Toll-like receptors as transducer of inflammatory signals in glia: the astrocyte-microglia connection

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ABSTRACT

In physiological conditions glia in the central nervous system (CNS) can produce and release protective factors such as anti-oxidant molecules and neurotrophic factors (Sofroniew et al., 2010). Events that impinge on CNS homeostatic balance can induce local inflammatory responses (Carson et al., 2006). Reactive glia can participate producing pro-inflammatory mediators such as chemokines, cytokines, purines and free radicals.

Toll-like receptors (TLRs) are involved in injury responses of nervous system tissue and in neuropathic pain. Here we have investigated the cross-talk mechanisms between glial cells in the CNS making use of an in vitro cellular model, evaluating how glia respond to TLR agonists based on cytokine synthesis and release as well as TLR mRNA/protein expression as readouts.

In order to analyze specific molecular parameters involved in the genesis and maintenance of inflammation, purified microglia and astrocyte-enriched cultures were generated from cerebral cortex of 1-2 day-old rat pups. For some experiments the enriched astrocyte cultures were purified by treatment with L-leucyl-L-leucine methyl ester (L-LME), which selectively depletes cultures of microglia. Activation of microglia and astrocytes (± L-LME) was achieved by treatment with lipopolysaccharide (LPS, TLR4 agonist); zymosan (TLR2 agonist) and poly(I:C) (TLR3 agonist) for 6 and 24 hours.

Gene expression analysis (Real Time-polymerase chain reaction) revealed the ability of microglia to induce mRNA coding for interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). In contrast, purified (nominally microglia-free) astrocyte cultures were not responsive to TLR agonists - unlike their astrocyte-enriched counterpart. Mediator production and release into the culture medium (analysed by ELISA)
confirmed that microglia themselves respond to pathogenic stimuli. Utilizing flow-cytometric analysis we evaluated the expression of TLR receptors on the cell surface (TLR2/4) or in endosomal membranes (TLR3) after 1, 6 or 24 hours of stimulation with TLR agonists.

Non-neuronal cell responsiveness to pathogenic stimuli is almost always linked to the production of inflammatory mediators. In this context we asked if the apparent inability of purified astrocytes to express a pro-inflammatory phenotype was dependent on the absence of the relevant TLR. Using confocal microscopy, stimulation with LPS conjugated with a fluorochrome showed the presence of TLR4 on the astrocyte cell surface and Western blot analysis revealed the presence of the co-receptors MD2 and CD14. As consequence, purified astrocytes have been studied in flow cytometry to evaluate alteration in TLR protein expression. Moreover, we reconstituted the inflammatory profile in astrocyte cell cultures by adding fixed numbers of purified microglia (10% of contaminating cells final). Although the latter 'co-cultures' express pro-inflammatory cytokines after TLR agonist stimulation the absolute levels are inferior to those measured in enriched astrocytes (<5% of contaminating microglia).

To further address the issue of whether microglial cell activation in the presence of astrocytes results from either physical interaction between cell membranes or chemical induction mediated by the release of mediator(s) into the culture medium, a "Transwell insert" system was used. The astrocyte/microglia co-culture paradigm described here may provide a useful starting point to elucidate the molecular mechanisms underlying astrocyte- and microglia-specific responses pertaining to, although not limited to, CNS inflammation, especially where TLR activation plays a role.
Nel sistema nervoso centrale (SNC), le cellule gliali in condizioni fisiologiche producono e rilasciano sostanze protettive come molecole anti-ossidanti e fattori neurotrofici (Sofroniew et al., 2010). Tutti gli eventi che alterano l’equilibrio omeostatico inducono una risposta infiammatoria locale (Carson et al., 2006). La glia reattiva partecipa producendo mediatori dell’infiammazione come chemochine, citochine, purine e radicali liberi.

I recettori Toll-like (TLRs) sono coinvolti nelle risposte da danno indotto a carico del tessuto nervoso e nel dolore neuropatico.

Nel nostro studio abbiamo investigato i meccanismi di comunicazione tra le cellule della glia attraverso la realizzazione di un modello cellulare in vitro idoneo alla valutazione della risposta gliale al trattamento con agonisti dei TLRs, valutando sia l’espressione di molecole associate all’attivazione dei recettori sia la modulazione genica/proteica degli stessi TLRs.

Per poter valutare meglio la genesi e la progressione dello stato infiammatorio, colture di microglia purificata e colture arricchite in astrociti (≥95%) sono state ottenute dal sacrificio di ratti neonati di 2 giorni e dalla successiva dissezione corticale. Per i nostri esperimenti le colture arricchite di astrocyti sono state trattate con L-leucil-L-Leucina estere metilico (L-LME) al fine di ottenere una coltura purificata di astrocyti (≥99%).

L’attivazione della microglia e degli astrocyti (± L-LME) è stata indotta dal trattamento con lipopolisaccaride (LPS, agonista del TLR4), zymosan (agonista del TLR2) e poli(I:C) (agonista del TLR3) per 6 e 24 ore.

L’analisi dell’espressione genica (in Real Time PCR) ha permesso di dimostrare la capacità delle cellule della glia di indurre la trascrizione di mRNA codificante per interleuchina-1β (IL-1β), interleuchina-6 (IL-6) e tumor necrosis factor-α (TNF-α). La coltura purificata di astrocyti non
risponde al trattamento con agonisti TLRs, diversamente dalla coltura arricchita in astrocti in cui persiste una piccola percentuale di cellule della microglia.

La produzione e il rilascio nel terreno di coltura di mediatori dell’infiammazione (dosaggio ELISA) confermano che la microglia risponde allo stimolo patogenico. Inoltre le analisi di citofluorimetria hanno permesso di valutare l’espressione dei TLRs sulla membrana cellulare (TLR2/4) e sulla membrana degli endosomi (TLR3) dopo 1 ora, 6 ore e 24 ore di trattamento.

La responsività delle cellule non-neuronali ad uno stimolo lesivo viene solitamente valutata sulla base della capacità delle cellule di produrre mediatori pro-infiammatori. Alla luce di queste evidenze abbiamo voluto chiarire se l’apparente assenza di responsività della coltura purificata di astrocti, dipendesse da alterazioni a carico della struttura recettoriale. Utilizzando la microscopia confocale, abbiamo marcato le cellule con LPS-coniugato con un fluorocromo dimostrando la presenza del TLR4 sulla superficie cellulare degli astrociti e le analisi di Western Blot hanno permesso di confermare anche la presenza dei co-recettori CD14 e MD2.

In particolare, lo studio sugli astrociti purificati è stato approfondito mediante citofluorimetria per valutare le alterazioni a carico dell’espressione proteica dei TLRs.

Un’ulteriore batteria di esperimenti è stata condotta ripristinando il profilo infiammatorio aggiungendo un numero fisso di cellule di microglia (per un totale del 10% di cellule contaminanti) ad una coltura purificata di astrocti. Sebbene la ri-aggiunta di microglia su un monostrato di astrocti purificati (≥99%) ripristini il profilo infiammatorio della coltura, in termini di valore assoluto la quantità di citochine prodotte e rilasciate è comunque inferiore ai valori misurati nella coltura arricchita in astrocti (in cui la contaminante microgliale è ≤5%). Per meglio chiarire se l’attivazione microgliale in
presenza di astrociti dipendesse da il contatto fisico tra le membrane cellulari oppure da fattori chimici abbiamo allestito un sistema “Transwell”.
Il paradigma descritto della co-coltura astrociti/microglia protrebbe rappresentare un utile punto di partenza per chiarire i meccanismi molecolari che sottendono le specifiche risposte delle singole popolazioni cellulari all’infiammazione, non solo del SNC, specialmente in tutti quei meccanismi che prevedono il coinvolgimento dei recettori TLRs.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>I</td>
</tr>
<tr>
<td>Riassunto</td>
<td>III</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>VII</td>
</tr>
<tr>
<td>List of abbreviation</td>
<td>XI</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. The central nervous system</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1. The role of microglia in the CNS</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1.1. Identification of microglia populations</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1.2. Physiological properties of microglia</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2. The role of astrocytes in the CNS</td>
<td>7</td>
</tr>
<tr>
<td>1.1.2.1. Identification of astrocytic populations</td>
<td>8</td>
</tr>
<tr>
<td>1.1.2.2. Physiological properties of astrocytes</td>
<td>8</td>
</tr>
<tr>
<td>1.1.3. Microglia and astrocyte interplay</td>
<td>10</td>
</tr>
<tr>
<td>1.1.4. Inflammation</td>
<td>12</td>
</tr>
<tr>
<td>1.2. Toll-like receptors</td>
<td>13</td>
</tr>
<tr>
<td>1.2.1. TLR function, localization and signaling</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2. TLR expression in microglia and astrocytes</td>
<td>22</td>
</tr>
<tr>
<td>1.2.2.1. TLR2</td>
<td>22</td>
</tr>
<tr>
<td>1.2.2.2. TLR3</td>
<td>23</td>
</tr>
<tr>
<td>1.2.2.3. TLR4</td>
<td>23</td>
</tr>
<tr>
<td>1.2.2.4. Other TLRs</td>
<td>24</td>
</tr>
</tbody>
</table>
1.2.3. TLRs in CNS health and disease 26
  1.2.3.1. Alzheimer disease 28
  1.2.3.2. Multiple sclerosis 28
  1.2.3.3. Spinal cord injury 30
  1.2.3.4. Neurogenesis, learning and memory 31

2. THESIS AIMS 33

3. MATERIALS AND METHODS 35
  3.1. Primary culture of mixed glial cells 35
    3.1.1. Primary culture of purified microglial cells 36
    3.1.2. Isolation of a purified population of astrocytes 36
    3.1.3. Treatment with TLR agonists 37
  3.2. Immunophenotype analysis 38
    3.2.1. Flow Cytometry 38
    3.2.2. Immunofluorescence 39
  3.3. Gene expression analysis and protein release 40
    3.3.1. Total RNA extraction 41
    3.3.2. RNA spectrophotometric quantification 43
    3.3.3. First-Strand cDNA Synthesis 44
    3.3.4. Real Time-Polymerase Chain Reaction (qRT-PCR) 44
      3.3.4.1. Primer design 44
      3.3.4.2. qRT-PCR analysis 45
      3.3.4.3. Statistical analysis 46
    3.3.5. Protein expression analysis 47
      3.3.5.1. Total protein extraction 47
      3.3.5.2. Western blot analysis 48
3.3.6. Cytokine enzyme-linked immunosorbent assay (ELISA)

4. RESULTS

4.1. Cellular and biochemical characterization of glia cell population from cortex
   4.1.1. Immunophenotypic characterization
   4.1.2. Morphological and molecular characterization of astrocyte-enriched and purified cultures

4.2. TLR agonists-dependent pro-inflammatory profile in purified microglia
   4.2.1. Cytokine gene expression modulation after LPS, zymosan and poly(I:C) treatment
   4.2.2. IL-1β release after LPS, zymosan and poly(I:C) treatment
   4.2.3. TLRs gene expression and protein modulation
      4.2.3.1. TLR modulation in microglia challenged with LPS
      4.2.3.2. TLR modulation in microglia challenged with zymosan
      4.2.3.3. TLR modulation in microglia challenged with poly(I:C)

4.3. TLR agonists-dependent cytokine expression in astrocytes: influence of microglia

4.4. LPS binding to TLR4

4.5. LPS, zymosan and poly(I:C) modulate TLR expression in purified astrocytes
   4.5.1. TLR modulation in astrocytes challenged with LPS
4.5.2. TLR modulation in astrocytes challenged with zymosan 81
4.5.3. TLR modulation in astrocytes challenged with poly(I:C) 84
4.6. Reintroduction of microglia restores purified astrocytes responsiveness to TLR agonists 87
4.7. Microglia-astrocyte communication: evaluation of possible mechanism 90

5. DISCUSSION 93
6. REFERENCES 99
7. Publications 117
List of abbreviations

AD: Alzheimer's disease
AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ATP: Adenosine triphosphate
cAMP: Cyclic adenosine monophosphate
CD: Cluster of differentiation
CNS: Central nervous system
CR3: Complement receptor type 3
DAMP: Damage-associated molecule pattern
FSC: Forward scatter
GABA: gamma-aminobutyric acid
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GFAP: Glial fibrillary acid protein
Iba1: Ionized calcium binding adaptor molecule 1
IFN-γ: Interferon-gamma
IL-1β: Interleukine-1 beta
IL-6: Interleukine-6
iNOS: Inducible Nitric oxide synthase
KO: Knock-out
L-LME: L-leucyl-L-leucine methyl ester
LPS: Lipopolysaccharide
LTA: Lipotheicoic acid
MS: Multiple sclerosis
NF-Kb: Nuclear Factor kB
NO: Nitric oxide
P2X: Purinergic receptor type X
P2Y: Purinergic receptor type Y
PAMP: Pathogen-associated molecular pattern
PGN: Peptidoglycan
PRR: Pattern recognition receptor
ROS: Reactive oxygen species
SCI: Spinal cord injury
SSC: Side scatter
TLR: Toll-like receptor
TNF-a: Tumor necrosis factor-alpha
1. INTRODUCTION

1.1. The central nervous system

The central nervous system (CNS) integrates information it receives from, and coordinates and influences the activity of all parts of the body. This phenomenon, named homeostasis, is fundamental for the organism’s survival. The CNS in its most fundamental state is composed of neurons and glia. Neurons use their electrical properties to transmit information by means of electrical and chemical signals. They are organized in circuits and are usually considered the most important cellular component of the brain.

Glia lack electrical properties and have always been considered as supporting cells for neurons. We now know that glial cells are not only heterogeneous, but also play critical roles in supporting the health of neurons as well as possessing signaling properties in their own right. CNS and peripheral nervous system glia can be distinguished on the basis of morphology, function and location. For example in mammals, glia are classified as astrocytes, oligodendrocytes (and the related Schwann cells) and microglia.

The presence of glial cells is conserved across the animal kingdom, from man to also the simplest invertebrate, no doubt a reflection of their fundamental importance to the organism. There is a correlation between animal size and the percentage of glial cells in brain. For example, in Drosophila 25% of the brain is made up of glia; this proportion rises to 65% in mouse and about 90% in man (Allen and Barres, 2009).
1.1.1. The role of microglia in the CNS

The CNS has been considered as immune-privileged because of the presence of resident macrophagic cells, the microglia. The latter can be easily distinguished from other glial cells by origin, morphology, gene expression pattern and function (Sajo and Glas, 2011). Microglia are considered to derive from cells of monocytic lineage and invade the brain early in development (Farber and Kettenmann, 2005). Of the total CNS glial cell content, some 5-20% comprises microglia, depending on the specific brain region (Sajo and Glas, 2011; Lawson et al., 1990).

In the healthy brain, microglia are characterized by ramified branches that emerge from the cell body and communicate with surrounding neurons and other glial cells. This morphology represents the “resting” state. Upon a change in the brain environment, microglia quickly adopt an “ameboid” activated phenotype and migrate to the site of injury, proliferate and release pro-inflammatory mediators including cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide (NO). When prolonged, such cellular activation may contribute to neurodegeneration and neoplastic disease progression (Fig. 1.1). Microglia are considered also as central players of CNS immune responses (Sajo and Glas, 2011; Farber and Kettenmann, 2005).
1.1.1.1. Identification of microglia populations

Since microglia and macrophages exhibit the same lineage origin, they share many antigenic markers. For this reason the lack of unique microglial antigens has hindered until now their identification. Immunohistochemistry

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**Figure 1.1 Microglial activity states throughout the activation process.** Microglia in the surveillance state (traditionally termed 'resting' state)—constantly survey their environment for signals that would indicate a potential threat to CNS homeostasis. The appearance of such ‘activating’ signals (infection, trauma or cell impairment) or loss of constitutive ‘calming’ signals triggers a transition to an alerted state. Cells hence further commit to distinct reactive phenotypes, constituted by transcriptional profiles and non-transcriptional changes, and enter their executive phase (for example, release of cytokines and chemokines, phagocytic activity).

