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Foreword

During these three years of my PhD course, I had the possibility to work on different projects that I have carried out at the University of Cagliari, Department of Biomedical Sciences, under the supervision of Prof. P. La Colla, and at the Rocky Mountain Laboratories (National Institute of Allergy and Infectious Diseases, Laboratory of Persistent Viral Diseases, Neuroimmunology Section) under the supervision of Dr. K. E. Peterson.
In this thesis is described the work that I did at the RML.

The results obtained during these researches led to the submission of the following manuscripts:

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Table of contents

Abstract

Both astrocytes and microglia are often activated in association with diseases of the central nervous system (CNS), including virus-induced neurological disease. Understanding how activation alters the transcriptome of these cells may offer valuable insight in regards to how activated cells mediate neurological damage. Furthermore, identifying common and unique pathways of gene expression during activation may provide new insight into the distinct roles these cells have in the CNS during infection and inflammation. In the current study, we utilized microarray analysis to examine the response of astrocyte and microglia to specific innate immune stimuli. Since virus infections often induce activation of toll-like receptor 7 (TLR7), we used the TLR7 agonist imiquimod that has been shown to induce activation of both microglia and astrocytes in vivo and in vitro. We found that microglia generate a much stronger response to TLR7 activation than astrocytes, with the upregulation and downregulation of substantially more genes in microglia than that observed in astrocytes. We identified genes that were uniquely upregulated by each cell type and examined these genes for upregulation by other factors including virus infection. These data provide new insights into innate immune activation of astrocytes and microglia that will be useful in characterizing the cellular responses of these genes during infection or insult of the CNS.

1. Introduction

1.1. Neuroinflammation

The term neuroinflammation is used to describe the immune responses within the Central Nervous System (CNS) associated with a large number of neurodegenerative diseases [1]. Neuroinflammation is a common response to viral, bacterial and parasitic infection or injury of the CNS and is dependent on the initiation of innate immune responses [2-5]. The inflammatory response may induce beneficial effects such as phagocytosis of debris and apoptotic cells, initiate repair processes, limit virus replication through production of type I interferons and recruitment of virus-specific T cells to the CNS [6, 7]. However, neuroinflammation can also result in production of neurotoxic factors that contribute to the development and severity of neurodegenerative pathology and can also lead to the recruitment of virus-infected cells to the CNS [8].

The CNS has limited interactions with the peripheral immune system due to the lack of lymphatics vessels and the presence of tight junctions at the blood–brain and blood–cerebrospinal fuid (CSF) barriers that limit the influx of cells and protein to the CNS [9-11]. As such, peripheral immune cells have limited access to the CNS. In addition, the brain does not have a resident population of dendritic cells to trigger innate immune responses. Thus, cells intrinsic to the brain, including brain macrophages and microglia and astrocytes play important roles in the innate immune response to pathogen infection in the CNS. These cells are often referred to as glia cells, and their activation termed gliosis.

1.2. Glial Cells

Glial cells are defined as the non-neuronal cells of the cells of the CNS and can be divided into two groups: microglia and macroglia. Macroglia include oligodendrocytes, the myelin forming cells, and astrocytes, the most abundant and heterogeneous population of glial cells, and the major ‘housekeeping’ cells of the CNS [12]. Macroglial cells are derived from the embryonic neuroectoderm. Microglia are the resident “immune cell” of the brain and are derived from the yolk sac early in development [13].

Despite their differences in origin, astrocytes and microglia are often found activated together in cases of neurodegeneration. Proinflammatory cytokines and chemokines produced by both astrocytes and microglia are common finding among virus infections in the CNS, including HIV infection in adult and pediatric patients. Astrocytes and microglia are recognized as active participants in various pathological conditions such as viral, bacterial and parasitic infection or

injury of the CNS or chronic neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Multiple Sclerosis and acquired immune deficiency syndrome dementia [2-5, 14-16]. The strong correlation between neurologic disease and the intensity of the innate immune response suggests that several proinflammatory cytokines and/or chemokines may contribute to the disease process. Astrocytes and microglia often produce different cytokines during virus infections. However, it is unclear whether this difference is due to the infection/stimuli or different responses of the cells to the same stimuli.

