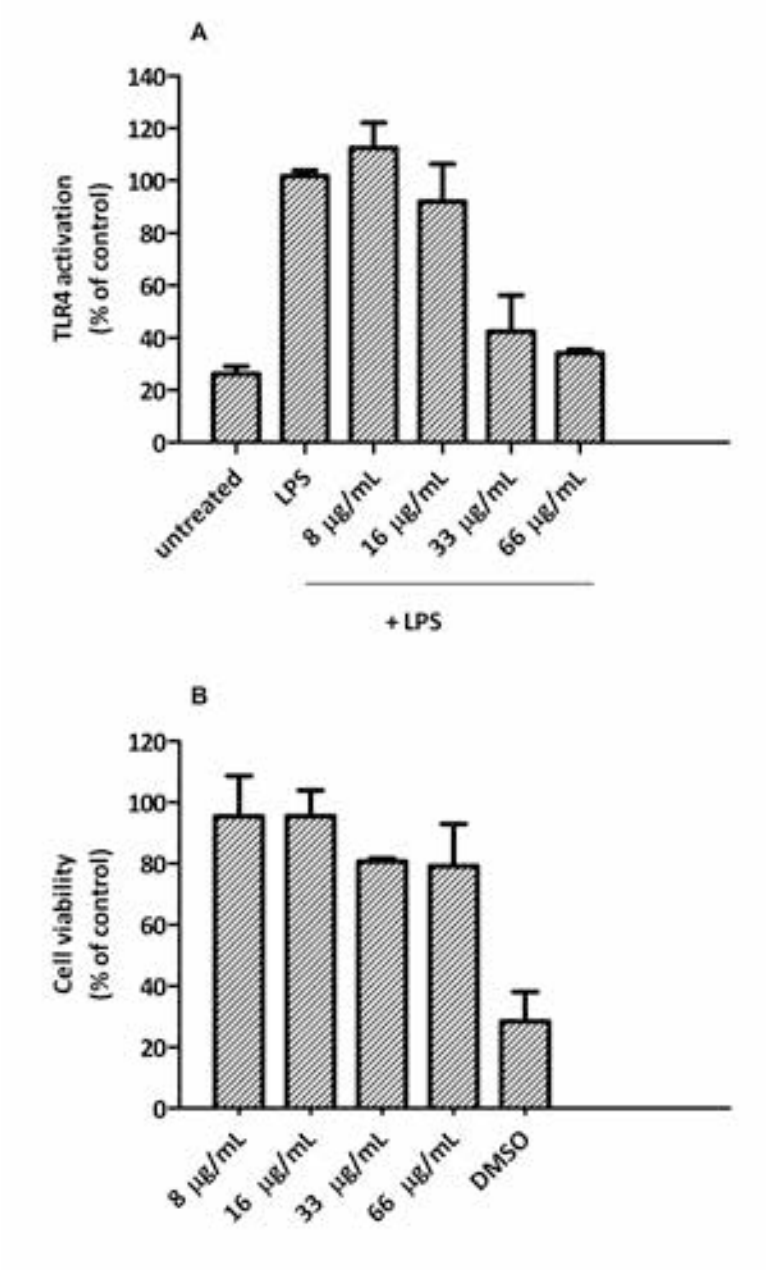

Fig.2. A) Dose-dependent inhibition of TLR4 activation in HEK-Blue-4™ cells by phenolic extract from EVOO. The results were normalized on positive control (cells treated with only LPS) and derived from three independent experiments. B) MTT assay of total phenolic extracts on HEK-Blue-4™ cells. The results

were normalized on negative control (cells treated with only PBS), while 10% DMSO is used as a positive control inducing cell lethality. Data are derived from three independent experiments.