Some microglia may not retransform to a completely naive status but remain in a 'post-activated' state. These cells could retain subtle changes, for example, in transcriptional activity that affect their sensitivity to constitutive (calming) signals or alter responses to subsequent stimulation. Post-activated microglia could thus have acquired some experience (indicated as memory in the figure by a floppy disk icon) (Hanash et al., 2007).
or immunofluorescence techniques can be applied to brain slices to detect in vitro microglial cells. The downside of these procedures, however, is that they fail to distinguish microglia from macrophages. Antibodies raised against complement receptor type 3, CR3 (Graeber et al., 1989; Kingham et al., 1999) recognize the complex CD11b/CD18 (also named OX42), expressed by rat and mouse microglia and usually used as a classical marker of microglia (Robinson et al., 1986). Alternatively, the cluster of differentiation type 68 (CD68) (also named ED-1), a lysosomal protein, can be used to identify microglia (Graeber et al., 1990; Slepko & Levi 1996; Kingham et al., 1999; Hooper et al., 2005). High levels of CD68 expression are associated with macrophages and activated microglia, while low levels of expression are associated with quiescent ramified microglia (Graeber et al., 1990; Slepko & Levi 1996; Kingham et al., 1999).

Microglial identification is often performed using flow cytometry, which enables one to determine differences as well as quantify antigen expression levels. Ramified parenchymal microglia possess the phenotype CD11b+, CD45low (Ford et al., 1995; Becher & Antel 1996; Kingham et al., 1999; Hooper et al., 2005) while other CNS and peripheral macrophages exhibit the phenotype CD11b+, CD45high. Moreover, ionized calcium binding adaptor molecule 1 (Iba1), a protein that mediates Ca²⁺ signals, can be used to selectively detect microglia.

Brain microglia reportedly exhibit antigen heterogeneity (Ito et al., 1998; Perry et al., 1985). For example, an antibody raised against OX-42 was described to react with ramified but not perivascular microglia (Graeber et al., 1988). By contrast, an anti-ED2 antibody reacted with perivascular but not ramified microglia (Raeber et al., 1989). This heterogeneity can be a reflection of different subpopulations of microglia. Ito and colleagues
demonstrated that Iba1 recognizes ramified microglia throughout the white and grey matter and perivascular microglia in the brain, suggesting that different subpopulations of microglia share a common pattern of Iba1 expression (Ito et al., 1998).

1.1.1.2. Physiological properties of microglia

Microglia cell cultures have been used to identify surface membrane receptors and channels. Classical neurotransmitters engage microglial receptors in physiologic conditions and trigger increases in $\text{Ca}^{2+}$ concentration, a transient increase in $\text{K}^+$ conductance or open an intrinsic ionic pore.

Different subtypes of glutamate receptors are expressed by microglia: these include α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate. Their activation induces the release of tumor necrosis factor-α (TNF-α) (Noda et al., 2000) but can also reduce microglia activation by lipopolysaccharide (LPS) (Taylor et al., 2002), a component of the outer membrane of Gram-negative bacteria. Further, microglial cell γ-aminobutyric acid (GABA)$_B$ receptors can control $\text{K}^+$ conductance and modulate the immune response reducing the release of interleukin-6 (IL-6) in cells stimulated with LPS (Farber and Kettenmann, 2005, Kuhn et al., 2004).

Adenosine triphosphate (ATP) is a nucleoside important not only as a key energy substrate of cells but also as a cell signaling molecule. Purinergic P2Y and P2X receptors are expressed on the cell surface (Farber and Kettenmann, 2005). In microglia ATP can trigger complex responses upon binding its target receptors. In particular, activation of P2X receptor
subtype 7 in LPS-'primed' microglia can induce the release of interleukin-1β (IL-1β (Facci et al., 2014; Ferrari et al., 1997; Honda et al., 2001).

Microglia, moreover express β₁- and β₂- adrenergic receptors. Stimulation with norepinephrine increases intracellular cyclic adenosine monophosphate concentration and subsequent mRNA level of IL-1β (Tanaka et al., 2002).

Several different in vitro models have been used to characterize microglia in pathologic conditions. The most common of these involves exposing cells to LPS, which can trigger the release of numerous neurotransmitters and molecules associated with inflammation such as chemokines, cytokines and ROS (Table 1.1) (Farber and Kettenmann, 2005).

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<th>Receptor</th>
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<tr>
<td>Purinergic</td>
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<td>GABA-B</td>
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<td>Beta-adrenergic</td>
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<td>AMPA</td>
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Table 1.1. The effect of neurotransmitter receptor stimulation on LPS-induced release of mediators (Farber and Kettenmann, 2005)

The same pro-inflammatory mediators released by activated microglia can recognize specific receptors expressed on their own plasma membrane and thus act in an autocrine fashion (Farber and Kettenmann, 2005).
1.1.2. The role of astrocytes in the CNS

Rudolf Virchow was the first to introduce the term “neuroglia”. He defined these cells as small and round-shaped, which filled-up the extracellular space and were part of the connective tissue. While the term neuroglia is still used, our knowledge of these cells has dramatically changed (Wang and Bordey, 2008). In mammals, astrocytes are generated during gliogenesis that begins late in embryonic development and continues during the neonatal and postnatal period. Since different astrocyte lineages can be distinguished, it is possible to postulate that not all originate in the same manner (Wang and Bordey, 2008).

Astrocytes are found throughout the entire CNS in a contiguous and non-overlapping manner. There are no CNS regions devoid of astrocytes or closely related cells (Sofroniew and Vinters, 2009). Because of their morphology and anatomical location, astrocytes can be divided into two main subtypes, called protoplasmic and fibrous. The former are located in grey matter, while the latter are found throughout the white matter (Sofroniew and Vinters, 2009).

Both cell subtypes make contacts with blood vessels. In particular, synapses are usually enveloped by the processes of protoplasmic astrocytes, while nodes of Ranvier are tightly wrapped by the processes of fibrous astrocytes (Sofroniew and Vinters, 2009). It has been estimated that in hippocampus and cerebral cortex several hundred dendrites from multiple neurons are contacted by branching processes of a single astrocyte (Sofroniew and Vinters, 2009; Bushong et al., 2002; Halassa et al., 2007; Ogata et al., 2002).
1.1.2.1. Identification of astrocytic populations
Glial cells can be characterized using specific molecular markers. Astrocytes are commonly identified by the presence of intermediate filaments (glial fibrils). The major component of glial fibrils, glial fibrillary acid protein (GFAP) has become a typical marker in immunohistochemical techniques. However, this astrocytic marker has limitations. GFAP can be clearly detected only in reactive astrocytes during pathological events. It cannot be considered as an absolute marker because of its low detection level in all non-reactive astrocytes in healthy brain. To overcome this impasse, double-staining with multiple markers is possible (Sofroniew and Vinters, 2009). Large-scale genetic analysis of the astrocyte transcriptome allowed one to identify a large number of molecules typical of these cells compared to neurons and oligodendrocytes (Sofroniew and Vinters, 2009; Chahoy et al., 2008).
An alternative way to characterize astrocytes is by the use of flow cytometry, whose high sensitivity permits the detection of low protein expression. The immunophenotype of astrocytic cells can be well-characterized based on different expression levels in GFAP-positive cells related to specific parameters of dimension and cell surface complexity (forward and side scatter, FCS and SSC).

1.1.2.2. Physiological properties of astrocytes
Astroglia were at one time considered to be in a certain sense the brain's 'glue', providing structural support for neurons. We now know that the story is far more complex, whereby glial cells are active players in CNS well-being. Astrocytes outnumber other cells in CNS.
The different subtypes of astrocytes share a common set of biophysical characteristics. These macroglia all express potassium ($K^+$) and sodium ($Na^{2+}$) channels, and are able to evoke inward currents without generating action potentials - unlike neurons. For this reason astrocytes are referred to as “passive” cells, but not necessarily “silent” or unresponsive (Sofroniew and Vinters, 2009; Wang et al., 2008). An intriguing property of astrocytes is their ability to generate intracellular calcium ($Ca^{2+}$) waves that can be considered as a form of astrocyte excitability. Cell behaviors will be determined necessarily by intracellular ion species and concentration. Ionic movements can be triggered by activity-dependent transmitter release from neurons. Likewise, it can elicit the release of gliotransmitters from astrocytes, thereby altering neuronal excitability (Sofroniew and Vinters, 2009; Halassa et al., 2007; Perea et al., 2009; Shigetomi et al., 2008).

Neighboring astrocytes can communicate with each other by means of ion waves traversing gap junctions. Gap junction coupling of astrocytes into multicellular networks participates both in normal function and CNS disorders (Sofroniew and Vinters, 2009; Nedergaard et al., 2003; Shigetomi et al., 2008; Volterra et al., 2005). The above phenomenon is now believed to allow astrocytes to play a direct role in synaptic transmission.

Astrocytes express also a repertoire of cell surface metabotropic and ionotropic receptors. Furthermore, they express receptors for growth factors, chemokines, steroids and receptors involved in innate immunity (Wang et al., 2008). It is important to keep in mind that astrocytes are also a critical component of the blood-brain barrier, which can become compromised in various neuropathologies - especially when inflammation is involved (Fig. 1.2).
1.1.3. Microglia and astrocyte interplay
Microglia are the principal resident immune cell type in the CNS ('brain macrophages') and as such represent the first line of defense, constantly surveilling their environment to detect pathogens and injury. Activation takes place immediately after the detection of exogenous substances, such as LPS, or pro-inflammatory mediators, such as IL-1β (Davalos et al., 2005). Microglia undergo rapid proliferation in order to increase their number for the upcoming "battle". Indeed immunostaining at this stage shows an upregulation of OX42 (Kim and de Vellis, 2005). Their migration to the injured area is accompanied by promotion of cell proliferation through the secretion of pro-inflammatory factors. All these events allow microglia to destroy the invading exogenous agent, remove potential harmful debris, and secrete growth factors promoting tissue repair to return to homeostasis (Fig. 1.2) (Kreutzberg, 1996).

![Figure 1.2. Activity states of microglia.](image)

*Left panel:* Microglia in normal tissue constantly screen their environment (1). Equipped with receptors for a plethora of molecules, they can immediately sense signs of disturbed structural and functional integrity. Neurons may also deliver signals which keep microglia in this surveillance mode, indicating normal function (2). Besides parenchymal microglia, perivascular macrophages are in closer association with blood vessels (3). Subsets of circulating monocytes may replenish perivascular cells. *Center panel:* (4). The response is probably supported by neighboring astrocytes releasing, for example, purinoreceptor ligands (5). Microglia can produce neurotrophic factors to support endangered neurons (6). Disruption of ongoing communication through calming signals would allow an endangered neuron to call for microglial assistance (7). Such neurons can also emit signals indicating disturbed functions using molecules that are not usually released (at all or at critical
concentrations; (8)). Microglial cells may be able to limit further damage and restore normal homeostasis. Right panel: stronger insults to the CNS (infectious challenge or significant tissue injury) may trigger more drastic changes in the functional phenotype of microglia. Excessive acute, sustained (chronic) or maladaptive responses of microglia may lead to substantial impairment of neurons and glia (9). Failure of protection and an active contribution to damaging cascades have been attributed to activated glial cells in many pathologic scenarios in the CNS (Hanish et al., 2007).

Activation of microglia and astrocytes arises at different stages. For instance in neuropathic pain, microglia are activated earlier than are astrocytes. The latter respond more slowly to spinal nerve injury (Colburn et al., 1997). In particular, pro-inflammatory cytokines released by activated microglia can facilitate the activation of astrocytes. This process may rely mainly on IL-1β as mediator because of its fast release and ability to induce other inflammatory mediators (John et al., 2005). Once activated, the levels of intracellular Ca^{2+} in astrocytes would increase and spread to other, neighboring astrocytes (Fig. 1.3) (Liu et al., 2011). Further, Ca^{2+} waves generated in activated astrocytes can result in rapid motility and morphological changes (Scemes, 2000) which can propagate to neighboring microglia (Schipke et al., 2002).

![Figure 1.3. Schematic representation of Ca^{2+} waves.](image)

ATP mediates the propagation of elevated intracellular calcium. It can contribute to astrocyte-astrocyte communication and distant microglia activation (Liu et al., 2011)
Blocking purinergic receptors with antagonists, such as suramin, can fully prevent this propagation (Guan et al., 1997) – pointing to ATP as the principal mediator (Cotrina et al., 1998). Purinergic receptors are highly expressed on microglia, thus placing ATP in a position to mediate astrocyte-to-microglia communication (Honda et al., 2001; Noremberg et al., 1997; Shigemoto-Mogami et al., 2001; Suzuki et al., 2004). In addition, microglia respond to cytokines released from ATP-stimulated cells (Bianco et al., 2005; Hide et al., 2000). Astrocytes propagating long-distance Ca\(^{2+}\) waves can contribute to microglia activation at a distance (Nedergaard and Dirnagl, 2005, Liu et al., 2011).

1.1.4. Inflammation

Inflammation and neurological diseases are intimately connected. Although fundamentally a protective cellular response aimed at removing injurious stimuli and initiating the healing process, when prolonged, inflammation overrides the bounds of physiological control and eventually becomes destructive. The degree and extend of inflammation is a function of the interplay between the initiating insult (pathogen and/or tissue trauma) and the immune system (Carson et al., 2006; Lo et al., 1999; Medzhitov et al., 1998). Inflammation increasingly surfaces as a key element in the pathobiology of chronic pain and neuropathic pain, neurodegenerative diseases, stroke, spinal cord injury, and perhaps even neuropsychiatric disorders (Carson et al., 2006; Melchior et al., 2006; Herbert et al., 2005; others). A plethora of pro-inflammatory cytokines, eicosanoids, and other immune neurotoxins, have been found in cerebrospinal fluid and/or affected brain regions of patients with neurodegenerative disorders.
(Nagatsu et al., 2000). Glia provide a link also between neuroinflammation and neuropathic pain (Thacker et al., 2007): microglia, in particular, show increased activity in multiple pain processing pathways in response to peripheral injury (Gao and Ji, 2010).

A key advance in the field of pain research has been the insight that not only neurons, but also immune-derived non-neuronal cells, such as microglia, as well as other non-neuronal cells (astrocytes, mast cells), are involved in pain signaling. The participation of these non-neuronal cells allows for the transmission of pain messages from peripheral nociceptors to the spinal and supraspinal levels. Further, these cell types are able to communicate with each other – as noted above. This has important implications for pain treatment, as pain pharmacology has traditionally targeted neurons while ignoring these non-neuronal elements and their interactions. This, no doubt, accounts for the fact that current analgesics predominately modulate pain transduction and transmission in neurons and have limited success in controlling disease progression. Understanding how glia respond to pathogens will be important in the design of more efficacious anti-inflammatory therapeutics.

1.2. Toll-like receptors

The immune system preserves homeostasis and protects the organism against disease. To function properly, an immune system must detect a wide variety of external agents, known as pathogens, and distinguish between self and non-self. In many species, the immune system can be subdivided in two branches: the innate and the adaptive immune systems. The former is cell-mediated and represents the first line of defense, while the latter is
defined as humoral and is responsible for the production of antibodies and formation of long-lived memory cells (Kelian, 2005).

Microglia, together with macrophages, dendritic cells, natural killer cells and neutrophils are components of the innate immune system and express a predetermined subset of germline-encoded receptors, called pattern recognition receptors (PRRs). PRRs are a large family of receptors classified as membrane-bound, cytoplasmic or secreted. Toll-like receptors (TLRs) are cell surface PRRs and are considered crucial environmental-sensing molecular motifs termed pathogen-associated molecular patterns (PAMPs) and endogenous molecules termed damage-associated molecular patterns (DAMPs) (Hanke and Kelian, 2011).

