First identification of Toll-like receptor-4 in avian brain: evolution of lipopolysaccharide recognition and inflammation-dependent responses

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Abstract
In this work, we examine the effects of lipopolysaccharide (LPS) treatment on nerve cells of chick embryo used as a universal avian model. We demonstrate that LPS leads to a dramatic cell loss in primary cultures of both glia and neurons, isolated from chick embryos. Toxic effects appear to be mediated by the Toll-like receptor (TLR)-4 complex, expressed in both glial and neuronal cells, since after TLR-4 silencing by RNA interference experiments LPS-induced cytotoxicity was prevented. The role of nitric oxide in LPS-induced cell damage has also been investigated. These results demonstrate, for the first time in avian nerve cells, the surface expression of TLR-4 and its role as a pattern recognition receptor involved in LPS-induced cell responses in a similar manner to that observed in mammals.

Keywords: Glial cells; nervous cells; lipopolysaccharide; nitric oxide; TLR-4

Introduction
The evolutionary ancient innate immune system represents the first line of host defence against a number of pathogens. Cells of the natural immune response are able to recognize microbial molecular motifs with high specificity through a series of genetically and stable conserved cell membrane receptors. In particular, receptors related to the Drosophila toll gene, and therefore referred to as Toll-like receptors (TLRs), seem to play a central role in the initiation of cellular innate immune responses. These receptors belong to a multigene family encoding important pattern recognition receptors that have been recognized in a number of invertebrate and vertebrate genomes. [1-4] Among these, Toll-like receptor (TLR)-4 is the receptor required for the signal transduction induced by lipopolysaccharide (LPS), the main cell membrane constituent of Gram-negative bacteria. [3,4]

It has long been established that lower vertebrates, including fish and amphibians, exhibit resistance to toxic effects induced by LPS, probably due to the lack of a TLR-4 ortholog. In this context, it has recently been reported that in zebrafish TLR-4 does not recognize LPS, negatively regulating the TLR signalling pathway, supporting the idea of a divergent functional evolution of this receptor. [5]

Recent analyses of chicken genome sequences have revealed the presence of TLRs in this animal although their role has not been well clarified. Moreover, a recent report demonstrated that the expression of TLR-4 in peritoneal macrophages of passerine birds was found to be positively enhanced after Gram-negative treatment. [6]

Several authors agree that many effects induced by LPS are mediated by TLR-4 expressed on the surface membrane of both immune and nonimmune cell types, [7,8] The cellular signalling events consequent to LPS stimulation by endotoxin receptor engagement consist
of the release of reactive oxygen and nitrogen intermediates, proinflammatory cytokines and chemokines that can result cytotoxic rather than specifically bactericidal for the host. Recently, a relationship between systemic infection and the worsening of many diseases of the central nervous system (CNS) has been documented, although the mechanisms inducing neuronal loss are not yet well established.\(^9,10\) In this context TLRs, constitutively expressed in different anatomical areas of the CNS, seem to be involved in neurodegenerative disorders.\(^11,12\)

In this study, we have identified, for the first time in a bird model represented by the chick embryo, TLR-4 surface expression on primary nerve cells and its role in toxicity mechanisms induced by in vitro LPS treatment.

**Materials and methods**

**Chick nerve cell cultures**

Ten-day chick embryo brains were stripped of the meninges and mechanically dissociated with a Pasteur pipette. The cells were gently pelleted and resuspended in MEM (GIBCO, Invitrogen, Italy). Cell viability was 95% as determined by trypan blue exclusion. In order to obtain glia-enriched primary cultures, the cell suspension was plated on noncoated six-wells tissue culture plates and on noncoated four-wells tissue culture plates with glass slides. The culture medium was MEM (GIBCO) supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% foetal calf serum. To obtain neuron-enriched cultures, the dissociated cells were plated at a density of 1.5 \(\times\) 10⁵ into six-wells culture plates and of 25 \(\times\) 10⁵/15-mm well (Nunc) into four-wells culture plates on glass coverslips both coated with 10 µg/mL poly-D-lysine. Culture medium was Neurobasal medium supplemented with B27 (Gibco) and 0.5 mM glutamine. The day after plating, 50% of the medium was changed and thereafter the medium was changed every 3 days.