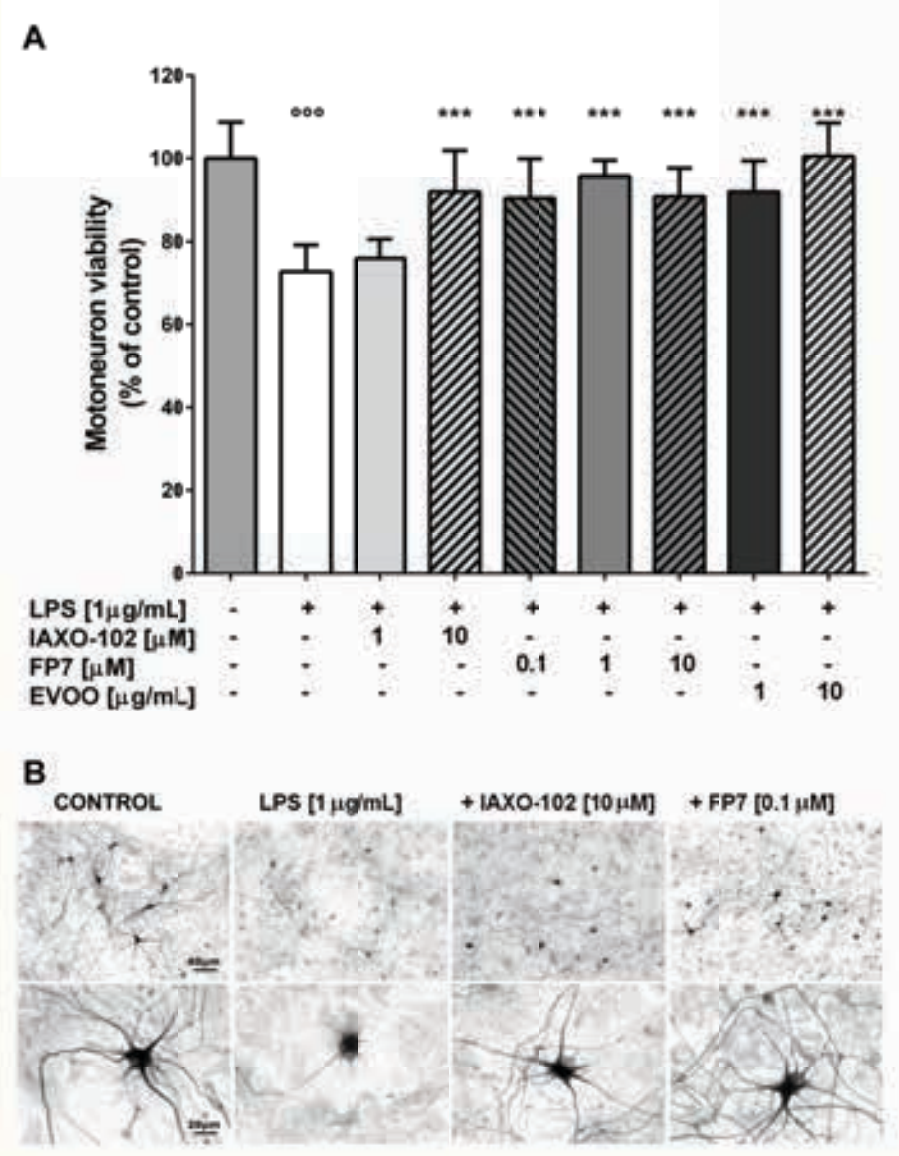

Fig.3. Motoneuron death in cocultures stimulated by LPS: protective effects of TLR4 antagonists. Motoneuron death induced by LPS (alone or in co-treatment with TLR4 antagonists) was assessed in motoneuron/glia cocultures after 24 hours of exposure. A): Bars represent mean percentage ± standard deviation of motoneuron death in the different treatment condition compared to control. Data from at least three independent experiments were analyzed. °°°$p<0.001$ vs control; ***$p<0.001$ vs LPS. One-way Anova and Tukey's test. B): Low- (upper line) or high- (lower line) magnified representative pictures of SMI32-positive

motoneurons maintained in control conditions or treated with LPS alone or in combination with 10 μM IAXO102 or 0.1 μM FP7.