PAMPs are a heterogeneous group of molecules originating from pathogens that range from lipids to lipopeptides, proteins, and nucleic acids (Table 1.2) (Kawai and Akira, 2006).

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbial Components</strong></td>
</tr>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>Lipopeptides</td>
</tr>
<tr>
<td>Lipopeptides</td>
</tr>
<tr>
<td>LTA</td>
</tr>
<tr>
<td>PGN</td>
</tr>
<tr>
<td>Proteins</td>
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<tr>
<td>Lipoprotein</td>
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<tr>
<td>Flagellin</td>
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<tr>
<td>CpG-DNA</td>
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<tr>
<td>ND</td>
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<tr>
<td>Fungus</td>
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<tr>
<td>Zymosan</td>
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<tr>
<td>Phospholipomannan</td>
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<tr>
<td>Mannan</td>
</tr>
<tr>
<td>Glucuronicylmannan</td>
</tr>
<tr>
<td>Parasites</td>
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<tr>
<td>IL-1R-related</td>
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<tr>
<td>Glycoinositolphospholipids</td>
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<tr>
<td>Nematodes</td>
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<tr>
<td>Protein-internal molecule</td>
</tr>
<tr>
<td>Viruses</td>
</tr>
<tr>
<td>DNA</td>
</tr>
<tr>
<td>dsDNA</td>
</tr>
<tr>
<td>ssRNA</td>
</tr>
<tr>
<td>Envelope proteins</td>
</tr>
<tr>
<td>Hemagglutinin protein</td>
</tr>
<tr>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1.2. Pathogen-associated molecular patterns and TLRs. PAMPs originate from bacteria, fungi, parasites and viruses (Akira et al., 2006).
DAMPs activate the innate immune system through TLRs (Table 1.3) (Karico et al., 2004; Tsan and Gao, 2004), and can initiate and perpetuate immune response in the non-infectious inflammatory response, in contrast to PAMPs. The former can simultaneously control homeostasis and/or disease progression. Until now their regulatory mechanism has remained unclear (Kelian, 2008).

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Endogenous ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>HSP60, 70, Gp96</td>
</tr>
<tr>
<td></td>
<td>HMGBl</td>
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<tr>
<td></td>
<td>(β-defensin1)</td>
</tr>
<tr>
<td></td>
<td>Surfactant protein A, D</td>
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<tr>
<td></td>
<td>Eosinophil-derived neurotoxin</td>
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<tr>
<td></td>
<td>Gangliosides</td>
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<tr>
<td></td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid fragment</td>
</tr>
<tr>
<td>TLR3</td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>HSP60, 70, 22, Gp96</td>
</tr>
<tr>
<td></td>
<td>HMGBl</td>
</tr>
<tr>
<td></td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>Fibronectin extra domain A</td>
</tr>
<tr>
<td></td>
<td>Tenascin-C</td>
</tr>
<tr>
<td></td>
<td>Surfactant protein A, D</td>
</tr>
<tr>
<td></td>
<td>β-defensin2</td>
</tr>
<tr>
<td></td>
<td>S100A8, 9 (MRP8, 14)</td>
</tr>
<tr>
<td></td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td></td>
<td>Lactoferrin</td>
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<tr>
<td></td>
<td>Gangliosides</td>
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<tr>
<td></td>
<td>Serum amyloid A</td>
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<tr>
<td></td>
<td>Oxidised LDL</td>
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<td></td>
<td>Saturated fatty acids</td>
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<td></td>
<td>Hyaluronic acid fragment</td>
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<tr>
<td></td>
<td>Hepatic sulfite</td>
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<tr>
<td>TLR7</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Chromatin-IgG complexes</td>
</tr>
<tr>
<td></td>
<td>DNA immune complexes</td>
</tr>
</tbody>
</table>

Table 1.3. Damage-associated molecular patterns. Endogenous TLR ligands originate after cell disruption (Lee et al., 2013)

After ligand engagement TLRs undergo conformational changes that allow them to interact with five different adaptor molecules: MyD88, Mal, TRIF, TRAM and SARM. In turn, this leads to activation of a phosphorylation cascade and signal transduction, culminating in induction of the nuclear
factor kB (NF-KB), mitogen-activated protein kinase (MAPKs) and/or interferon (IFN)-regulatory factor (IRF) signalling pathways. The end result is the modulation of expression levels of immune response genes (Hanke and Kelian, 2011).

These cells participate in both innate and adaptive immune response. Indeed they allow the neuroinflammation induction secreting pro-inflammatory mediators and regulating T-cell responses (Aloisi et al., 2001; Hanish et al., 2002).

The TLR family includes 13 receptors in rodent and 11 in human (Hanke and Kelian, 2011). All CNS cell types express these receptors but at different levels. Indeed microglia, as immune-competent cells, express all currently known TLRs, whereas other neural cells (e.g. astrocytes) express a more limited TLR repertoire (Fig. 1.4) (Hanke and Kelian, 2011). The presence of TLRs in both microglia and astrocytes is necessary for the amplification of pro-inflammatory responses. Table 1.4 provides a summary of what is currently known about TLR expression/function in neurons and glia (Kelian, 2005).
Figure 1.4 Expression of TLR family members in CNS cells

Table 1.4. TLR expression in CNS cell types. Consequences of TLR stimulation might relate to those already known from immune cell populations. However, differences regarding receptor complex organization, signaling and associated downstream effects might reveal yet unknown effects (Hanke and Kelian, 2011).

1.2.1. TLR function, localization and signaling

TLRs are transmembrane receptors composed of an extracellular domain with a ligand-binding site and an intracellular domain with a signaling Toll-IL-1 receptor (TIR) site. The TLR family members show considerable sequence divergence which allows them to recognize a wide range of
chemical structures. The TIR domain, in contrast, is highly conserved between family members and recognizes the TIR domain containing adaptor molecules. The localization and trafficking of TLRs within the cell is an important mechanism to sense signals from the external environment. At the same time, ligand binding represents a negative feedback to avoid excessive activation of TLR signaling (Fig. 1.5). Trafficking of extracellular TLR2/4 and intracellular TLR3/7/9 have been extensively investigated (McGettrick and O'Neill, 2010).

**Figure 1.5. TLR Trafficking and signaling**
PAMP engagement induces conformational changes of TLRs that allow homo- or heterophilic interactions of TLRs and recruitment of adaptor. TLR5 uses MyD88 and activates NF-kB resulting in induction of inflammatory cytokines. TLR2 is also expressed within the endosome and can induce type I IFN via IRF3 and IRF7 in response to viruses. TLR4, which is expressed on the cell surface, initially transmits signals through NF-kB and then is transported into phagosomes, where it activates IRF3 signaling to induce type I IFN.
TLR3, TLR7, and TLR9 are localized mainly to the endoplasmic reticulum in the steady-state and traffic to the endosomal compartment, where they engage their ligands. TLR3 activates the TRIF-dependent pathway to induce type I IFN and inflammatory cytokines. TLR7 and TLR9 activate NF-κB and IRF7, respectively (Kawai and Akira, 2011).

Initial studies using cell lines transfected with TLR4 showed no responsiveness to LPS. It was later determined that an additional molecule, named MD2, was needed for LPS signaling. MD2 is physically bound to TLR4 forming a complex, and recognizes the lipid A portion of LPS, the biologically active component (Shimazu et al., 1999; Viriyakosol et al., 2000). TLR4 is currently the best-characterized TLR. Together with MD2 and CD14, a glycosylphosphatidyl inositol (GPI)-anchored receptor belonging to PRRs, these form a complex that binds LPS.

A single LPS molecule can regulate TLR4 cell surface expression at two levels: the amount of TLR4 moving from Golgi to plasma membrane; the amount of TLR4 moving from plasma membrane to endosomes. In resting human monocytes TLR4 protein can be detected both in Golgi and on the cell surface (Husebye et al., 2006; Latz et al., 2002). MD2 has an essential role in regulating TLR4 distribution and, as a consequence, in LPS responsiveness (Nagai et al., 2002; Shimazu et al., 1999). There is good evidence to confirm that a continuous cycling of TLR4 between Golgi and plasma membrane occurs after LPS engagement. This cycle leads to interaction with molecular transducers and finally cytosolic NF-κB activation (Verstrepen et al., 2008; Windheim et al., 2008). Within 15 minutes after LPS binding, TLR4 translocates from cell surface to endosome via a clathrin-dependent dynamic process (Husebye et al., 2006; Palsson-McDermott et al., 2009). It was initially thought that this receptor movement served to allow its degradation (Husebye et al., 2006), although
this does not now appear to be the case. Indeed, TLR4 internalization is essential for a functional signaling pathway. While blocking TLR4 trafficking had no effect on NF-kB signaling, phosphorylation of IRF3 was completely abolished. Upon LPS stimulation, MyD88 translocates rapidly to the plasma membrane where it co-localizes with Mal and TLR4 in lipid rafts. This interaction leads to activation of the MyD88-dependent NF-kB pathway (Botelho et al., 2000). During endocytosis Mal dissociates from TLR4, which allows the receptor to remain in contact with TRAM and activate the IRF3 pathway.

TRIF is expressed in the cytosol of resting cells. Upon LPS stimulation, it relocates within 30 minutes to plasma membrane lipid rafts and early endosomes with TLR4. It subsequently co-localizes with TRAM and CD14 forming a complex that leads to IRF3 activation (Tanimura et al., 2008; Palsson-McDermott et al., 2009; Honda et al., 2004; Wong et al., 2009). These results proved clearly that internalization of TLR4 serves not merely to down-regulate receptor signaling, but also to allow activation of the TRAM-TRIF pathway and IFN-γ synthesis (Kagen et al., 2008).

Nilsen and colleagues (2008) used resting monocytes to demonstrate TLR2 expression on the cell surface, in early endosomes, and late endosomes/lysosomes. Further studies using cell lines detected this receptor also in Golgi. The activity of TLR2 is strictly related to the co-expression of CD14. Indeed, peptidoglycan (PGN) or lipotheicoic acid are first recognized by CD14; this complex then interacts with TLR2/TLR1 and/or TLR2/TLR6 heterodimers. These data have been confirmed using TLR2 knock-out (KO) or CD14 KO mice, and suggest a common functional mechanism for these two receptors (Kelian et al., 2008). As with TLR4,
upon stimulation TLR2 accumulates in lipid rafts and in phagosomes (Triantafilou et al., 2002; Ozinsky et al., 2000; Takahashi et al., 2008; Underhill et al., 1999). Likewise, antigens bound to TLR2 are presented to the major histocompatibility complex (MHC) class II to induce proliferation of mouse C-specific human CD4+ T cell clones (Schjetne et al., 2003). TLR2 internalization is required for the production of IFNγ but not TNFα. These data support the idea that TLR2 and TLR4 have distinct subcellular locations and mediate two signaling pathways (Barbalat et al., 2009).

TLR3/7/8/9 are nucleic acid-sensing TLRs and are localized to the endoplasmic reticulum (ER), and possess retention signals located in different sites depending on the TLR (Leifer et al., 2006; Nishiya et al., 2005). It remains unclear if other extracellular TLRs have an export signal or simply lack the retention signal (McGettrick and O’Neill, 2010). Upon stimulation TLR3/7/9 move from the ER to endosomes (Johnsen et al., 2006; Latz et al., 2005). Delivery of internalized nucleic acid (DNA, single-stranded RNA, double-stranded RNA) to the endosome is pivotal to interaction with these TLRs (Kawai and Akira, 2010). Further, also LPS treatment can trigger TLR7 and TLR9 movement from ER to endosomes, even though it is not a specific ligand for these two receptors (Johnsen et al., 2006; Yi et al., 1998).

Self-derived nucleic acids do not activate innate immune responses under physiological conditions, as they are normally degraded by serum nucleases before being bound by TLRs in the endolysosomes. For this reason the intracellular localization of nucleic acid-sensing TLRs is fundamental for avoiding contact with self-nucleic acid (Barton et al., 2006).
1.2.2. TLRs expression in microglia and astrocytes

1.2.2.1. TLR2

TLR2 is constitutively expressed on microglia and its expression is up-regulated by agents such as PGN and lipotheicoic acid (Kelian et al., 2008; Laflamme et al., 2001, 2003). Given microglia heterogeneity in the CNS and the fact that subpopulations of microglia can differ in their receptor pattern, there is much interest in elucidating TLR distribution. For example, in primary cultures of cortical mouse astrocytes, cells obtained from TLR2 KO animals were protected against PGN, demonstrating the pivotal role of TLR2 in recognizing *S. aureus* during infection (Kelian et al., 2005).

TLR2 expression in primary astrocytes from mice is rather consistent across studies, although some inconsistencies have been reported using cells from other sources, e.g. human. Some reports describe the presence of TLR2 mRNA (Bsibsi et al., 2002), but others failed to detect the receptor on the cell surface (Farina et al., 2005). Using *In situ hybridization* in mouse brain stimulated with LPS or cytokines, some reports show the presence of TLR2 mRNA in microglia but not astrocytes (Rivest, 2003; Owens, 2005). These conflicting data may be due to species differences, route of administration of PAMP *in vivo*, and the extent of astrocyte purity in *in vitro* studies. Another issue to consider is the length of time during which astrocytes are co-cultured with microglia before purification. Indeed, it is possible that astrocytes are more reminiscent of the *in vivo* interactions between these cells (Kelian, 2008).
1.2.2.2. TLR3

Several studies have demonstrated the expression of TLR3 in microglia (Bsibsi et al., 2002; Olson and Miller, 2004). Treatment of these cells with a mimetic of the natural ligand, poly(I:C), induces the production of IL-1β, IFNγ and IL-6. In contrast with other TLRs, the synthetic agonist cannot induce up-regulation of the receptor (Olson and Miller, 2004). Using astrocytes purified from human white matter, Bsibsi and colleagues (2002) demonstrated receptor expression by measuring release of pro-inflammatory mediators after treatment with poly(I:C).

1.2.2.3. TLR4

As mention before, TLR4 is crucial for the recognition of LPS, a Gram-negative cell wall component. In early studies, stimulation with LPS activated both TLR4 and TLR2 (Yang et al., 1998; 1998). It was later discovered that lipoprotein impurities in the LPS preparation were responsible for TLR2 activation. Removal of these contaminants eliminated the stimulatory effect on TLR2, but not TLR4 (Hirshfeld et al., 2000; Tapping et al., 2000). Microglia express TLR4 on their cell surface (Kitamura et al., 2001; Qin et al., 2005). Microglial cell activation can occur in a TLR4-independent manner with high doses of LPS, indicating the engagement of lower-affinity receptors (Kelian, 2008; Perera et al., 1997). CD14 interacts with TLR4 to maximize LPS responsiveness (in effect, a co-receptor). Primary microglia express CD14 as demonstrated using CD14 KO mice. This receptor is essential for low-dose LPS responseiveness (Esen and Kelian, 2005).
Protracted LPS stimulation (i.e. 72 hrs) is able to induce apoptosis in microglia via IFN-γ release. This autocrine stimulation is driven by TLR4 activation (Jung et al., 2005). Under these conditions other factors are released, including NO, superoxide and other cytokines. Collectively these factors are capable of inducing cell death also in susceptible neurons and oligodendrocytes when co-cultured with microglia. These results were confirmed using primary cultures from TLR4-deficent mice (Lenhardt et al., 2004).

In contrast to microglia, TLR4 expression on astrocytes remains an open question. Several groups have demonstrated cell surface TLR4 in vitro (Farina et al., 2005) or in vivo (Laflamme and Rivest, 2001; Lehnardt et al., 2002; 2003), while others detected low, constitutive expression of TLR4 in astrocytes that increased upon cell activation (Bsbisi et al., 2002; Bowman et al., 2003). The divergent results for TLR4 expression can probably be explained in the same way as for TLR2 discrepancies already discussed. In addition, the sensitivity of TLR detection methods applied may be a factor.