**Morphological analysis**

We typed the cells by using specific Abs directed against cellular proteins of neurons (Polyclonal anti-neurofilament NF-L, rabbit IgG, Proteus, Biosciences, Inc., Italy) or glial cells (Monoclonal anti S-100 β chain, mouse IgG, Sigma-Aldrich, Italy) followed by treatment with a goat anti-rabbit IgG FITC-conjugated secondary Ab (Molecular Probes, Invitrogen), and a goat anti-mouse IgG TRITC-conjugated secondary Ab (Sigma-Aldrich). Cells were observed with a confocal microscope with a Leica 63× oil immersion lens.

**Cell culture treatments**

Cell cultures were treated with 10 µg/mL LPS of *Salmonella typhimurium* (Sigma-Aldrich) for different incubation times (24, 48, and 72 h).

**Nitric Oxide (NO) production**

The concentration of stable nitrite, the end product of nitric oxide (NO) generation, was determined by the method described by Ding et al.\(^13\) Briefly, nerve or glial cells, isolated and cultured as indicated above, were treated for different times (24, 48, and 72 h) with 10 µg/mL LPS. At the end of treatment, culture supernatants were collected and incubated with the Griess reagent (Vetrotecnica, Padova, Italy) (1:1 v/v) for 10 min at room temperature (RT). The absorbance of supernatants was spectrophotometrically measured at 540 nm and the NO₂⁻ concentration was determined by extrapolation from a NaNO₃ standard curve and expressed as nanomolar per litre. To avoid interference by nitrates possibly present in the medium, in each experiment, the absorbance of the unconditioned medium was assumed as the “blank.”

**Electrophoresis**

After treatment, cells were harvested and lysed in ice-cold lysis buffer (0.5 M Tris–HCl, 1.86 g/mL EDTA, 1 M NaCl, 0.001 g/mL Digitonin, 4 U/mL Aprotinin, 2 µM Leupeptin, and 100 µM PMSF). Lysates were centrifuged at 13,800 g for 20 min at 4°C. The protein concentration was determined by the Bradford method.\(^14\) Protein samples were diluted with sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 10% w/v SDS, 5% β2-mercaptoethanol, 0.05% w/v bromophenol blue) and then boiled for 3 min. Proteins (25 µg/lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on 10–12% Sodium Dodecyl Sulphate (SDS) precast polyacrylamide gels (BioRad Laboratories, CA, USA).

**Western blotting**

After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer [20 mM Tris/150 mM glycine, pH 8, 20% (v/v) methanol] was used for membrane saturation and blotting. Specific proteins were detected using rabbit polyclonal anti human TLR-4 (1:200) or rabbit anti iNOS/NOS TYPE II (1:200), according to the manufacturer’s instructions (Santa Cruz Biotechnology, DBA Italia, Milan, Italy). The binding of antibodies was detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG followed by chemiluminescence detection (Pierce, Perbio, Rockford, USA).
**Immunofluorescence staining**

To investigate the surface expression of TLR-4, nerve or glial cells were plated onto four-well tissue culture plates stimulated with LPS (10 μg/mL) at 37°C for various times. After three washes with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde (PFA) for 15 min. Then, cells were incubated with mouse monoclonal anti-TLR-4 (1:50), for 1 h at RT and then incubated overnight at 4°C. After incubation, cells were kept for 1 h at RT and then washed and treated with goat anti-mouse IgG TRITC conjugated (1:200) (Molecular Probes) for 2 h at RT. The neurofilament of cells was stained with polyclonal anti-neurofilament NF-L, rabbit IgG (Proteus, Biosciences Inc.), diluted 1:100, while anti S-100 Ab rabbit IgG (Affinity Bioreagents, Alexis, Italy) was used to show glial cells. The secondary Ab used was goat anti-rabbit Alexa 488 labelled (Molecular Probes). Nuclei were stained with the TO-PRO (1:8000) (Molecular Probes).