LPS-mediated motoneuron death was counteracted by TLR4 antagonists

Treatment with 1 μg/mL LPS induced motoneuron death by about 30% (31.2 ± 8.2 %, $p<0.001$ vs CTR) after 24 h incubation (Fig.3A and representative pictures of LPS-injured motoneurons in B). When the cocultures were pretreated with IAXO102, 2 hours before LPS exposure, there was a significant decrease in motoneuron death (to 7.9 $\pm$ 9.8 %, $p<0.001$ vs LPS; Fig.3A) by 10 μM concentration (representative pictures of rescued motoneurons in Fig.3B). FP7 showed a higher efficacy in reducing motoneuron death, since it significantly counteracted the LPS neurotoxicity down to 100 nM (% MN death were reduced to 9.5 ± 9.4, 4.4 ± 3.8, 9.2 ± 6.9 respectively by 0.1/1/10 μM; $p<0.001$ vs LPS for all the concentrations tested, Fig.3A and representative pictures in B). Treatment with EVOO significantly reduced the motoneuron death both by 1 and 10 μg/mL ($p<0.001$ vs LPS for 1 or 10 μg/mL EVOO; Fig.3A).

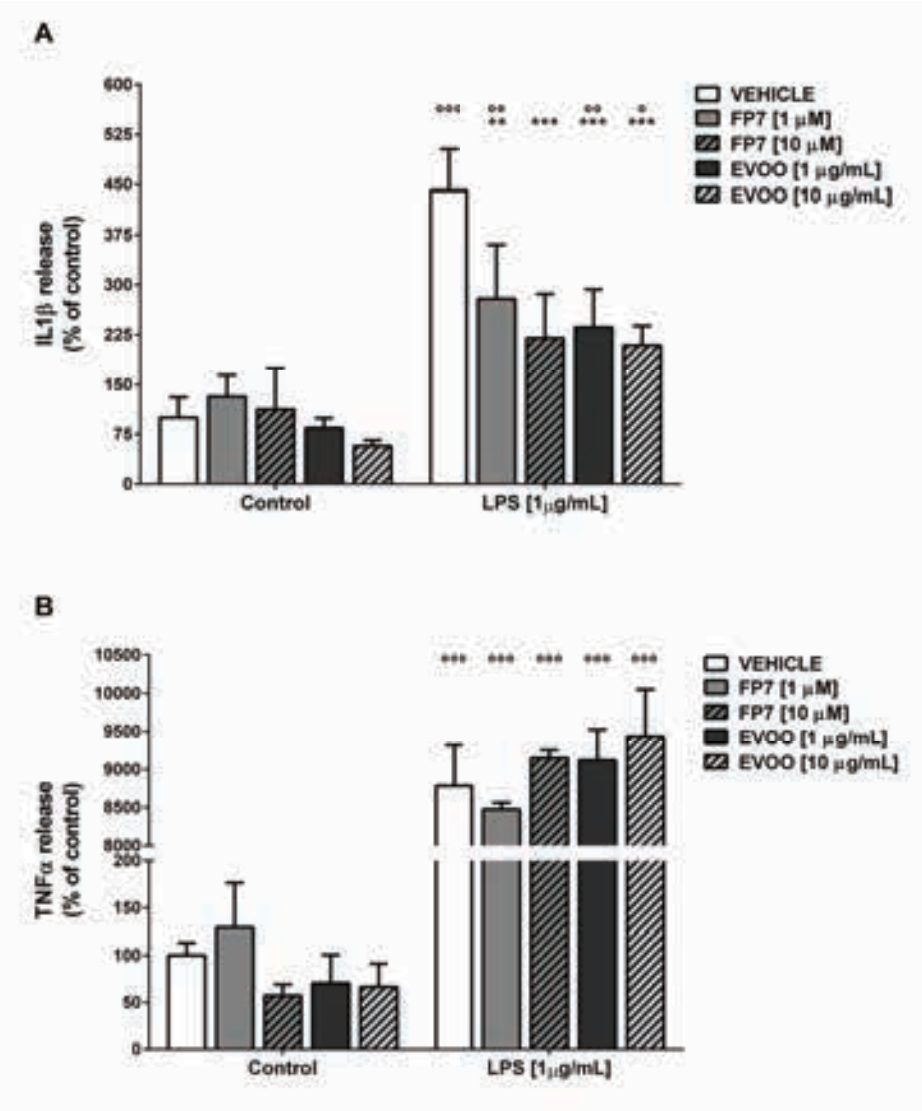

Fig.4. FP7 and phenols from EVOO reduced the IL-1β release by LPS-activated microglia. Purified microglia culture were treated with LPS, 1 or 10 μM FP7, 1 or 10 μg/mL EVOO, or cotreated with LPS + TLR4 antagonists. IL-1β(A) or TNF-α (B) concentrations in culture medium was assessed after 24 hours by ELISA assay. Bars represent the mean percentage ± standard deviation of cytokine concentrations in medium, normalized to control. Data from at least three independent experiments were analyzed. °°°$p<0.001$ vs control; **$p<0.01$, ***$p<0.001$ vs LPS. Two-way Anova and Bonferroni's test.