**1.2.2.4. Other TLRs**

Numerous studies have been conducted to detect the presence of other TLRs. The available data are equivocal, given the lack of functional studies, for example, using KO mice.

TLR1 and TLR6 form a heterodimer with TLR2 and mediate Gram-positive recognition (Ozynsky et al., 2000; Takeuchi et al., 2002; 2003). They are both expressed on microglia (Bsbisi et al., 2002; Kelian et al., 2002; Olson and Miller, 2004) and astrocytes (Carpentier et al., 2005).

TLR5 binds flagellin, a monomer of bacterial flagella. Astrocytes and microglia express this receptor on their surface (Bowman et al., 2003;
Carpentier et al., 2005; Bsibsi et al., 2002; Olson and Miller, 2004). In particular Bowman and colleagues (2003) demonstrated that astrocytes treated with flagellin release IL-6 and up-regulate expression of TLR5, its target, but also TLR2 and TLR4. TLR7 and TLR8 share a high degree of primary sequence homology: each binds GU-rich single stranded RNA (ssRNA). Since mammalian RNA contains GU-rich sequences, these TLRs can be involved as an autoimmune trigger in patients with systemic Lupus erythematosus, who show high levels of auto-antibodies against RNA (Lau et al., 2005). While no studies have been conducted treating glia with TLR7/8 agonists, there is evidence supporting their expression in microglia and astrocytes (Bsibsi et al., 2002; Olson and Miller, 2004; Carpentier et al., 2005).

TLR9 mediates its responses when binding to bacterial DNA, viral DNA and synthetic oligodeoxynucleotides containing unmethylated CpG motifs. Several studies have reported TLR9 expression on rodent primary microglia in culture. Astrocytes are also activated by TLR9 agonists. TLR9 activation in microglia results in the production of numerous pro-inflammatory mediators and activation of inducible NO synthase. These effects have not been verified in primary human astrocytes (Kelian, 2008).

TLR10 is an orphan receptor, and is capable of forming heterodimers with TLR1 and TLR2 (Hasan et al., 2005). TLR11 is involved in uropathogenic bacterial identification, for example, E. coli (Zhang et al., 2004). Considering the limited pattern expression of TLR10 and TLR11, it seems unlikely - although this remains to be completely excluded - that either one is to be found in CNS glia (Kelian, 2008).
1.2.3. TLRs in CNS health and disease

The discovery of TLRs in mammals has greatly improved our understanding of immune system reactions to diverse pathogens. Although the classical role of TLRs has been linked to the removal of microbial agents, these PRRs in reality impact several aspects of CNS homeostasis and non-infectious diseases/damage (Hanke and Kelian, 2011) (Table 1.5).

### Table 1.5

<table>
<thead>
<tr>
<th>(a)</th>
<th>Healthy CNS</th>
<th>TLR(s)</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memory</td>
<td>3</td>
<td>TLR3-KO mice exhibit enhanced hippocampal-dependent working memory</td>
<td></td>
</tr>
<tr>
<td>Neurogenesis</td>
<td>2, 3 and 4</td>
<td>TLR-KO mice exhibit impaired (TLR2) or enhanced (TLR3 and TLR4) neurogenesis</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b)</th>
<th>CNS insult</th>
<th>TLRs</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial meningitis</td>
<td>2, 4 and 9</td>
<td>TLRs are necessary for eliciting maximal antibacterial immunity</td>
<td></td>
</tr>
<tr>
<td>Bacterial abscess</td>
<td>2 and 4</td>
<td>TLRs are necessary for eliciting maximal antibacterial immunity</td>
<td></td>
</tr>
<tr>
<td>Viral infection</td>
<td>3 and 9</td>
<td>TLRs function to restrict viral infection and neuronal injury</td>
<td></td>
</tr>
<tr>
<td>Parasitic infection</td>
<td>1, 2 and 9</td>
<td>TLRs contribute to parasite clearance, but may also exacerbate disease severity</td>
<td></td>
</tr>
<tr>
<td>Neuronal injury</td>
<td>2 and 4</td>
<td>TLR2 and TLR4 have been implicated in mediating neuronal death during stroke</td>
<td></td>
</tr>
<tr>
<td>SCI</td>
<td>2 and 4</td>
<td>TLR2 stimulation causes significant axonal and myelin damage</td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>2, 4, 5, 7 and 9</td>
<td>TLR-deficient mice demonstrate increases in diffuse Aβ and TLR deposits</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.5. TLRs role in the healthy (a) and diseased (b) brain

For instance, beyond pathogen recognition TLRs in the brain are activated in several pathogenic conditions such as following neuronal cell injury. TLR2- and TLR4-KO mice have been used to demonstrate a role for both PRRs in mediating neuronal cell death after stroke (Lenhardt et al., 2007; Ziegler et al., 2007; Hyakkoku et al., 2010). During experimental ischemia, microglia show increased TLR2 expression. The mechanism leading to neuronal cell death, however, remains unclear. It will be important to understand if TLR2 over-expression is a consequence of inflammation or
has a functional impact on disease. TLR2- and TLR4-KO mice have been utilized to evaluate parameters such as infarct size, neurological deficits and neuronal cell damage (Tang et al., 2007). The endogenous ligand(s) that trigger TLR activation after stroke have yet to be identified. Considering the extensive inflammation and neuronal cell injury, it is not unreasonable to presume that a mixture of elements released after cell death are responsible (Hanke and Kelian, 2011).

Another interesting aspect of the role of TLRs in neuropathology is that of painful neuropathy. TLR4 has a critical role in the induction phase of behavioral hypersensitivity. Using two different TLR4 mutant mouse strains, Tanga et al. (2004, 2005) showed these animals to have an attenuated mechanical allodynia and thermal hypersensitivity in comparison to wild-type mice. Moreover there was a decreased expression of activated microglial markers and a reduction in pro-inflammatory mediator release.

Making use of MyD88- and TLR2-KO mice to dissect the TLR signaling pathway, Owens and colleagues (2005) investigated the connection between glial responses and axonal degeneration in the hippocampal dentate gyrus. In this model axonal terminals located in the entorhinal cortex are transected to reproduce the pathology (Jersen et al., 1997, 1999; Finsen et al., 1999). Axotomy in MyD88-KO mice led to a reduction in both macrophages and lymphocytes infiltrating the hippocampus. In addition, microglia that normally increase at specific times post-lesion, were reduced in hippocampus of TLR2-KO mice. These results are consistent with a role for TLR2 in inflammatory response following injury. However, neither TLR4-KO nor MyD88-KO mice showed a complete inhibition of behavioral hypersensitivity or axonal inflammation. It is thus possible that additional receptors or factors elicit neuroinflammation (Owens et al., 2005).
1.2.3.1. Alzheimer disease (AD)
Aging is a physiological event often associated with a progressive increase in basal neuroinflammation state and innate immune receptor expression (Letiembre et al., 2009; Letiembre et al., 2007; Miller et al., 2007). It is not still clear why all these changes occur, although it may be an adaptive response to aging. Indeed, there is a correlation between the size of amyloid β (Aβ) plaques and the level of pro-inflammatory cytokines in the AD brain. Further, activated microglia surround and envelop the Aβ deposit, forming complexes between cell surface receptors such as CD36 and CD47, and Aβ (Bornemann et al., 2001; Bolmont et al., 2008). There is some evidence to suggest the involvement of TLRs in this complex formation. Aβ may trigger microglial TLR4-TLR6, releasing cytokines such as IL-1β, IL-10, IL-17 and TNF-α. Jin and colleagues (2008) have found a correlation between TLR4 signaling and AD progression. In another study, TLR2/TLR4-double-KO mice receiving active Aβ immunization showed a reduction in pro-inflammatory mediator release and a less severe impairment of cognitive function (Vollmar et al., 2010).
It is conceivable that TLRs have a dual role in the progression or attenuation of AD, which might be a function of the burden and biochemical composition of Aβ but also the extent of neuronal cell pathology (Hanke and Kelian, 2011). Whether or not TLRs may come forward as new potential therapeutic targets remains a speculative proposition.

1.2.3.2. Multiple sclerosis
Multiple sclerosis (MS) is a chronic neurodegenerative disease affecting brain and spinal cord and is characterized by autoimmune demyelination and progressive axonal degeneration (Hanke and Kelian, 2011). There is good
evidence to suggest that infections can provoke clinical relapses in MS patients (Sibley et al., 1985; Rapp et al., 1995; Buljevac et al., 2002). The best characterized PAMPs involved in these phenomena are PGN and pertussis toxin, which enable inappropriate immune-mediated recognition of self-antigens. As consequence antigen-presenting cells do not recognize self-myelin antigens. Molecular mechanisms are not completely clarified but probably involve TLRs on antigen-presenting cells (Segal et al., 2000; Ichikawa et al., 2002; Kerfoot et al., 2004; Waldner et al., 2004; Visser et al., 2005).

Experimental autoimmune encephalomyelitis (EAE) is the most common animal model for human MS. Pertussis toxin is necessary to induce the disease because of its ability to alter blood-brain barrier permeability to allow immune cell entry into the CNS (Racke et al., 2005). In this model the first effect is modulation of adhesion molecule (P-selectin)-dependent rolling of leukocytes to the cerebral vascular endothelium and blood-brain barrier impairment (Kerfoot et al., 2004). This cellular recruitment can be attenuated in TLR4-deficient mice. Although these data suggest TLR involvement, more experiments are needed. It may be possible that unknown environmental factors contribute to establishment of EAE in the animal model, given the observed variability in the study of Kerfoot et al., (2004).

Human TLR4 genetic variants have been taken into consideration to determine disease involvement (Reindl et al., 2003; Kroner et al., 2005). Two mutations occur with high frequency: D299G, T399I. The former alters the TLR4 extracellular domain, resulting in defective signaling (Arbour et al., 2000; Lorenz et al., 2001). In MS patients the TLR4 polymorphism does not appear to influence either incidence or progression.
of the pathology (Reindl et al., 2003; Kroner et al., 2005). In addition to TLR4, also TLR2 participates in the innate immune response in the EAE model. In some brain areas, levels of TLR2 expression are higher than those of TLR4 (Zekki et al., 2002). Accordingly, PGN can induce clinical disease (Visser et al., 2005).

The above studies illustrate how TLRs can provide a link between infectious disease and uncontrolled immune response (Kelian et al., 2008).

1.2.3.3. Spinal Cord injury

Spinal cord injury (SCI) frequently occurs after motor/vehicle accidents, falls, sporting/recreation accidents and violence (Beers et al., 2006; Jackson et al., 2004). Spinal cord compression can result from traumatic SCI as well as non-traumatic events, such surgical intervention, tumor invasion or degenerative bone diseases (Prasad et al., 2005; Shedid et al., 2007; Babb et al., 2006). During trauma, axon and myelin damage is delayed 24-48 hours post-injury. Strategies to attenuate neurodegeneration have been oriented to blocking secondary injury cascades such ischemia, excitotoxicity and inflammation (Dusart et al., 1994; Fehlings et al., 2001). Injecting the TLR2 agonist zymosan into the spinal cord elicits production of neurotoxic mediators in the brain (Popovich et al., 2002). Several studies have demonstrated that TLR2, but not TLR4 causes significant axonal and myelin damage, even though the latter can induce macrophage activation (Schonberg et al., 2007).

It is clear that TLRs influence SCI pathology and repair, post-traumatic inflammation, neuronal cell survival and axon regeneration. These receptors can be considered as potential targets for modulating SCI to facilitate reparative processes. Understanding how TLRs control neural and glial
progenitor cell fate will be critical in designing cell replacement therapies for SCI and other neurological disorders (Thuret et al., 2006; Bradbury et al., 2006).

1.2.3.4. Neurogenesis, learning and memory

Neural progenitor cells (NPCs) express many immune-relevant molecules necessary to interact with an inflamed CNS microenvironment (Ji et al., 2004; Ni et al., 2004; Kim et al., 2006; Keohane et al., 2010; Ben-Hur et al., 2003). Specific brain areas associated with the generation of new neurons are the sub-ventricular zone of the lateral ventricles and the sub-granular zone of the hippocampal dentate gyrus. Adult NPCs in these areas express TLR2 and TLR4 (Okun et al., 2010; Rolls et al., 2007). These PRRs control proliferation and differentiation of NPCs in opposing ways (Covacu et al., 2009): TLR2-KO mice display impaired hippocampal neurogenesis whereas TLR4-KO animals show enhanced proliferation and neuronal cell differentiation. Like with TLR4-KO, TLR3-KO mice display increased hippocampal neurogenesis, hippocampal cornus hammonis 1 and dentate gyrus volumes (Okun et al., 2010). As these experiments were conducted in the absence of infectious stimuli, it remains unclear what signals are responsible for the observed phenotypes (Hanke and Kelian, 2011). Other physiological functions such as learning and memory can be related to TLR activation. Cognitive impairment is often associated with systemic inflammation (Cunningham et al., 2009), although the precise connection between TLRs and behavioral/cognitive functions remains to be elucidated (Hanke and Kelian, 2011).
2. THESIS AIMS

In physiological conditions glia in the CNS can produce and release neuroprotective factors such as anti-oxidants and neurotrophic factors (Sofroniew et al., 2010). It is not uncommon for the healthy brain to respond to stress and insults by transiently up-regulating inflammatory processes which are kept in check by endogenous protective elements (Carson et al., 2006). Indeed, inflammation is fundamentally a protective cellular response aimed at removing injurious stimuli and initiating the healing process. When prolonged, however, inflammation overrides the bounds of physiological control and eventually becomes destructive. Upsetting this homeostatic balance, however, can result in disease or exacerbation of initiating factors that result in disease. Inflammation increasingly surfaces as a key element in the pathobiology of neurodegenerative diseases, stroke, spinal cord injury, neuropathic pain, and perhaps even neuropsychiatric disorders.

From a mechanistic point of view, TLRs expressed by immunocompetent cells like microglia are thought to be critically involved in neuroinflammation. A number of questions remain open to investigation, including TLR expression by astrocytes (which are involved also in neuroinflammation) (Hanke and Kelian, 2011), cross-talk between microglia and astrocytes in inflammation, and the potential for activation of one TLR isoform to influence expression/activity of other TLR isoforms.

This research project was organized around 3 main objectives:

1. To establish and characterize cultures of microglia and astrocytes from neonatal rat cortex, utilizing immunocytochemical, molecular biological, and fluorescence-activated cell sorting techniques. This will include the application of methodology to purify astrocyte cultures, which will allow to observe their behaviors when presented
with and inflammatory challenge in the absence of contaminating microglia.

2. To investigate how glia respond to TLR agonists through the analysis of TLR mRNA expression and their presence on cell surface (TLR2/4) or the endosome membrane (TLR3).

3. To investigate the cross-talk mechanisms between glial cells in the CNS by: analyzing inflammatory mediator expression at the gene and product level in purified microglia, astrocyte-enriched cultures, and the effect of cultured microglia with purified astrocytes; establishing a “Transwell insert” system to examine the nature of interactions between the two types of glia, measuring the production of IL1β.
3. MATERIALS AND METHODS

3.1. Primary culture of mixed glial cells

Solutions:

- Papain (Worthington (Lorne))
- DNase I bovine pancreas 4 mg/mL (Sigma)
- L-cysteine 24 mg/mL (Sigma)
- Trypsin inhibitor 100 mg/mL (Sigma)
- Bovine serum albumin 5 mg/mL (Sigma)
- Poly-L-lysine 1 mg/mL in Borate Buffer 0.15 M pH 8.4 (Sigma)
- L-leucyl-L-leucine methyl ester 50 mM (L-LME) (Sigma)

Procedures:

Mixed glial cell cultures were routinely prepared using postnatal day 1-2 rat pups of both sexes CD strain, Sprague-Dawley). Experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92), and were approved by the Institutional Animal Care and Use Committee. The cerebral hemispheres were removed and the meninges peeled off. The dissected cortical tissue was minced and incubated with a solution containing papain (140 µL), DNase I (30 µL) and L-cysteine (30 µL) in L-15 medium (+L-glutamine, L-amino acids; Invitrogen) for 1 hour in a 37°C water bath. Upon completion of the enzyme incubation step the supernatant was replaced with ovomucoid solution (30 µL of trypsin inhibitor, 30 µL of DNase I and, 30 µL of bovine serum albumin in L-15 medium) for 5 minutes at in a 37°C water bath.