**Confocal laser microscopy**

Slides were visualized using a Leica TCS SP2 confocal microscope (Leica, Solms, Germany) with a 63× oil immersion lens. Alexa Fluor 488 and TRITC were excited at 488 and 540 nm, respectively, then detected between 506–538 nm and 570–573 nm, respectively. Optical sections (0.5 μm) were merged and projected with Bio Rad software.

**RT-PCR**

The expression of the TLR-4 gene was analysed by RT-PCR. Briefly, total cellular RNA was extracted from nerve or glial cells by the Trizol isolation reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed in a final volume of 20 μL containing 3 μg of total RNA, 40 U of RNase Out (Invitrogen), 40 μL of oligo dT with 0.5 mM dNTP (PCR Nucleotide Mix, Roche Diagnostics, Milan, Italy), 40 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Roche Diagnostics). The reaction tubes were incubated at 37°C for 59 min, and then at 95°C for 5 min and at 4°C for 55 min. cDNA obtained was then amplified by a thermal cycler (Eppendorf, Milan, Italy) under the following conditions: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35 cycles of amplification). The reaction tube contained, in a final volume of 50 μL, 2 μL of cDNA, 200 μM dNTP (PCR Nucleotide Mix, Roche Diagnostics), 4 U TaqDNA Polymerase (Roche Diagnostics), 5 μL of MgCl₂ buffer stock solution, and 50 pmol of primers specific for a 361 bp fragment of the gene coding for TLR-4 [(F) 5’ TTGAACCTTCTGAAATGGGT 3’; (R) 5’ AGGGAGTTTCTCCTTCTCT 3’] (NCBI accession number NM_021297.1) or primers specific for a 450 bp fragment of the gene coding for GADPH [(F) 5’ ACCACGTCCCATGCCATC 3’; (R) 5’ TCCACACCTGTTGCTGTGA 3’] (NCBI accession number NM_021297.1), used as housekeeping gene control.

The resulting products were separated with agarose gel (1.2%), stained with ethidium bromide and sequenced to verify whether the correct product was amplified.

**Cytotoxicity assay**

Evaluation of LPS cytotoxicity on nerve and glial cultures was quantified using neutral red uptake test (Sigma-Aldrich). The cells (2 × 10⁴/well) were growing in 96-well plates in the presence of appropriate medium for the selection of neurons or glia.

A PBS 1× solution of neutral red 1 mg/mL was added to LPS-treated cells in order to reach a final concentration of 50 μg/mL. Experiments included untreated cells used as controls. Cells were incubated for 2 h at 37°C and then after washing, acid alcohol (1% acetic acid in 50% ethanol) was added for 15 min to extract neutral red from living cells. The eluted stain intensity (optical density) was measured at 540 nm in a Victor Multiplate reader (Wallac, PerkinElmer, Monza, Italy). The surviving cell number was directly related to the absorbance of the eluted stain.

**Treatment of cell cultures with small interfering RNA**

Cell cultures (60–70% confluence) were submitted to TLR-4 specific small interfering RNA (siRNA) using a commercial kit (Santa Cruz Biotechnology) according to the manufacturer’s protocol. Cells were incubated with 60–80 pmols of siRNA for 5–7 h at 37°C, using a transfection reagent. Then, cells were added with 1 mL of complete medium (2×) and incubated for 24 h. The day after, medium was replaced with fresh culture medium and cells were incubated for 48 h before treatment with endotoxin.