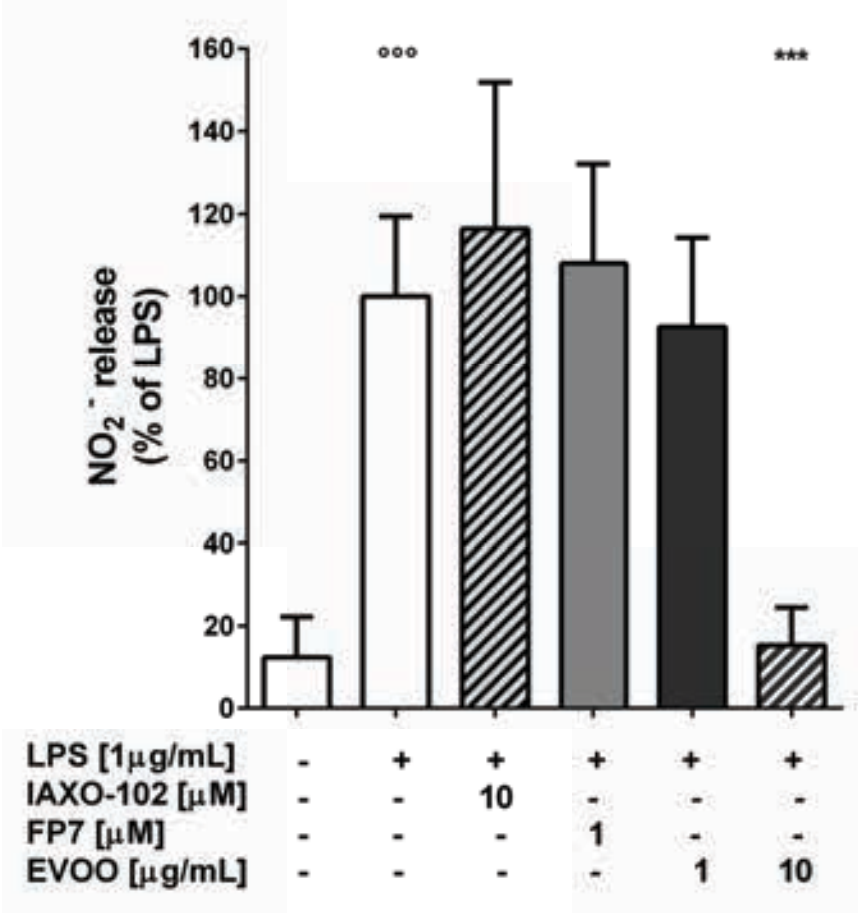

Fig.5. Nitric oxide release in coculture medium after exposure to LPS ± TLR4 antagonists. Motoneuron/glia cocultures were treated with LPS, alone or in cotreatment with the TLR4 antagonist, for 24 h. The nitrite release in culture medium was assessed by Griess reagent assay. Bars represent the mean ± standard deviation (in percentage) of nitrite compared to LPS treatment condition (set as 100% of nitrite release). Data from at least three independent experiments were analyzed. ***$p<0.001$ vs LPS. Oneway Anova and Tukey's test

Effects of TLR4 antagonists on cytokine and nitric oxide release induced by LPS in spinal cord cultures

In order to identify possible mechanisms underlying the neuroprotective effects of the tested TLR4 antagonists against LPS toxicity, two inflammatory events known to be elicited by LPS exposure have been investigated: i) microglia activation and ii) nitric oxide release in motoneuron/glia cocultures.