Cerebral cortices were plated in 75-cm² poly-L-lysine-coated tissue culture flasks (BD Falcon) at a density of 1.5 brains per flask and grown in high-
glucose Dulbecco’s modified Eagle’s medium (DMEM) (+4.5 g/L glucose, L-glutamine, pyruvate; purchased from Invitrogen) with 2 mM glutamine, 50 units/ml penicillin/50 µg/ml streptomycin, 50 µg/ml gentamycin and 10% fetal bovine serum (FBS) (glia growth medium). Culture medium was changed after 24 hours.

3.1.1. Primary culture of purified microglial cells

Rat microglia were isolated from the mixed glial cell cultures as previously described (Rosin et al., 2004). Microglia were isolated between days 7-10 by shaking the flasks on an orbital shaker at 200 rpm for 1 hour (37°C). The culture supernatant (containing mainly microglia) was transferred to sterile petri plastic dishes (Sterilin, Bibby-Sarstedt) and incubated for 45 minutes at 37°C (5% CO₂, 95% air) to allow differential adhesion of microglia. The adherent microglial cells (>99% pure) were detached by mechanically scraping into glia growth medium and replated in this same medium, on poly-L-lysine-coated (10 µg/mL) microwell culture plates or dishes.

3.1.2. Isolation of a purified population of astrocytes

The attached cell monolayer remaining after shaking to recover microglia was used as a source of highly enriched astrocytes (>95%). To eliminate residual microglia, astrocyte monolayers were incubated 1 hour with 50 mM L-LME, a lysosomotropic agent (Hamby et al., 2006) dissolved in growth medium. Culture medium was exchanged for fresh medium, and allowed to recover for 1 day in growth medium prior to experimentation. Cultures were visually inspected to ensure microglial lysis. Care must be taken, as longer exposure times to L-LME can lead to astrocytic toxicity.
In some cases cell culture inserts were used to establish astrocyte/microglia co-cultures. Enriched astrocytes were seeded into a poly-L-lysine-coated 24-well plate (3 x 10^5 cells per well) in culture medium. Twenty-four hours later some cultures were treated with 50 mM L-LME for 60 minutes, as described above. In parallel, 24-well culture inserts were seeded with 5 x 10^4 microglia in culture medium (0.4 ml per insert), and placed in a 24-well plate (notched for inserts) in this same medium (0.8 ml/well). Transwell cell culture inserts are convenient, easy-to-use permeable support devices; the suspended design allows for undamaged co-culturing of cells in the lower compartment. The porous transwell membrane allows for communication between the chambers, and for passage of microglia-derived factors to the lower chamber containing astrocytes and vice versa. The following day, inserts were transferred to the 24-well plate of astrocytes. The distance between the astrocyte monolayer and microglia on the insert membrane is 1 mm, according to the manufacturer's description. At this time LPS (100 ng/ml final) was added to either the upper or lower chamber (0.4 ml and 0.8 ml final volume, respectively), and incubation continued for another 24 hours. The culture medium was then collected, and cells lysed, as described below. The IL-1β content of culture supernatants and lysates was determined by ELISA, as described below.

3.1.3. Treatment with TLR agonists

**Solutions:**

- LPS-EB Ultra-Pure 100 ng/mL (Invivogen)
- Zymosan 10 µg/mL (Invivogen)
- Poly(I:C) 50µg/mL (Sigma)
LPS-EB Ultra-Pure is a selective agonist for TLR4, zymosan binds TLR2 and poly(I:C) is a synthetic analogue of double stranded-RNA (dsRNA). Agonists were added in DMEM + 10% FBS. Cells were treated for 6 or 24 hours for gene expression and cytofluorimetric analysis. For western blot analysis cells were treated in DMEM without serum for 15, 30 or 60 minutes. After treatments cells were collected and processed as described in the following subsections.

3.2 Immunophenotype analysis
3.2.1 Flow Cytometry

Solutions:

- BD CytoFix/CytoPerm (BD Biosciences)
- BD CytoFix (BD Biosciences)

Procedures:
The cortical cell suspension was fixed with BD CytoFix or fixed/permeabilized with BD CytoFix/CytoPerm at 4°C for 20 minutes. Purified microglia and enriched astrocytes were stained for flow cytometric analysis using different markers. The Immunophenotypical characterization was performed using the following antibodies:

- mouse anti- GFAP (Cell Signaling)
- rabbit anti-Iba1 (Wako)
- rabbit anti-TLR2 (Santa Cruz)
- rabbit anti-rat TLR4 (Santa Cruz)
- rabbit anti-rat TLR3 (Santa Cruz)
For indirect staining, Alexa Fluor®488 anti-rabbit or anti-mouse secondary antibodies (Life Technologies) were utilized. Samples labelled with isotypic or secondary conjugated antibodies were prepared as controls. Data were acquired using a flow cytometer FACSCanto II (BD Biosciences) and then analyzed with the Substruction tool of Summit 4.3 software (DAKO-Beckman Coulter).

3.2.2 Immunofluorescence

**Solutions:**

- L-leucyl-L-leucine methyl ester 50 mM (L-LME)
- Poly-L-lysine 1 mg/mL in borate buffer 0.15 M pH 8.4 (Sigma)
- 4 % paraformaldehyde (PFA)
- Phosphate buffer saline (PBS) 10X
  - NaCl 80 g
  - KCl 1g
  - Na\textsubscript{2}HPO\textsubscript{4} 5.75 g
  - KH\textsubscript{2}PO\textsubscript{4} 1 g
- 0.05% Triton-X 100 (0.05% PBS-T)
- 10% Normal goat serum (NGS)
- 4’-6’-diamidino-2-phenylindole (DAPI) 100 ng/ml
- Fluoromount-G

**Procedures:**

Enriched astrocytes were seeded on poly-L-lysine-coated 12-mm diameter cover-glasses (Menzel-Gläser, Menzel GmbH, Germany) placed in the wells of a 12-well multiwall plate, at a density of 500,000 cells per well, using glia cell growth medium and allowed to adhere overnight. The next day the cells
were treated with 50 mM L-LME for 1 hour, and allowed to recover for 1 day in growth medium. Cells were fixed with 4% PFA (Sigma-Aldrich), at 4°C for 30 minutes. After fixation, cells were washed 3x10 minutes in PBS-1X pH 7.4. Cells were then permeabilized and blocked with 0.05% PBS-T/10% NGS for 1 hour at room temperature. Finally the cells were incubated overnight with primary antibody (Ab).

The following Abs were used:
- anti-GFAP (Sigma-Aldrich) for astrocytes
- anti-Iba 1 (Wako, Japan) for microglia.

Cells were washed with PBS-1X 3 times for 10 minutes and subsequently incubated for 1 hour at room temperature with an anti-mouse AlexaFluor 595 or anti-rabbit Alexa Fluor488 Ab (1:500, Molecular Probes, Life Technologies). Nuclei where visualized by incubating for 2 minutes with DAPI (Boehringer Mannheim, Germany). Cover glasses were mounted onto glass slides using Fluoromount-G (Southern Biotech, USA), and images were acquired on a Leica DMI4000 B microscope equipped for immunofluorescence (Leica Microsystems GmbH, Wetzlar, Germany) using a Leica DFC 480 digital camera (Leica Microsystems GmbH, Wetzlar, Germany).

3.3 Gene expression analysis and protein release

Cells were seeded in poly-L-lysine-coated 24-well plates at a density of 250,000 astrocytes per well and 25,000 microglia per well using glia cell growth medium. Cells were stimulated to produce and release pro-inflammatory mediators using one of the following TLR agonist: LPS; Zymosan; Poly(I:C)) for 6 and 24 hours. Supernatants were collected for ELISA assay and cells were lysated for total RNA extraction.
Total RNA extraction

Total RNA was isolated from cells using the ReliaPrep™ RNA Cell Miniprep System (Promega), according to the manufacturer's instructions. Purity and integrity of the isolated RNA is critical for its effective use in applications such as reverse transcription PCR (RT-PCR) and reverse transcription quantitative PCR (RT-qPCR). In recent years, RT-PCR and RT-qPCR have emerged as powerful methods to identify and quantitate specific mRNAs from small amounts of total RNA and mRNA. The ReliaPrep™ RNA Cell Miniprep System has been designed to supply the need for methods to rapidly isolate high-quality RNA, substantially free of genomic DNA contamination, from small amounts of starting material. The ReliaPrep™ RNA Cell Miniprep System provides a fast and simple technique for preparing purified and intact total RNA from cultured cells. The system also incorporates a DNase treatment step designed to substantially reduce genomic DNA contamination, which can interfere with amplification-based methodologies.

Solutions:

- **BL + TG Buffer**
  
  4 M Guanidine thiocyanate
  
  0.01 M Tris (pH 7.5)
  
  2% 1-Thioglycerol

- **Column Wash Solution**

- **DNase I incubation mix:**
  
  24 µl of Yellow Core Buffer
  
  3 µl 0.09 M MnCl
  
  3 µl of DNase I enzyme

- **Yellow Core Buffer**
  
  0.0225 M Tris (pH 7.5)
  
  1.125 M NaCl
0.0025% yellow dye (w/v)

- **RNA Wash Solution**
  
  60 mM potassium acetate,  
  10 mM Tris-HCl (pH7.5 at 25°C)  
  60% ethanol

- **Nuclease-Free Water**

- **95% ethanol, RNase-free**

- **100% isopropanol, RNase-free**

- **Phosphate buffer saline (PBS) 1X**

Lysates may be prepared directly in the culture dish by the addition of BL + TG buffer directly to the dish for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Cells lysates were collected in a sterile centrifuge tube and 100% isopropanol added (30 µL) and mixed by vortexing for 5 seconds. Lysates were then transferred to the ReliaPrep™ Minicolumn and placed into a collection tube (all provided by kit). The minicolumns with their bound nucleic acids were then centrifuged at 12,000 × g for 30 seconds at 20°C-25°C. The binding reaction occurs rapidly due to disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the column, RNA wash solution (500µl) was then added to the minicolumn and the column centrifuged at 12,000 × g for 30 seconds. To each minicolumn was applied 30 µl of freshly prepared DNase I incubation mix directly to the membrane inside the column and incubation carried out for 15 minutes at room temperature. This step allows for digestion of contaminating genomic DNA. After this incubation, column wash solution (200 µl) was added to the minicolumn to purify the bound total RNA from contaminating salts, proteins and cellular components. Samples were centrifuged at 12,000 × g for 15 seconds. RNA wash solution (500 µl) was added to the minicolumn and centrifuged at 20°C-25°C.
12,000 × g for 30 seconds. This wash and centrifugation step was repeated again, but with 300 µL of column wash solution and a 2-minute spin at 12,000 × g. The minicolumn was transferred to the elution tube, and nuclease-free water was added to the membrane (30 µL). Final elution was performed by centrifugation at 14,000 × g for 1 minute. The elution tube containing the purified RNA was stored at −80°C until use.

3.3.2. RNA spectrophotometric quantification

Total RNA yield and purity was determined measuring 1 µL samples with the NanoDrop2000 spectrophotometer (Thermo Scinetific) at a wavelength of 260 nm.

Total RNA isolated with the ReliaPrep™ RNA Cell Miniprep System was substantially free of DNA and contaminating protein. The purity of the sample was evaluated by the determination of its optical density at 260 and 230 nm, corresponding to the absorption wavelength of contaminants. The absorbance of RNA samples at 260 nm and 280 nm, diluted in nuclease-free water, was used to evaluate protein contamination (A260/A280 ratio). Pure RNA exhibits an A260/A280 ratio of 2.0.

However, it should be noted that, due to variations between individual starting materials and in performing the procedure, the expected range of A260/A280 ratios for RNA may be 1.7–2.1. Using this protocol, the RNA usually exhibited an A260/A230 ratio of 1.8–2.2. A low A260/230 may indicate guanidine contamination that can interfere with downstream processing.
3.3.3. First-Strand cDNA Synthesis

Retrotranscription (RT) reaction mixture was prepared in a final volume of 10 µl.

<table>
<thead>
<tr>
<th>Mix components</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>random primers</td>
<td>250 ng</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>5 mM</td>
</tr>
<tr>
<td>Total RNA</td>
<td>5 µg</td>
</tr>
</tbody>
</table>

The mixture was heated to 65°C for 5 minutes and incubated on ice for at least 1 minute for primer annealing. The reaction was performed adding in the tubes:

<table>
<thead>
<tr>
<th>First-Strand Buffer 5X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiothreitol (DTT) 0.1 M</td>
</tr>
<tr>
<td>RNase OUT Recombinant Ribonuclease Inhibitor 40 U/mL</td>
</tr>
<tr>
<td>SuperScript™ III Reverse Transcriptase (200 U/mL)</td>
</tr>
</tbody>
</table>

RT reaction was performed at 50°C for 70 minutes and inactivated by heating at 75°C for 15 minutes. The cDNA was stored at -20°C until use.

3.3.4. Real Time-Polymerase Chain Reaction (qRT-PCR)

3.3.4.1. Primer design

One of the most important steps in selective amplification of a cDNA target is primer design. Nowadays many tools are available on-line. For our
experiments, primers were designed on-line on www.pubmed.com using “Primer-BLAST”. Forward primer (For) binds the Leader strand of double-stranded DNA while reverse primer (Rev) binds the Lagging strand of double-stranded DNA.

The following primer pairs were used:

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAPDH For</td>
<td>5’-CAAGGTCATCCATGACAACCTTGT-3’</td>
</tr>
<tr>
<td></td>
<td>GAPDH Rev</td>
<td>5’-GGGCCATCCACAGTCTTCTG-3’</td>
</tr>
<tr>
<td>IL-1ß</td>
<td>IL-1ß For</td>
<td>5’-TGTGGCAGCTACCTATGTCT-3’</td>
</tr>
<tr>
<td></td>
<td>IL-1ß Rev</td>
<td>5’-GGGAACATCACACACTAGCA-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNF-α For</td>
<td>5’-CATCTTCTCAAAACTCGAGTGACAA-3’</td>
</tr>
<tr>
<td></td>
<td>TNF-α Rev</td>
<td>5’-TGGGAGTAGATAAGGTACAGCCC-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6 For</td>
<td>5’-TCACAGAAGGAGTGCTAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>IL-6 Rev</td>
<td>5’-GCTTAGGCATAGCACACTAGG-3’</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR2 For</td>
<td>5’-TCCATGTCTGGTTGACTGG-3’</td>
</tr>
<tr>
<td></td>
<td>TLR2 Rev</td>
<td>5’-AGGAGAAGGGGCACACGAGAC-3’</td>
</tr>
<tr>
<td>TLR4</td>
<td>TLR4 For</td>
<td>5’-GATTGCTCAGACATGGCAGTTC-3’</td>
</tr>
<tr>
<td></td>
<td>TLR4 Rev</td>
<td>5’-CACCTGCAGGTTAGGTGTTTCTGCTAA-3’</td>
</tr>
<tr>
<td>TLR3</td>
<td>TLR3 For</td>
<td>5’-TGAAACTACGGCGATGCAG-3’</td>
</tr>
<tr>
<td></td>
<td>TLR3 Rev</td>
<td>5’-AGGCGATTTCCTTCCCCGA-3’</td>
</tr>
</tbody>
</table>

3.3.4.2. qRT-PCR analysis
Total RNA was extracted from cells using the ReliaPrep™ RNA Cell Miniprep System (Promega), according to the manufacturer’s instructions. RT was performed with Superscript III reverse transcriptase (Life Technologies). The qRT-PCR is a real time PCR reaction, performed in a MX 3000P, Stratagene.
The PCR cycling conditions were 4 minutes of denaturation followed by 50 cycles of:

<table>
<thead>
<tr>
<th></th>
<th>Temp (°)</th>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>94</td>
<td>30</td>
</tr>
<tr>
<td>Denaturation</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>30</td>
</tr>
</tbody>
</table>

At the end of the amplification there was a dissociation thermal profile of 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds. Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. Dissociation curves were generated for each primer pair showing single product amplification.