**Densitometric analysis**

The bands obtained after immunoblotting were submitted to densitometric analysis using ID image analysis software (Kodak Digital Science). Results were expressed as arbitrary units.

**Statistical analysis**

The statistical significance of differences between groups was analysed by Student’s t-test.
Results

Morphological and immunochemical analysis of neuron-enriched cultures

Chick nerve cultures were morphologically characterized by confocal microscopy observation. Specific Abs directed against cellular proteins of neurons (Polyclonal Ab anti-neurofilament NF-L) or glial cells (Monoconal Ab anti S-100 β chain) showed an enriched content of neurons (90%) or glial cells (90%) in the respective experiments. These results were confirmed by western blotting analysis performed on cell lysates obtained as previously described, indicating only the presence of the specific neurofilament or glial protein (data not shown).

NO production and iNOS expression in LPS-stimulated cells

Chick nerve cells (neurons and glial cells) treated in vitro with LPS (10 μg/mL) for different times (24, 48, and 72 h) induced a significantly enhanced NO production when compared with untreated cells (Figure 1A and 1B). NO production occurred in a time-dependent manner both in neurons and glial cells, with a maximal response of 72 h after cell treatment with endotoxin. Endotoxin stimulation in the presence of 1400 W, the NO synthase inhibitor, determined a reduction of the NO production almost to basal levels, suggesting that the NO production in LPS-stimulated cells is iNOS mediated (data not shown).

These data have been confirmed by immunoblotting analysis for iNOS expression. Figure 2A and 2B shows the presence of a 130 kDa protein, corresponding to the molecular weight of the iNOS enzyme, in the cell lysates obtained from cell cultures of neurons and glia exposed to LPS. The same time-dependent increase of protein levels was observed in both endotoxin-treated neuron and glia cultures. A basal expression of iNOS was observed in untreated cells, explaining the basal NO production observed in cell cultures. Figure 3 shows that LPS stimulation in the presence of tosyl phenylalanyl chloromethyl ketone (TPCK), the specific NF-kB inhibitor, determined a reduction both of NO release (Figure 3A) and iNOS expression (Figure 3B), suggesting an involvement of NF-kB in the LPS-induced responses in our in vitro model.

Effect of LPS treatment on cell viability

Microscopic observation of LPS-treated cells demonstrated evident cell suffering in comparison with

**Figure 1.** Nitric oxide production by LPS-stimulated cell cultures performed with the Griess reaction as described in the Materials and Methods section. Neurons (A) or glial cells (B) were incubated with medium (control) or LPS (10 μg/mL), for 24, 48, and 72 h at 37°C. Results are expressed as means ± SE of three independent experiments.

**Figure 2.** iNOS expression in LPS stimulated cell cultures. Immunoblotting detection of iNOS in neurons (A) or in glial cells (B) incubated with medium alone (control) or stimulated with LPS (10 μg/mL), for 24, 48, and 72 h. β-actin was used as loading control. Results are reported as densitometric analysis of iNOS expression, after normalization against β-actin. Values (means±SE of three experiments) are expressed as arbitrary units.
untreated cells. Cell damage resulted more evident in neurons than in glial cells, these last showing a greater resistance to endotoxin treatment. We quantitatively examined the effect of LPS treatment on cell viability by neutral red assay. Figure 4 shows a 50% reduction of viability in neuron cultures and a loss of 10% viability in glial cells; as observed in the figure, the percentage of LPS-treated neurons appeared significantly (P < 0.05) reduced in comparison with untreated cells (control), starting from 48 to 72 h of endotoxin exposure, whereas glial cultures exhibited no significant reduction of the cell viability percentage after endotoxin treatment.

Expression of TLR-4

Confocal laser microscopy observation detected the presence of the TLR-4 receptor on the surface membrane of both glial and neuronal cells (data not shown). RT-PCR confirmed the expression of TLR-4 mRNA. A transcript of the predicted size (361 bp) was detected in both glia and neurons, as reported in Figure 5A.