To study microglia activation, changes in pro-inflammatory cytokine release were assessed in purified microglia cultures. LPS treatment induced increase in IL-1β (Fig.4A) and TNF-α (Fig.4B) release in culture medium ($p<0.001$ vs control for both cytokines). Co-treatment with FP7 (Finteraction (DFn, DFd): $F(2, 18) = 9.156$, $p<0.01$) or EVOO ($F(2, 18) = 11.93$, $p<0.001$) significantly prevented the LPS-mediated IL-1β release (Fig.4A). No significant effects were observed for TLR4 antagonists on TNF-α release (Fig.4B).

To investigate the effects of the tested compounds on LPS-induced cell oxidative stress, the release of NO species in neuron/glia coculture medium was assessed. Treatment with 1 μg/mL LPS induced increase in NO release in culture medium after 24 hours, compared to control conditions ($p<0.001$, One-way Anova and Tukey's test; not shown). Pretreatment with 10 μg/mL EVOO almost completely prevented the NO release by LPS-stimulated cultures (15.3 $\pm$ 9.3 %, $p<0.001$ vs LPS; Fig 4). IAXO102 and FP7 (tested at the protective concentrations observed in motoneuron death assessment) were not effective on NO release induced by LPS (Fig.5).

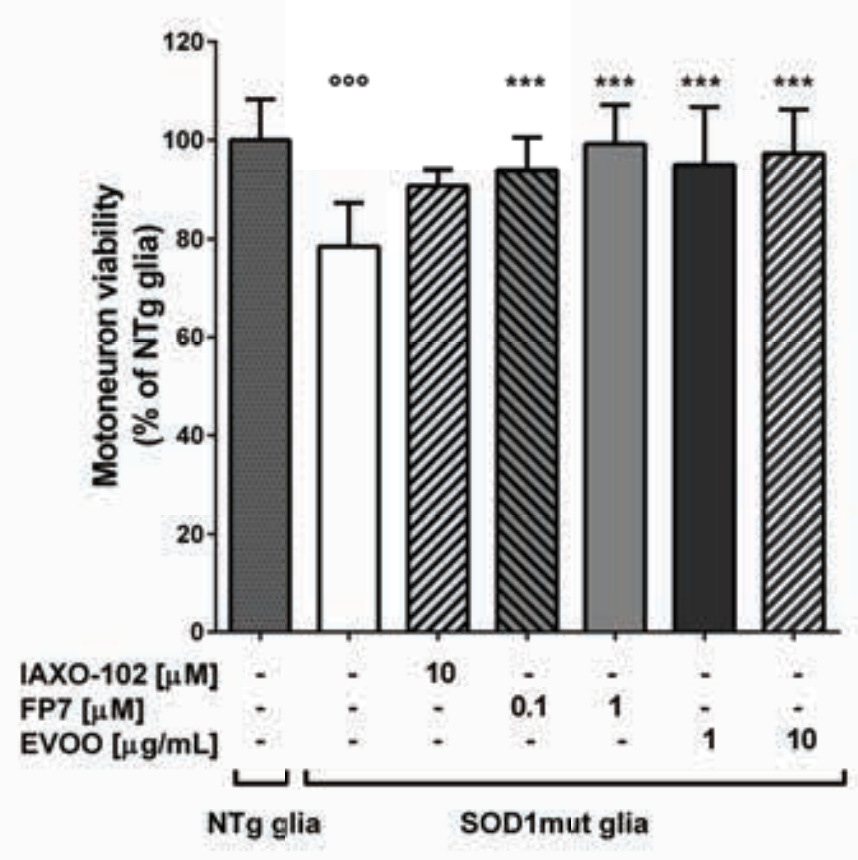

Fig.6. Motoneuron death induced by SOD1mut glia is counteracted by TLR4 antagonists. Motoneuron death assessed in motoneurons/glia cocultures with wild type or SOD1mut glia after 24 hours. Bars represent mean ± standard deviation of the percentage of motoneuron death compared to untreated wild-type motoneuron/glia cocultures (control). Data from at least three independent experiments were analyzed. °°° p<0.001 vs control; ***p<0.001, **p<0.01, *p<0.05 vs untreated motoneuron/SOD1mut glia cocultures. One-way Anova and Tukey's test.