### 3.3.4.3. Statistical analysis

Data are given as mean ± SEM. Statistical analyses to determine group differences were performed either by two-sample equal variance Student’s t-test, or by one-way analysis of variance, followed by Dunnett’s or
Bonferroni’s post-hoc tests for comparisons involving more than two data groups. Significance was taken at $p<0.05$.

3.3.5 Protein expression analysis

3.3.5.1. Total protein extraction

Solutions:

- NP40 Lysis Buffer (Invitrogen)
  - 50 mM Tris, pH 7.4
  - 250 mM NaCl
  - 5 mM EDTA
  - 50 mM NaF
  - 1 mM Na$_3$VO$_4$
  - 1% Nonidet P40 (NP40)
  - 0.02% NaN$_3$

- Proteinase inhibitor cocktail (Sigma)

- Pefablock 0.1 M (Roche)

- Working solution
  - NP40 lysis buffer
  - Proteinase inhibitor cocktail 1:10
  - Pefablock 0.1 M 1:100

Cell lysis with mild detergent is commonly used for cultured animal cells. Working solution was added for 30 minutes to adherent cells (on ice) and then the extract was transferred to a centrifuge tube. Lysates were centrifuged at 12,000 x g for 10 minutes at 4°C. After centrifugation, the supernatant was collected and stored at -80°C until use for protein determination and western blot analysis.
3.3.5.2. Western blot analysis

Protein quantification was conducted using the BCA Protein Assay Reagent Kit (Pierce) according to the manufacturer’s protocol. Protein samples (10 µg) were separated on a Mini-PROTEAN ® Precoat Gel (Biorad) with a 4-15% gradient for 90 minutes at 140V. Proteins were electrophoretically transferred from the gel onto polyvinylidene difluoride (PVDF, Millipore) membranes overnight at 4°C at 25V. Membranes were blocked with 3% bovine serum albumin (Sigma Aldrich) and then incubated overnight at 4°C with one of the following primary antibodies:

- GAPDH (working dilution 1:200; Santa Cruz)
- β-Actin (working dilution 1:25000; Sigma)
- TLR4 (working dilution 1:300; Abcam)
- CD14 (working dilution 1:200; Santa Cruz)
- MD2 (working dilution 1:1000; Abcam)

The membranes were washed and then incubated for 1 hour with the appropriate secondary antibody (goat anti-rabbit or goat anti-mouse, BioRad) conjugated to horseradish peroxidase (HRP) at a dilution of 1:4000. Developing has been performed using an enhanced chemiluminescence substrate (Sigma). Immunoreactivity was visualized using the VersaDoc Imaging System and protein expression normalized to GAPDH or β-actin for band density quantification.

3.3.6 Cytokine enzyme-linked immunosorbent assay (ELISA)

Solutions:

- Coating antibody working solution

Coating antibody stock solution (1:100)
Coating buffer

- Detection antibody working solution
  - Detection antibody stock (1:200)
  - Assay diluent

- Avidin-HRP working solution
  - Avidin-HRP stock solution (1:2000)
  - Assay diluent

- Color development solution
  - Color development Reagent A:B (1:2)

- Stop solution
  - H$_2$SO$_4$ (2 M)

- PBS-T
  - PBS
  - Tween-20 (1:2000)

Procedure:

Cells were seeded in poly-L-lysine-coated 24-well plates at a density of 250,000 astrocytes per well and 25,000 microglia per well using glia cell growth medium. Cells were stimulated to produce and release pro-inflammatory mediators in medium containing TLR agonist (LPS; Zymosan; Poly(I:C)). Cell supernatants were harvested after 24 hours and cytokine release was assayed by ELISA according to the manufacturer's instructions (Antigenix America, Huntington Station, NY, USA).

Multi-well plates were pre-coated with coating antibody working solution. Supernatants were incubated in these pre-coated multi-well plates at room
temperature for 2 hours and then washed using PBS-T. Detection antibody working solution was added to the plates and incubation continued for another 2 hours. After washing, Avidin-HRP working solution was added, the plates were incubated at room temperature for 30 minutes, followed by addition of color development solution. After a further 30-minute incubation the reaction was stopped by adding 2N H₂SO₄ and absorbance measured at a wavelength of 540 nm. The amount of cytokine was quantified using a standard curve.
4. RESULTS

PART 1

4.1 Cellular and molecular characterization of glial cell populations from cortex

4.1.1. Immuno-phenotypic characterization

Glial cell preparations were subjected to flow cytometric analysis to determine expression of cell type-specific (astrocytes and microglia) surface markers by immunoreactivity. Cells were defined by cell count versus GFAP expression level (Fig. 4.1, first column), by granularity characteristics (side scatter, SSC) versus Iba1 expression level (Fig. 4.1, second column) and finally by size (forward scatter, FSC) versus granularity (SSC) (Fig. 4.1, third column). The basic scatter dot blot was used to exclude cell debris from the analysis and the regions containing astrocytes and microglia were identified. By gating the individual subpopulation (determined by GFAP expression, low or high), the level of Iba1 expression was examined.

In the mixed glial cell population, GFAP-negative staining corresponds to microglia. Microglia (defined as GFAP-negative cells) can be also morphologically identified as the smaller cells and with less surface complexity compared to the GFAP-positive subpopulation (Fig. 4.1, row A). The percentage of these two cell groups are about 90% GFAP⁺ and 10% GFAP⁻.

In the CNS microglia comprise 5-20% of all glial cells, depending on the specific brain region (Sajo and Glas, 2011; Lawson et al., 1990). Our characterization confirms data available in the literature. This mixed glial cell population can be used as a starting point for the isolation of purified microglia and astrocyte-enriched subpopulations.
Purified microglia were obtained by detaching this population from the starting mixed glial cell preparation, as described in "Materials and Methods". Iba1 staining in back-gating analysis is shown in dot blot graphs (fig. 4.1, row B, middle and right panels). These results confirm the isolation of a population with essentially 100% of cells expressing Iba1. Astrocyte-enriched cultures were immunostained for GFAP (Fig. 4.1, row C). By gating on GFAP expression (as for the mixed cell preparation) we obtained 95% GFAP-positive cells; the remaining 5% of GFAP-negative cells correspond to microglial cell contamination.

**Fig. 4.1.** Flow cytometric analysis of glial cell cultures with cell-type specific markers. Analysis of mixed glial cell cultures shows that Iba1-positive cells (microglia-specific marker) correspond to about 10% of all the events recorded GFAP-positive cells (astrocyte-specific marker) represent the most abundant population in the sample (row A). Analysis of purified microglia shows that Iba1-positive cells comprise essentially 100% of events recorded; there is no signal for the astrocyte marker GFAP (row
B). Analysis of enriched astrocyte cultures shows that GFAP-positive cells represent 95% of all events recorded (row C).

4.1.2. Morphological and molecular characterization of astrocyte-enriched and purified cultures

The above-described astrocyte-enriched cultures (≥95%) were next subjected to analysis by indirect immunofluorescence using cell-type specific antibodies. Astrocytes were again identified by their expression of GFAP, while microglia were immunostained with Iba-1 (Fig. 4.2, upper row). In order to study astrocyte behaviors without potential interference from contaminating microglia, L-LME was used to deplete cultures of residual microglia. L-LME is a lysosomotropic agent which enters cells via receptor-mediated endocytosis. L-LME undergoes a condensation process catalyzed by dipeptidyl peptidase I, also known as cathepsin C (Thiele and Lipsky, 1990) in lysosomes. Condensation of L-LME leads to lysosomal rupture and DNA fragmentation in dipeptidyl peptidase I-expressing immune cells, like microglia. L-LME was employed initially to destroy macrophages (Thiele et al., 1983) and, more recently, to deplete microglia from neural cultures including astrocytes (Giulian et al., 1993; Guillemin et al., 1987) and oligodendrocytes (Hewett et al., 1999). Hamby et al. (2006) demonstrated that exposing confluent cortical astrocytes to 50-75 mM L-LME for 60-90 minutes effectively depleted microglia from the high-density astrocyte monolayers, as evidenced by the selective depletion of microglial-specific markers. The resulting purified astrocyte monolayers appeared morphologically normal 24 hours after L-LME treatment.

To verify the effect of L-LME treatment in our cultures, enriched cortical astrocytes were first incubated 60 minutes with 50 mM L-LME, and then processed for immunofluorescence analysis and Iba-1 gene expression by
RT-PCR. Immunostaining of these cortical astrocytes shows a confluent carpet of GFAP\(^+\) cells, interspersed with a few Iba1\(^+\) immunoreactive cells (Fig. 4.2, lower row): the resulting astrocyte purity was judged to be ≥99%.

Fig. 4.2. Cortical astrocyte-enriched cultures were characterized by immunostaining using GFAP (red) for astrocytes and Iba1 (green) for microglia (Upper panels). L-LME treatment reduces markedly Iba1-positive microglia while GFAP-positive astrocytes remain abundant (lower panels). In these images, nuclei are colored blue with DAPI, which forms fluorescent complexes with natural double-stranded DNA.

Microglia but not astrocytes are reported to express the mRNA for Iba-1. Indeed, elimination of residual microglia from the L-LME-treated enriched astrocyte cultures was confirmed by the loss of Iba-1 gene expression (Fig. 4.3). For all Real Time-PCR analyses, the amount of gene product was calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90%-100%. The term “fold-increase” is defined as the cDNA ratio between target gene and reference gene (GAPDH) normalized to untreated control.
Fig. 4.3. Astrocyte cultures were characterized by Iba1 mRNA levels. L-LME treatment in a purified-astrocyte culture (≥99%) leads Iba1 mRNA level to a 0.17-fold difference compared to control (enriched-astrocyte culture, ≥95%).

PART 2

4.2 TLR agonist-dependent pro-inflammatory profile in purified microglia

4.2.1 Cytokine gene expression modulation after LPS, zymosan and poly(I:C) treatment

Microglia are the brain's macrophages which serve specific functions in defense of the CNS against microorganisms, removal of tissue debris in neurodegenerative diseases or during normal development, and in autoimmune inflammatory disorders of the brain (Zielasek and Hartung, 1996). Microglia express functional TLR2 (Kim et al., 2007) and TLR3 (Ribes et al., 2010; Obata et al., 2008) in addition to TLR4. TLR signalling pathways may be involved in neurodegenerative disorders (Okun et al., 2011).
In cultured microglia, soluble inflammatory mediators such as cytokines and bacterial products like LPS are capable of inducing a wide range of microglial cell activities, e.g. increased phagocytosis, chemotaxis, secretion of cytokines, activation of the respiratory burst and induction of nitric oxide synthase (Zielasek and Hartung, 1996).

Given the complexity of studying glial cell activation in vivo, for these experiments we used well-characterized cultures of purified microglia to examine their responses to zymosan (TLR2 activator) (Ozinsky et al., 2000) and poly(I:C), an activator of TLR3 (Alexopoulou et al., 2001). Commercial sources of LPS are frequently contaminated by other bacterial components, such as lipoproteins, thus activating TLR2 as well as TLR4 signalling. The Ultra-Pure LPS-EB preparation used here (referred to as 'LPS') only activates the TLR4 pathway (InvivoGen).

As reported in the following figures, engagement of each of the three TLRs resulted in IL-1β, IL6 and TNF-α gene induction.

In all cases, the fold-difference in the level of normalized gene target in treated cells is expressed relative to control samples (CTR) and reported as mean ± standard error (SEM) where CTR is equal to 1.

Cultures were stimulated with 100 ng/ml of LPS for 6 and 24 hours. IL-1β, IL-6 and TNF-α transcripts are up-regulated after treatment versus control. mRNAs show a peak induction at 6 hours of treatment (277.37 ± 26.67 vs CTR ± 0.29 for IL-1β; 79.01 ± 7.44 vs CTR ± 0.24 for IL-6; 11.67 ± 1.31 vs CTR ± 0.21 for TNF-α). At 24 hours all three mRNAs are still up-regulated (170.91 ± 6.97 vs CTR ± 0.12 for IL-1β; 64.67 ± 5.08 vs CTR ± 0.42 for IL-6; 8.38 ± 1.11 vs CTR ± 0.14 for TNF-α) (Fig. 4.4).
Fig. 4.4 Purified microglia were challenged with LPS (100 ng/ml) and processed for mRNA expression by RT-PCR. IL-1β, IL-6 and TNF-α mRNA were quantified after 6 hours (left panels) and 24 hours (right panels) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: *p<0.05; **p<0.01; ***p<0.001.

Moreover, cultures were stimulated with 10 µg/ml of zymosan for 6 and 24 hours. mRNAs for IL-1β, IL-6 and TNF-α are up-regulated after treatment versus control (1874.46 ± 111.08 vs CTR ± 0.06 for IL-1β; 134.57 ± 7.31 vs CTR ± 0.42 for IL-6; 91.53 ± 7.78 vs CTR ± 0.15 for TNF-α).
At 24 hours all three mRNAs are still up-regulated (180.48 ± 14.40 vs CTR ± 0.31 for IL-1β; 40.29 ± 7.17 vs CTR ± 0.29 for IL-6; 7.82 ± 1.23 vs CTR ± 0.24 for TNF-α) (Fig. 4.5).

Fig. 4.5 Purified microglia were challenged with zymosan (10 µg/ml) and processed for mRNA expression by RT-PCR. IL-1β, IL-6 and TNF-α mRNA were quantified after 6 hours (left panels) and 24 hours (right panels) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*: p≤0.01**: p≤0.001***
Finally, cultures were stimulated with 50 µg/ml of poly(I:C) for 6 and 24 hours. mRNAs for IL-1β, IL-6 and TNF-α are up-regulated after treatment versus control (563.91 ± 49.36 vs CTR ± 0.27 for IL-1β; 90.02 ± 16.36 vs CTR ± 0.44 for IL-6; 34.55 ± 3.05 vs CTR ± 0.06 for TNF-α).

At 24 hours all three mRNAs remain up-regulated (108.31 ± 11.18 vs CTR ± 0.06 for IL-1β; 78.40 ± 25.04 vs CTR ± 0.39 for IL-6; 6.79 ± 2.39 vs CTR ± 0.10 for TNF-α) (Fig. 4.6).
Fig. 4.6 Purified microglia were challenged with poly(I:C) (50 µg/ml) and processed for mRNA expression by RT-PCR. IL-1β, IL-6 and TNF-α mRNA were quantified after 6 hours (left panels) and 24 hours (right panels) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***

4.2.2. IL-1β release after LPS, zymosan and poly(I:C) treatment

In the brain, IL-1β is mainly produced by activated microglia (Giulian et al., 1986; Van Dam et al., 1995). LPS is a potent activator of IL-1β transcription/translation (Chauvet et al., 2001) and this process occurs in primary cortical microglia, as well (Barbierato et al., 2013).