Immunoblot analysis (Figure 5B) revealed a single band at the known size of TLR-4 (approximately 90 kDa). TLR-4 expression resulted significantly increased after 72 h LPS treatment in neuronal cells, whereas glial cells did not exhibit significant differences after endotoxin treatment in comparison with controls.

Involvement of TLR-4 complex in functional responses of cells

Preliminary experiments were performed to explore an involvement of the TLR-4 complex in functional responses of cells submitted to LPS treatment. Interestingly, TLR-4...
blockade using specific antibodies determined a significant reduction of NO release in both glial and neuronal cells (data not shown). Since TLR-4 is reported to be primarily involved in the signal transduction pathway in immune cells,\textsuperscript{15–17} we performed silencing experiments in order to explore the role of this endotoxin receptor in functional cell responses. Interestingly, siRNA TLR-4 led to a significant reduction of NO release in LPS-treated

Figure 5. Expression of TLR-4. (A) RT-PCR analysis of TLR-4 mRNA expression in neurons (N) or glial cells (G). Experiment was repeated thrice, using GADPH as housekeeping gene. (B) Immunoblot analysis of TLR-4 neurons or glial cells incubated with medium alone (control) or stimulated with LPS (10 μg/mL), for 24, 48, and 72 h. Results are reported as densitometric analysis of TLR-4 expression, after normalization against β-actin. Values (means ± SE of three experiments) are expressed as arbitrary units.

Figure 6. Effect of TLR-4 siRNA on NO production in LPS stimulated cell cultures. (A) Neurons (left) or glial cells (right) were incubated with medium alone or siRNA TLR-4 (controls), or with LPS (10 μg/mL), or siRNA TLR-4 + LPS for 24, 48, and 72 h at 37°C. (B) Viability tested by neutral red assay. Percentage of cell viability in neurons or glial cells incubated with medium alone (controls) or with LPS (10 μg/mL) for 24, 48, and 72 h at 37°C, evaluated as optical density (570 nm) in neurons and glial cells, TLR-4 treated (dotted lines) or untreated siRNA (continuous lines). Values are expressed as means ± SE of three experiments.
Discussion

Our experimental investigation shows that in chick nerve cell populations, LPS is able to induce inflammatory responses, which occurs through an increased production of NO, the molecule known to be a mediator of inflammation. Both in neurons and in glial cells, stimulation with LPS (10 μg/mL) leads to a significant increase in NO production in the culture medium by 24 h of treatment, reaching the highest level after 72 h of treatment. The production of NO, however, achieves higher concentrations in neurons when compared with glia. We also observed that LPS is able to up-regulate iNOS expression in both cell types. The induction of iNOS is primarily associated with inflammatory responses that can be triggered by various factors. It has previously been reported that human neurons, in addition to other cells of the CNS, can be induced to express iNOS both in vivo and in vitro.

The production of NO is involved in cell death through necrosis or apoptosis, which occurs in situations of ischemia or neurodegenerative diseases, but is also crucial because, as a physiological product, it guarantees a correct differentiation of neural cells during brain development, capable of halting proliferation and thus inducing differentiation and growth arrest. Since we used an experimental model represented by embryo-isolated primary cultures, the basal production of NO observed in untreated neurons and glia could serve to ensure a fine regulation of the balance between proliferation and cell differentiation.

Our morphological analysis provides, for the first time in birds, evidence of TLR-4 expression in both cell populations examined. Moreover, the gene silencing experiments suggest an involvement of the TLR-4 receptor in triggering inflammation response, since in TLR-4 silenced cells a significant reduction of NO release is observed after stimulation with endotoxin, as well as a significant reduction of LPS-induced cytotoxicity.