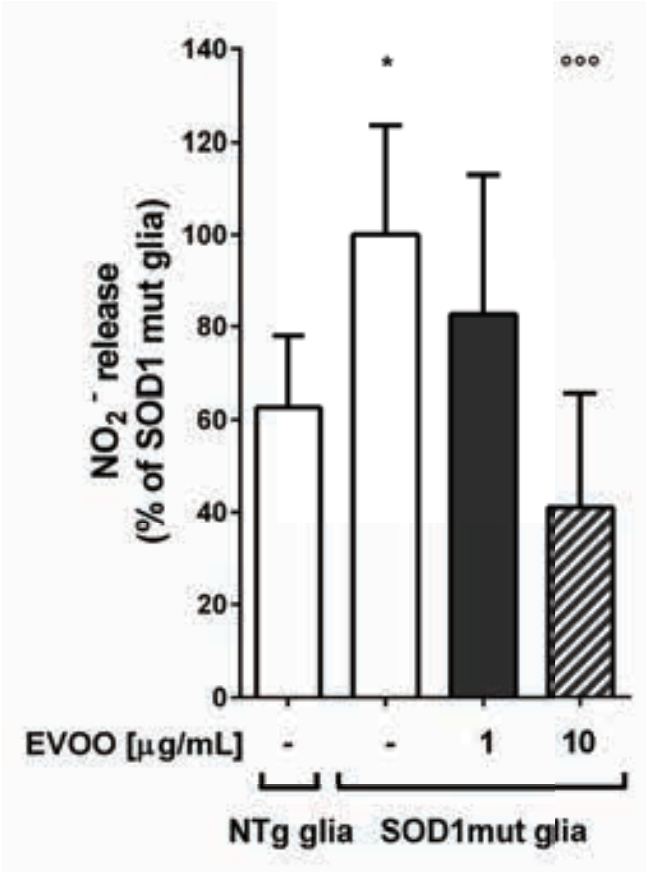

Fig.7. EVOO inhibited the nitric oxide release induced by SOD1 mut glia. Cocultures of motoneurons with NTg or SOD1 mut glia were established. Chronic exposure to 1 or 10 μM EVOO (from 1 to 7 DIV) was performed, and the nitrite release in culture medium was assessed by Griess reagent assay. Bars represent the mean ± standard deviation (in percentage) of nitrite compared to untreated MNs/SOD1 mut glia (set as 100% of nitrite release). Data from at least three independent experiments were analyzed. *p<0.05 vs MNs/NTg glia; °°°p<0.001 vs untreated MNs/SOD1 mut glia. One-way Anova and Tukey's test.

TLR4 antagonists reduced the SOD1mut glia-driven motoneuron death

Cocultures of wild-type motoneurons with glia obtained from SOD1 G93A or NTg mouse embryos were established. When cocultured with SOD1mut glia instead of NTg glia, increase in motoneuron death was observed (19.9 $\pm$ 4.4 %, $p<0.001$ vs MNs/NTg cocultures, One-way Anova and Tukey's test; Fig 5) 1 week after the culture establishment. This neurotoxic effect was prevented when 10 μM IAXO102 ($p< 0.01$), 0.1 – 1 μM FP7 ($p<0.001$) or 1- 10 μg/mL EVOO phenols ($p<0.05$ vs untreated MNs/SOD1mut glia cocultures, respectively) were added into the medium on the day after coculture establishment (Fig.6).

Phenols from EVOO reduced the nitric oxide release in motoneurons/SOD1mut glia cocultures

The release of NO was quantified in culture media from cocultures of motoneurons with NTg or SOD1mut glia. In the presence of SOD1mut glia an increase in NO medium levels was detected ($p<0.05$ vs MNs/NTg cocultures, One-way Anova and Tukey's test). Similarly to what observed for LPS-treated cultures, the exposure to 10 μg/mL of EVOO phenols significantly prevented the NO release measured in untreated MNs/SOD1 mut glia (Fig.7, $p<0.001$, One-way Anova and Tukey's test).

DISCUSSION

The present study originates from the reported evidence of an involvement of the TLR4 signaling in ALS pathogenic mechanisms, and as such it could represent a promising therapeutic target for the treatment of this devastating disease. In addition to the current knowledge, here we proved that synthetic and natural TLR4 antagonists are able not only to prevent the death of motoneurons induced by LPS but are also very effective in protecting motoneurons from the toxicity of microglia carrying the SOD1G93A mutation, typical of familial ALS.