Our experiments suggest that not only LPS but also treatment with other TLR ligands causes appreciable amounts of IL-1β to accumulate in the culture medium after 24 hours. In particular, LPS induces synthesis and release of 32.54 ± 10.01 pg/mL, zymosan induces 95.85 ± 6.55 pg/mL and poly(I:C) induces 84.81 ± 3.24 pg/mL of IL-1β (Fig. 4.7).
Fig. 4.7 Purified microglia were challenged with agonists of TLR2, -3 and -4 and processed for protein measurement by ELISA. Supernatants were collected and IL-1β quantified after 24 hours of treatment. Standards with known amounts of IL-1β were used to convert values into absolute concentration of IL-1β in pg/mL. Data are means ± SEM (triplicate culture wells).
4.2.3. TLR gene expression and protein modulation

4.2.3.1 TLR modulation in microglia challenged with LPS

TLR4 is expressed in a number of tissues, and is particularly pronounced among myelomonocytic cells (Munzio et al., 2000; Poltorak et al., 1998). Munzio et al. (2000) reported that LPS increased levels of TLR4 mRNA in human peripheral blood monocytes in an actinomycin D-dependent fashion, suggesting a transcriptional regulation. By contrast, Poltorak et al. (1998) demonstrated that TLR4 mRNA was constitutively expressed in RAW 264.7 macrophage cells, being rapidly and transiently suppressed by LPS treatment. Similarly, Nomura et al. (2000) observed that LPS treatment of mouse peritoneal macrophages lowered both TLR4 mRNA levels and surface TLR4 expression (Fan et al., 2014).

As TLR4 regulation in glia remains largely unexplored, we focused on gene expression analysis of TLRs in microglia challenged with 100 ng/mL LPS for 6 and 24 hours. After 6 hours of treatment, TLR4 mRNA level was significantly down-regulated versus control (0.10 ± 0.04 vs CTR ± 0.32) (Fig. 4.8A, top left panel) and the effect prolonged until 24 hours (0.42 ± 0.01 vs CTR ± 0.03) (Fig. 4.8A, top right panel). Unexpected, LPS modulates also TLR2, but rather inducing a strong *up-regulation* after both 6 hours (3.93 ± 0.37 vs CTR ± 0.20) (Fig. 4.8A, middle left panel) and 24 hours (2.28 ± 0.03 vs CTR ± 0.02) (Fig. 4.8A, middle right panel). Although TLR3 mRNA level did not change after 6 hours of LPS treatment (0.87 ± 0.07 vs CTR ± 0.07) (Fig. 4.8A, bottom left panel) a highly significant down-regulation was observed after 24 hours (0.26 ± 0.01 vs CTR ± 0.07) (Fig. 4.8A, bottom right panel).

The expression of TLR protein level was studied using flow cytometric analysis, identifying the percentage of positive cells and Mean Fluorescent Intensity (MFI). This parameter is closely related to the amount of
antibody bound to a specific target protein, in particular TLRs. The aim was to evaluate both the early (1 hour) effect of TLR ligand on the protein complex and the effect of prolonged exposure (samples analysis performed after 6 and 24 hours). The percentage of TLR labelled cells progressively decreased and MFI resulted significantly down-regulated (Fig. 4.8B).

Fig. 4.8A. Effects of LPS on TLR gene expression in purified rat cortical microglia. TLR mRNA levels were evaluated by Real-Time PCR after 6 and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett's multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
Fig. 4.8B. TLR expression in rat cortical microglia challenged with LPS: analysis by flow cytometry. Microglia were challenged with 100 ng/mL LPS for 1, 6 and 24 hours. Data reported in histograms are expressed as percentage of positive cells (red profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar chart graphs represent the relative MFI measured on the sample. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett’s multiple comparision test vs untreated cells: *p≤0.05; **p≤0.01; ***p≤0.001
4.2.3.2. TLR modulation in microglia challenged with zymosan

TLR gene expression was next analysed in microglia challenged with 10 µg/mL zymosan for 6 and 24 hours. As expected, zymosan modulates its target receptor, TLR2, inducing a marked and significant up-regulation after 6 hours (7.52 ± 1.00 vs CTR ± 0.04) (Fig. 4.9A, middle left panel) which persisted up to at least 24 hours (2.60 ± 0.39 vs CTR ± 0.09) (Fig. 4.9A, middle right panel).

TLR2 agonist activity regulates also TLR4 and TLR3 mRNA. Transcript levels for TLR4 are significantly down-regulated versus control at both 6 hours (0.26 ± 0.07 vs CTR ± 0.03) (Fig. 4.9A, top left panel) and 24 hours (0.33 ± 0.05 vs CTR ± 0.24) (Fig. 4.9A, top right panel). Likewise, TLR3 mRNA experiences a significant reduction 6 hours (0.43 ± 0.03 vs CTR ± 0.10) (Fig. 4.9A, bottom left panel) and 24 hours (0.24 ± 0.05 vs CTR ± 0.14) (Fig. 4.9A, bottom right panel) with LPS treatment.

In terms of TLR protein expression, flow cytometric analysis showed that the percentage of positive cells marked for TLR2 is not influenced by zymosan treatment, as confirmed by MFI, while TLR3 is progressively increased. TLR4 expression on the cell surface, as for TLR2, is not altered by zymosan treatment (Fig. 4.9B).
Fig. 4.9A. Effects of zymosan on TLR gene expression in purified rat cortical microglia. TLR mRNA levels were evaluated by Real-Time PCR after 6 and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett's multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
Fig. 4.9B. TLR expression in rat cortical microglia challenged with zymosan: analysis by flow cytometry. Data reported in histograms are expressed as percentage of positive cells (red profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar chart graphs represent the relative MFI measured on the samples. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett's multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
4.2.3.3. TLRs modulation in microglia challenged with poly(I:C)

Gene expression analysis of TLRs in microglia challenged with 50 µg/mL poly(I:C) for 6 and 24 hours was analysed. Unexpected, poly(I:C) does not modulate its target receptor, TLR3. A trend to decrease after 24 hours was observed, but was not statistically significant (6 hours treatment: $0.97 \pm 0.03$ vs CTR $\pm 0.14$; 24 hours treatment: $0.65 \pm 0.18$ vs CTR $\pm 0.10$) (Fig. 4.10A, bottom left and right panels). Poly(I:C), however, induced a highly significant increase in TLR2 gene expression after 6 hours ($5.32 \pm 0.42$ vs CTR $\pm 0.14$) and 24 hours ($2.83 \pm 0.73$ vs CTR $\pm 0.08$) (Fig. 4.10A, middle left and right panels, respectively). Transcript levels for TLR4 showed a statistically significant down-regulation after 6 hours ($0.11 \pm 0.01$ vs CTR $\pm 0.14$) (Fig. 4.10A, top left panel) and a non-significant trend after 24 hours ($0.68 \pm 0.22$ vs CTR $\pm 0.10$) (Fig. 4.10A, right column).

Flow cytometry revealed that poly(I:C) increased its target receptor, TLR3, after 1 hour. The percentage of positive cells for all 3 TLRs was significantly diminished after both 6 and 24 hours exposure to poly(I:C). These data were confirmed by MFI (Fig. 4.10B).
Fig. 4.10A. Effects of poly(I:C) on TLR gene expression in purified rat cortical microglia. TLR mRNA levels were evaluated by Real-Time PCR after 6 and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
Fig. 4.10B. TLR expression in rat cortical microglia challenged with 50 µg/mL poly(I:C) for 1, 6 and 24 hours: analysis by flow cytometry. Data reported in histograms are expressed as percentage of positive cells (red profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar chart graphs represent the relative MFI measured on the samples. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
PART 3

4.3. TLR agonist-dependent cytokine expression in astrocytes: influence of microglia

Numerous studies have described the use of astrocyte-enriched cultures to study their capability to elaborate inflammation-related molecules, e.g. cytokines, chemokines, and adhesion molecules. Oftentimes these reports assume that astrocytes are the cell type responsible for the observed effect, although this point can be questioned (Saura, 2007).

In view of the above, we decided to more fully characterize the role of astrocytes in our cultures, using a specific microglia toxin, L-LME (Thiele et al., 1983) to eradicate residual microglia. Confluent enriched astrocyte monolayers were treated for 1 hour with 50 mM L-LME (Hamby et al., 2006) followed 24 hours later by challenge with TLR ligands. As shown earlier, enriched astrocytes strongly respond to LPS (Fig. 4.11), zymosan (Fig. 4.12) and poly(I:C) (Fig. 4.13) treatment after both 6 and 24 hours with up-regulation of mRNA for the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Under these conditions, we verified that L-LME-treated astrocytes were unable to respond to the TLR ligands with induction of IL-1β and IL-6 mRNA expression, although TNF-α gene expression is not completely abolished (Figs. 4.11, 4.12, 4.13).

These observations are consistent with earlier findings (Barbierato et al., 2013). Because nominally microglia-free astrocytes are incapable of producing pro-inflammatory mediators following TLR2/3/4 activation, it is not unreasonable to assume that astrocytes per se are not the source of these molecules during inflammation.
Fig. 4.11 Responsiveness of enriched astrocytes to LPS (100 ng/ml) challenge before and after removal of microglia with L-LME: analysis of IL-1β, IL-6 and TNF-α mRNA expression by RT-PCR. Cells were processed after 6 hours (left column) and 24 hours (right column) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparision test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
Fig. 4.12 Responsiveness of enriched astrocytes to zymosan (10 µg/ml) challenge before and after removal of microglia with L-LME: analysis of IL-1β, IL-6 and TNF-α mRNA expression by RT-PCR. Cells were processed after 6 hours (left column) and 24 hours (right column) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparision test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
Fig. 4.13 Responsiveness of enriched astrocytes to poly(I:C) (50 µg/ml) challenge before and after removal of microglia with L-LME: analysis of IL-1β, IL-6 and TNF-α mRNA by RT-PCR. Cells were processed after 6 hours (left column) and 24 hours (right column) of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: *p≤0.05; **p≤0.01; ***p≤0.001.
4.4. LPS binding to TLR4

TLR4 is currently the best-characterized TLR. Together with MD2 and CD14, TLR4 forms a complex that binds LPS (Shimazu et al., 1999; Viriyakosol et al., 2000). Microglia express TLR4 on their cell surface (Kitamura et al., 2001; Qin et al., 2005) and CD14, as demonstrated using primary microglia from CD14 KO mice (Esen and Kelian, 2005). Together MD2 and CD14 interact with TLR4 to maximize LPS responsiveness.

In contrast to microglia, TLR4 expression by astrocytes remains an open question. Farina and colleagues (2005) have demonstrated TLR4 cell surface expression in vitro while other groups (Laflamme and Rivest, 2001; Lehnardt et al., 2002; 2003) described the presence of TLR4 in vivo.

Receptor functional analyses normally focus on the product of activation which, in our case, are cytokines. As L-LME-treated and nominally microglia-free astrocytes were unresponsive in terms of IL-1β, IL-6 and TNF-α gene expression upon TLR-ligand engagement, we wished to exclude the possibility of an alteration/absence for the cognate cell surface receptor complex.

Western blot analysis using specific antibodies against TLR4, CD14 and MD2 shows the presence of all members of the protein complex after L-LME treatment. No differences in protein expression were evident between enriched and L-LME-purified astrocytes (Fig. 4.14A).

Confocal microscopy shows co-expression of GFAP and TLR4 in both astrocyte-enriched cultures and in purified astrocytes (Fig. 4.14B). Moreover, we used a fluorescent conjugate of LPS from E. coli (Life Technologies, L-23351) to follow LPS binding and transport processes after 30 minutes of treatment. Cells were treated with LPS conjugated with Alexa Fluor 488 and GFAP, as astrocytic marker, to monitor real-time changes in cellular response to LPS (Fig. 4.14C).
Overall our experiments show the presence of the LPS receptor complex on the cell surface of cortical astrocytes and its ability to bind and internalize LPS after 30 minutes of exposure.

Fig. 4.14 Analysis of LPS receptor complex components using enriched and purified (L-LME-treated) rat cortical astrocytes. (A) Western blot analysis of TLR4, CD14 and MD2 expression. (B) Confocal microscopy shows co-expression of GFAP and TLR4 both in astrocyte-enriched and purified astrocytes. (C) LPS conjugated with Alexa Fluor 488 co-localizes with GFAP, after 30 minutes of treatment.
4.5. LPS, zymosan and poly(I:C) modulate TLR expression in purified astrocytes

4.5.1. TLR modulation in astrocytes challenged with LPS

Immune responses in the CNS are mainly attributed to microglia (Rivest, 2009), due to the capacity of these cells to present antigens (Gorina et al., 2011). However, astrocytes are the most abundant CNS cell type. Since we have demonstrated the presence of TLRs in purified astrocyte cell cultures, we asked whether TLR ligands are able to modulate features of immune responses regulating TLR protein expression, independent of cytokine production.

TLR gene expression was studied by Real-Time PCR using enriched astrocyte cell cultures without or with a prior exposure to L-LME to deplete the residual microglial cell population. Cells were challenged with 100 ng/mL LPS for 6 or 24 hours. TLR4 mRNA levels were significantly down-regulated both in enriched astrocytes (0.71 ± 0.03 vs CTR ± 0.03) and purified astrocytes (0.11 ± 0.01 vs CTR ± 0.03) at 6 hours (Fig. 4.15A, top left panel) and 24 hours (Fig. 4.8A, top right panel). In contrast to TLR4, LPS significantly up-regulated TLR2 mRNA levels after 6 hours in enriched as well as in purified astrocytes (Fig. 4.15A, middle left panel); this effect was still evident after 24 hours (Fig. 4.15A, middle right panel). LPS treatment significantly raised TLR3 mRNA at after 6 hours in enriched astrocytes only; there was a non-significant trend to increase in all other samples (Fig. 4.15A, bottom left and right panels). Interestingly, L-LME treatment reduced, but did not abolish basal TLR4 mRNA (0.22 ± 0.05 vs CTR ± 0.03 for TLR4; 0.44 ± 0.12 vs CTR ± 0.06 for TLR3; 0.13 ± 0.01 vs CTR ± 0.14 for TLR2).

Analyses of TLR cell surface expression of were performed using FACS utilizing purified astrocyte cultures challenged with LPS for 1, 6, and 24
hours. While the percentage of TLR4-positive cells increased already at 1 hour, the amounts of cell surface-expressed receptor did not change (Fig. 4.15B, bar chart). TLR3, but not TLR2 is modulated due to the treatment (Fig. 4.15B). These data demonstrate that the percentage of positive cells does not always follow the direction of the MFI, indicating a cellular regulation of the receptor exposure.
Fig. 4.15A. Effect of LPS (100 ng/ml) treatment on TLR gene expression in enriched and purified rat cortical astrocyte cell cultures. TLR mRNAs were quantified by Real-Time PCR after 6 hours and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical significance, calculated by Dunnett's multiple comparison test vs untreated cells: p≤0.05*: p≤0.01**: p≤0.001***
Fig. 4.158. Effect of LPS treatment on astrocyte expression of TLR proteins by FCM analysis. Enriched or purified rat cortical astrocytes were challenged with 100 ng/ml LPS for 1, 6 and 24 hours. Data reported in tracings are expressed as percentage of positive cells (blue profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar charts show the relative MFI values. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
4.5.2. TLRs modulation in astrocytes challenged with zymosan

Astrocytes were stimulated with zymosan (10 µg/mL) for 6 and 24 hours. Not unexpectedly, this TLR2 ligand modulates its target receptor, inducing a marked and significant up-regulation in purified astrocytes after 6 hours (10.85 ± 1.15 vs CTR ± 0.2) (Fig. 4.16A, middle left panel) and also after 24 hours (3.06 ± 0.16 vs CTR ± 0.2) (Fig. 4.16A, middle right panel). TLR2 agonist activity regulates also TLR4 and TLR3 mRNA. Transcript levels for TLR4 mRNA are down-regulated after 6 hours (0.0.12 ± 0.02 vs CTR ± 0.06) (Fig. 4.16A, top left panel) and 24 hours (0.37 ± 0.02 vs CTR ± 0.15) (Fig. 4.16A, top right panel). TLR3 mRNA does not statistically change in 6 hours (1.14 ± 0.08 vs CTR ± 0.07) (Fig. 4.9A, bottom left panel) but is significantly raised after 24 hours (1.55 ± 0.0 vs CTR ± 0.02) (Fig. 4.16A, bottom right panel).