Some studies have shown the involvement of TLRs in progressive neurodegenerative disorders. In particular, TLR-4 is considered to be the main signal transducer of LPS, since it has a transmembrane domain capable of activating the transcriptional factors involved in the cellular response. TLR-4 is highly expressed in microglia and is required to induce the cascade response to cytotoxic LPS. To support this, experiments performed in a murine model have shown that mutations in the TLR-4 gene lead to a nonworking protein product, are able to confer resistance to neuronal insults. These observations have allowed us to confirm, in birds, the same role for TLR-4 in inflammatory-based neurodegeneration already reported in higher vertebrates.

Based on three different techniques, RT-PCR, western immunoblot, and confocal microscopy, we evaluated the expression of this receptor in both cell types. RT-PCR and western-blot demonstrate that TLR-4 is expressed both in neuronal and glial cells, then confirmed by confocal analysis.

Several reports in literature suggest a critical role for this receptor in functional cell responses during inflammatory events. In this context, the Toll-mediated host defence system is seen to be conserved across a wide range of animal species, from Drosophila to Mammals. It is likely that TLRs originally had a developmentally correlated role which, in the course of evolution, acquired the significance of a pattern-recognition receptor involved in the host defence mechanisms. In fact, in fish, TLR-4 is not able to recognize LPS whereas, as reported by Vinkler et al., in birds TLR-4 expression is up-regulated after LPS binding. This supports the concept that the role of TLR-4 in the recognition of pathogenic agents arose later during evolution, as a result of selective pressures on TLRs.

In higher vertebrates, TLR-4 is clearly required for a significant induction of pro-inflammatory cytokines and other mediators of inflammation produced by LPS stimulation. It is required for signal transduction in monocytes and macrophages. Some authors have reported a central role of microglia in the induction of innate immune responses in the CNS, and that only these cells express TLR-4. Furthermore, a different expression ability has been emphasized as a distinctive feature between neurons and glia. From our experiments, it was apparent that TLR-4 is expressed both in glia and in neurons. This result is plausible, given the different experimental model that we used, especially because our study was conducted on chicken embryos at the tenth day of embryonic development and not on adults. Moreover, we observed that the expression of this receptor in glia does not undergo changes related to the time of endotoxin exposure, while the expression of TLR-4 increases in a time-dependent manner in neuronal cells. PCR confirmed the presence of the messenger for the TLR-4 receptor in both kinds of cells, and the messenger.
does not show quantitative changes between neurons and glia.

In our study, the role of TLR-4 in the cell response entity was investigated by gene silencing experiments. We saw a reduction in the production of NO in previously silenced neuronal and glial cells exposed to endotoxin for different times. Interestingly, in controls of both cell populations examined, treated for silencing without LPS exposure, we did not observe any change in NO levels. This confirms the hypothesis of a basal production of NO, independent of the external stimuli, probably involved in cell proliferation and differentiation as previously mentioned. In silenced glial cells, then stimulated with endotoxin, the production of NO is almost completely inhibited after 24 h, while at 48 and 72 h there was an increase of NO levels, although these were still significantly lower than in cells treated with LPS alone. This time-dependent increase may be explained in terms of a loss of silencing efficiency or even by alternative mechanisms of endotoxin internalization, which are activated and additional to the receptor-dependent mechanisms normally present in cells. These alternative mechanisms may consist of macropinocytosis phenomena,(20) the formation of vesicles coated by clathrin(30) and plasma membrane uncoated invagination involving the formation of caveolae and micropinocytosis,(31,32) Thanks to these mechanisms, we can deduce that TLR-4 is the main actor in the signal transduction triggered by bacterial endotoxin and that glia cells, as inflammatory competent cells, are more susceptible than neurons to a fine modulation acting through the receptor turnover.

In conclusion, our results represent the first evidence for a cytotoxic effect exerted by LPS in nerve cells of the chicken embryo, used as a universal avian model with the aim of evaluating the evolution of TLR-4 as a pattern recognition receptor involved in the host defence mechanisms.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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