EVOO phenols showed efficacy in reducing the release of nitric oxide from activated glia (either after LPS stimulation or by the presence of SOD1mutation), thus suggesting a putative mechanism of neuroprotection. LPS-induced nitric oxide release is however not affected by synthetic antagonists, thus suggesting that these compounds act through different mechanisms, including, although probably not exclusively, the modulation of pro-inflammatory cytokine release.

EVOO has recognized anti-oxidant and anti-inflammatory properties, and nitric oxide reduction in LPS-stimulated macrophages after treatment with the phenolic fraction of EVOO has been recently reported (23). We confirmed here that EVOO has similar protective effects also on the immunocompetent cells of the CNS.

A key role for microglia in mutated SOD1 (mSOD1)-induced motoneuron death has been proved both in SOD1G93A mice and in cell cultures. Microglia, as a component of the innate immune system, express CD14, TLR2 and TLR4 that mediate the immune response when activated. It has been reported that exogenous mSOD1 may be directly recognized by CD14 and in turn activate microglia leading to a neurotoxic cascade targeting motoneurons (8). However, this mechanism is not exclusive of the presence of SOD1 mutation since an overexpression of CD14 and TLR4 was also observed in sporadic ALS patients (7). We recently showed that a cyanobacteria-derived TLR4 antagonist was able to induce a significant anti-inflammatory and neuroprotective effects on LPS-stimulated spinal cord cultures and improvement in motor functional outcomes in a mouse model of spontaneous motoneuron degeneration (the Wobbler mouse)(15). The TLR4 antagonist, namely VB3323, however, has some limits in the therapeutic application since it is unknown if this molecule crosses the BBB, and the chemical composition, and consequently the toxicity profile of this molecule, cannot be exactly defined.

The advantages in the use of the synthetic compounds IAXO102 and FP7 as drug leads are i) their chemical purity, ii) their exactly defined chemical composition, and iii) the lack of toxicity.

Moreover, the hydrophilicity/hydrophobicity profile (logP) of both compounds can be varied by modifying their chemical structure with the aim of increasing their efficacy and of optimizing the pharmacokinetic properties. Phenols from EVOO are also interesting leads for drug development because of their lack of toxicity, although further studies are required to exactly define the active component(s) of the mixture.

Concerning the bioavailability of these compounds into the central nervous system, we previously demonstrated that IAXO102 is able to inhibit the activation of NF-κB and cytokines expression in the spinal cord of mice after a peripheral injection (21). Although this does not indisputably prove that the compound has reached the CNS, it is a clear proof of its anti-inflammatory effect *in vivo* in the CNS when systemically administered.

CONCLUSION

There is growing evidence suggesting that the peripheral immune system is also involved in ALS pathogenesis, especially by modulating the progression of the disease. It has been demonstrated that in SOD1G93A mice the inflammatory monocytes that express a polarized macrophage phenotype (M1 signature) are probably recruited into the CNS by activated microglia (25). In human ALS, the analogous monocytes expressing CD14+ CD16- also exhibit an ALS-specific microRNA inflammatory signature. These monocytes apparently undergo a migration into the CNS as their levels in the peripheral blood are significantly decreased in ALS patients with respect to other cells (26). Along this line, it has been recently reported that the deletion of TLR4 is able to significantly extend survival and transiently improve motor functional outcomes in a disease mouse model (14).

Based on these considerations, targeting the TLR4 signaling appears as a promising pharmacological approach in ALS. For what concerns the TLR4 antagonists proposed in this study, it seems

mandatory as next step to examine their effects *in vivo* in the mouse model of ALS, in order to assess potential therapeutic applications.

ACKNOWLEDGMENTS

Authors acknowledge Innaxon (UK) for providing IAXO-102. This study was financially supported by the Italian Ministry of University and Research (MIUR) PRIN 2010-11 project: "Italian network for the development of multivalent nanosystems," COST actions CM1102 "MultiGlycoNano" and BM1003 "Microbial cell surface determinants of virulence as targets for new therapeutics for Cystic Fibrosis", European Commission granted TOLLerant H2020-MSC-ETN-642157 project.

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