FCM analyses for protein expression show that the percentage of positive cells marked for TLR2 is strongly reduced by treatment, as confirmed also by MFI (Fig. 4.9B). TLR3 is not influenced by the treatment considering both the percentage of positive cells and MFI. TLR4 expression on the cell surface, as TLR2 decreases already after 1 hour and remains low until 24 hours (Fig. 4.9B).
Fig. 4.16A. Effect of zymosan on TLR gene expression in enriched and purified rat cortical astrocyte cell cultures. TLR mRNAs were quantified by Real-Time PCR after 6 hours and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical
Fig. 4.16B. Effect of zymosan on astrocyte expression of TLR proteins by FCM analysis. Enriched or purified rat cortical astrocytes were challenged with 10 μg/mL zymosan for 1, 6 and 24 hours. Data reported in tracings are expressed as percentage of positive cells (blue profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar charts show the relative MFI values. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett’s multiple comparision test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
4.5.3. TLR modulation in astrocytes challenged with poly(I:C)

Astrocytes were stimulated with poly(I:C) (50 µg/mL) for 6 and 24 hours and TLR gene expression analysed. Poly(I:C) treatment of purified astrocytes produced a significant, robust increase in TLR3 mRNA after 6 hours (3.56 ± 0.16 vs CTR ± 0.07) and 24 hours (2.18 ± 0.10 vs CTR ± 0.12) (Fig. 4.17A, bottom left and right panels, respectively). Further, poly(I:C) induced a strong and significant rise in TLR2 gene expression after 6 hours (11.24 ± 0.22 vs CTR ± 0.01) and after 24 hours (2.26 ± 0.15 vs CTR ± 0.15) (Fig. 4.17A, middle left and right panels, respectively). In contrast, transcript levels for TLR4 were statistically lower versus control after 6 hours (0.29 ± 0.05 vs CTR ± 0.11) (Fig. 4.17A, top left panel); the effect was not evident at the 24-hour time point (0.47 ± 0.03 vs CTR ± 0.19) (Fig. 4.17A, top right panel).

The effects of poly(I:C) on TLR protein expression were studied using FCM. The percentage of TLR3-positive cells after 6 hours was found to be decreased; this was confirmed by MFI. TLR3 expression on the endosomal membrane was not modulated by poly(I:C) treatment; these data are in agreement with MFI analysis (Fig. 4.17B). TLR2 and TLR4 were up-regulated after 6 hours of poly(I:C) treatment (Fig 4.17B).
Fig. 4.17A. Effect of poly(I:C) on TLR gene expression in enriched and purified rat cortical astrocyte cell cultures. TLR mRNAs were quantified by Real-Time PCR after 6 hours and 24 hours of treatment. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3 experiments. Statistical
Fig. 4.17B. Effect of poly(I:C) on astrocyte expression of TLR proteins by FCM analysis. Enriched or purified rat cortical astrocytes were challenged with 50 µg/mL poly(I:C) for 1, 6 and 24 hours. Data reported in tracings are expressed as percentage of positive cells (blue profiles) with respect to controls (grey profiles) prepared using secondary antibodies. Bar charts show the relative MFI values. Data are means ± SEM (duplicate culture wells) normalized to secondary antibody MFI. Statistical significance, calculated by Dunnett’s multiple comparison test vs untreated cells: p≤0.05*; p≤0.01**; p≤0.001***
4.6. Reintroduction of microglia restores purified astrocytes responsiveness to TLR agonists

The above results suggest that microglia-astrocyte interaction may be a necessary condition to elicit responsiveness to LPS, at least in terms of inflammatory mediator production. To test this possibility, we reconstituted a pro-inflammatory culture profile in cultures of L-LME-purified astrocytes by adding increasing numbers of purified microglia (9,000, 18,000, 36,000 cells) (Fig. 4.18). Interestingly, the reintroduction of microglia restored LPS, zymosan and poly(I:C) responsiveness (last three bars in each panel) in term of cytokine gene expression after 6 hours treatment (Fig. 4.18) and protein release (Fig. 4.19) after 6 hours treatment.

![Graphs showing cytokine gene expression analysis](image)

Fig. 4.18. Microglial cell addition to purified astrocytes restores a pro-inflammatory profile when presented with a subsequent challenge with TLR agonists for 6 hours. Cytokine gene expression analysis was carried out by Real Time PCR. Data are means ± SEM (triplicate culture wells) normalized to GAPDH levels, and are representative of 3
experiments. Statistical significance, calculated by Dunnett's multiple comparison test vs untreated cells: *p≤0.05; **p≤0.01; ***p≤0.001

Fig. 4.19. Recovery of a pro-inflammatory profile for purified astrocytes following addition of increasing numbers of microglial cells (CM) and challenge with TLR agonist. Cytokine release was measured by ELISA. Statistical significance, calculated by Dunnett's
The effect of microglial cell addition was examined in greater detail, choosing IL-6 release as an example. As Fig. 4.20 shows, equivalent numbers of microglia alone, when stimulated with LPS (100 ng/ml) for 24 hours released far less IL-6 than when cultured in the presence of L-LME treated (purified). However, cultures containing enriched astrocytes (approximate microglia content: 2,500 cells) generated an amount of IL-6 similar to that for L-LME-treated astrocytes with 20,000 microglia. This result suggests that the astrocyte/microglia 'co-culture' is more responsive when the two cell types develop together.

Fig. 4.20. Addition of microglia to L-LME-treated astrocytes restores LPS-induced IL-6 release. The same numbers of microglia were cultured in a parallel plate, treated with LPS as above and analysed for cytokine content after 24 hours. All data are means ± SEM (n=3).
4.7 Microglia-astrocyte communication: evaluation of possible mechanism

Earlier studies pointed to a lack of soluble astrocyte-derived factors as being responsible for imparting LPS sensitivity to microglia in terms of mediator release (Barbierato et al., 2013), suggesting instead a role for physical contact between these two cell populations. This question was examined further using a two-chamber cell culture system, in which an upper layer (insert) of microglia is separated from a lower layer of astrocytes by means of a porous membrane that allows for communication between the compartments. A 24-hour LPS incubation of astrocytes only resulted in a very small quantity of IL-1β release (Table 1) but much greater intracellular accumulation, which was reduced by >90% following L-LME treatment (Table 2). LPS addition to the microglia compartment also produced a small release of IL-1β but far more intracellularly; interestingly, the presence of LPS in the lower chamber also resulted in IL-1β release by microglia (most likely a result of trans-chamber LPS passage). LPS-treated microglia did not influence IL-1β expression by L-LME-treated astrocytes, either extracellularly or intracellularly. Although the intracellular content of IL-1β in microglia was greater in the presence of LPS- (and L-LME)-treated astrocytes compared to direct LPS treatment of the microglia (2218 ± 143 and 1407 ± 63 pg, respectively) values for IL-1β release in both cases were similar.
**TABLE 1: IL-1β Release (pg/chamber)**

<table>
<thead>
<tr>
<th>Insert</th>
<th>LPS</th>
<th>No L-LME</th>
<th>+ L-LME</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>63 ± 5</td>
<td>0 ± 6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>0 ± 0</td>
<td>−</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>0 ± 0</td>
<td>34 ± 4</td>
<td>−</td>
</tr>
</tbody>
</table>

**TABLE 2: Intracellular IL-1β (pg/chamber)**

<table>
<thead>
<tr>
<th>Insert</th>
<th>LPS</th>
<th>No L-LME</th>
<th>+ L-LME</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>148 ± 30</td>
<td>169 ± 53</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>1447 ± 42</td>
<td>140 ± 8</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>133 ± 9</td>
<td>0 ± 0</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>158 ± 13</td>
<td>−</td>
<td>1407 ± 63</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>146 ± 24</td>
<td>2218 ± 143</td>
<td>−</td>
</tr>
</tbody>
</table>
5. DISCUSSION

Toll-like receptors (TLRs) are best known for recognizing pathogens and initiating an innate immune response to protect the host. However, they also detect tissue damage and induce sterile inflammation upon the binding of endogenous ligands released by stressed or injured cells (Heiman et al., 2014) (Tables 2 and 3).

In the CNS, microglia are the best-characterized cell type expressing TLRs. They constantly survey their environment and can rapidly switch to an “activated” phenotype, producing factors that influence surrounding astrocytes. Upon coming into contact with a danger signal microglia undergo activation, a process which induces engagement of other immune system cells and repair. Left unchecked, protracted inflammatory stimuli lead to a strong release of pro-inflammatory factors and consequent neuron cell death (Glass et al., 2010). Astrocytes, the predominant CNS cell type, also become reactive following injury and have been implicated in the pathogenesis of CNS inflammation (Sofroniew and Vinters, 2010; John et al., 2005; Medeiros and Laferla, 2013) and neuropathic pain (Chen et al., 2012). As succinctly stated by Nathan and Ding (2010), "The problem with inflammation is not how often it starts, but how often it fails to subside".

The interplay between astrocytes and microglia and their associated pro-inflammatory environment is, no doubt, a key element in the pathogenesis of chronic pain and neuropathic pain, neurodegenerative diseases, stroke, spinal cord injury, and perhaps even neuropsychiatric disorders (Carson et al., 2006; Melchior et al., 2006; Herbert et al., 2005).
In the present study we used a series of TLR subtype-selective agonists (LPS for TLR4, zymosan for TLR2 and poly(I:C) for TLR3) to generate a glial cell-based experimental *in vitro* model of neuroinflammation, which can be applied to investigate the induction and release of pro-inflammatory mediators upon TLR activation.

Cortical purified microglia subjected to pathogenic stimuli responded already by 6 hours with the production of mRNAs coding for pro-inflammatory genes. After a longer stimulation period, all mRNAs were translated into the respective cytokine polypeptide which was released into the culture medium. Furthermore, these TLR ligands were capable of modulating the expression of both cell surface (TLR2/TLR4) and endosomal membrane (TLR3) TLRs. This modulation following TLR ligand presentation could be the consequence of several factors. Indeed, it was possible to observe both pre-existent protein level modulation (internalization/ligand-binding/receptor degradation/receptor exposition) and genetic regulation (up- or down-regulation of mRNAs coding for TLRs).

Intriguingly, not only did a given TLR ligand modulate its own receptor's expression, but also that of other TLRs as well. This last result proposes the existence of a cross-talk mechanism in the TLR pathway(s) which may have important consequences for how multiple TLR isoforms respond to stress/injury, for example as in neuropathic pain. Enriched astrocytes from rat cortex were responsive to all TLR agonists, as well, with induction of the genes for IL-1β, IL-6 and TNF-α.

The above findings obtained with *ex vivo* glial cell systems illustrate a widely-utilized approach to investigate activation of these cell types during inflammatory processes, and are often preferred over *in vivo* analysis.
because of the latter’s complexity. While microglia can be easily obtained as a highly purified (>99%) cell population (as demonstrated in this project), achieving highly purified astrocyte cultures is more difficult owing to minor, and variable, percentages of residual contaminating microglia (Saura, 2007).

In order to study astrocyte behaviours without potential interference from contaminating microglia, L-LME was used to deplete the enriched astrocyte monolayers of residual microglia. Microglia depletion was confirmed by the disappearance of Iba-1 gene and protein expression from these cultures.

L-LME treatment abolished TLR agonist induction and release of pro-inflammatory cytokines from cortical astrocytes. We asked if this unresponsiveness by nominally microglia-free astrocytes in terms of IL-1β, IL-6 and TNF-α gene expression, upon TLR-ligand engagement, could be due to possible alteration/absence of the cognate cell surface receptor complex. In contrast to microglia, TLR4 expression by astrocytes remains an open question. Farina and colleagues (2005) have demonstrated TLR4 cell surface expression in vitro while other groups (Laflamme and Rivest, 2001; Lehnardt et al., 2002; 2003) described the presence of TLR4 in vivo. In the present study, astrocytes stripped of microglia and incubated with a fluorescent LPS showed co-localization of immunoreactivity for GFAP and the TLR4-specific ligand. In addition, it was possible to demonstrate the presence of the TLR4 co-receptors MD2 and CD14. Moreover, purified astrocytes challenged with TLR agonists responded with a modulation of both its cognate receptor as well as other TLRs. These results are important in that they place astrocytes in the context of the inflammatory
It is worth stressing the point that numerous studies have described the use of astrocyte-enriched cultures to study their capability to elaborate inflammation-related molecules, e.g. cytokines, chemokines, and adhesion molecules (Saura, 2007). In the present study, we clearly demonstrate that oftentimes these reports wrongly assume that astrocytes are the cell type responsible for the observed effect. Rather, the effects may well be due to a minor population of contaminating microglia. To further emphasize this view, we performed experiments whereby fixed numbers of purified microglia (10% of contaminating cells final) were introduced to cultures of (L-LME) purified astrocytes. Doing so restored TLR responsiveness of the latter in terms of IL-1β, IL-6 and TNF-α gene expression.

The TLR agonist responsiveness of these microglia-astrocyte co-cultures was evident also at the level of mediator release. When an equivalent number of microglia alone was challenged with a given TLR agonist cytokine output (in terms of absolute amount) into the culture medium was surprisingly less than the amount released when the same number of microglia had been added to the astrocytes. These data show that astrocytes alone are unable to respond when challenged with exogenous TLR2/3/4 ligands. The fact that the response was more robust when microglia were in the presence of astrocytes suggests the existence of a synergism between astrocytes and microglia. It bears noting that although the 'co-cultures' express pro-inflammatory cytokines after TLR agonist stimulation, the absolute levels are inferior to those measured in enriched
astrocytes (<5% of contaminating microglia) - that latter having far fewer microglia than the numbers added to reconstitute the co-culture. Conceivably, microglia which are 'nurtured' by astrocytes may be more responsive to an inflammatory stimulus than cultures in which the microglia are chemically 'stripped' and then re-introduced. This tenet, if upheld \textit{in vivo} has important implications for how these two glial cell types may interact in pathology.

To further address the issue of whether microglial cell activation in the presence of astrocytes results from either physical interaction between cell membranes or chemical induction mediated by the release of mediator(s) into the culture medium, a "Transwell insert" system was used. In our study we pointed to a lack of soluble astrocyte-derived factors as being responsible for imparting LPS sensitivity to microglia in terms of mediator release, suggesting instead a role for physical contact between these two cell populations. The presence of LPS in the lower chamber resulted in IL-1β release by microglia (plated in the upper chamber, indicating trans-chamber LPS passage) but this release did not influence IL-1β expression by purified astrocytes, either extra- or intracellularly. The molecular basis for the observed astrocyte-microglia interaction remains to be clarified.

In conclusion, the astrocyte/microglia co-culture paradigm described here may represent a useful starting point to elucidate the cross-talk mechanisms underlying astrocyte- and microglia-specific responses after TLR activation during, although not limited to, CNS inflammation. To more fully understand how glial cells respond to inflammatory stimuli, future studies could explore intracellular signal transduction pathways.
Microglia themselves respond to TLR agonists, undergoing activation to release cytokines. Since purified astrocytes express TLRs - at least those evaluated in this study - but do not elaborate either transcription or translation for IL-1β, IL-6 and TNF-α, it is possible to speculate a different regulation of NF-kB, IRF3 or IRF7 signalling. It is even conceivable that transcription factor activation is under unknown control mechanisms or, alternatively, astrocytes might need a further signal(s) to induce activation. In spite of the large amount of data published to date, this field of research has much to reveal still.
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7. Publications