1.3. Microglia

Although originally thought to be derived from bone marrow monocytes, recent studies demonstrate that microglia are derived early during development from immature progenitors in the yolk sac. These cells persist in the CNS for the entire life of the organism with only limited turnover from bone-marrow derived monocytes [17].

In the healthy mature CNS, microglia have a ramified morphology, characterized by a small soma with fine cellular processes. This typical appearance, which is quite different from a classical macrophage, has been associated with microglia "resting" state. Infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity can evoke rapid and profound changes in the microglial cell shape, gene expression and the functional behavior which summarily is defined as "microglial activation" [18, 19]. When microglia becomes activated, they increase the size of their cell bodies, retract their processes and take an ameboid or macrophage-like morphology, attracted by endogenous and exogenous chemotactic factors using amoeboid-like movements they gather around sites of insult. At the site of damage, microglia phagocytize intracellular microorganism, removing dying cells and cellular debris [20, 21]. They also undergo rapid proliferation in order to increase their number. This activation may also lead to direct or indirect production of proinflammatory cytokines and chemokines which then contribute to the inflammatory environment. Chronically activated microglia overproduce soluble inflammatory mediators such as tumor necrosis factor (TNF), nictric oxide (NO) and interleukin-1 all of which have been demonstrated to enhance inflammation-induced oxidative stress in vulnerable neuronal populations [22-27].

Immune recognition molecules, such as MHC class II, can be identified on ramified (resting) microglia in the undisturbed brain, upon injury the molecules are highly upregulated and the expression of this complex is essential for interacting with T lymphocytes.[28-31].

Recent studies also indicate that microglia support and monitor synaptic function [32], and control synaptogenesis [33]. Microglia can also promote tissue repair by secreting growth factors thus

facilitate returning to homeostasis [34]. Thus, microglia are important for CNS development and maintenance.

Therefore, elucidation of molecular regulators of microglial responses that affect neuronal survival during chronic inflammatory stress is of great importance as it may reveal opportunities for novel anti-inflammatory strategies to prevent or delay onset of chronic neurodegenerative disease.

1.4. Astrocytes

Astrocytes, the most abundant glial cell are of neuroectodermal origin and are essential for brain homeostasis and neuronal function [35]. Astrocytes function as supportive cells in neural tissue, create the brain environment, build up the micro-architecture of the brain parenchyma and are responsible for a wide variety of complex and essential functions in the healthy CNS.

These star-shaped cells have a central cell body and long processes extending in all directions. Processes extending from different cells establish contacts with each other via gap junctions [36]. Moreover, their processes envelope synapses formed by neurons and also contact the exterior of capillary vessels. Astrocytes form 'end-feet' processes which surround blood capillaries and are thought to play a role in maintaining the integrity of the BBB. They regulate transendothelial cell migration across the blood–brain barrier (BBB) restricting the access of immune cells to the CNS parenchyma [37]. Astrocyte can control CNS homeostasis through the regulation of ion, neurotransmitter, metabolites and neuro-hormone concentrations, as well as the production of growth factors [38].

Astrocytes are emerging as key players in the regulation of synaptic activity throughout the brain. They regulate formation, maturation, maintenance, and stability of synapses thus controlling the connectivity of neuronal circuits. Astrocytes secrete numerous factors indispensable for synaptogenesis, and without astrocytes, formation of synapses would be greatly depressed [39-42]. Astrocytes control maturation of synapses through several signalling systems. Additionally, astrocytes have recently been shown to transmit signals to neurons that directly modulate synaptic strength or even contribute to synaptic transmission by release of transmitters that directly stimulate postsynaptic neurons [43]. Astrocytes are also involved in the synthesis and metabolism of neurotransmitters, modulating glutamate levels in the extracellular space and preventing glutamate-induced neurotoxicity [44, 45].

During pathological conditions of the brain, astrocytes are able to respond promptly to neuronal injury, undergoing changes in their phenotype. Upon activation, astrocytes increase production of glial fibrillary acidic protein (GFAP), undergo process extension and interdigitation, respond to protracted stress and insults by up-regulating inflammatory processes. The process by which these

cells become activated during infections and the downstream effects of astrocyte activation remain important questions in understanding pathogenesis in the CNS, indeed activated astrocytes release a wide array of pro-inflammatory molecules, including cytokines and chemokines that contribute to neuropathogenesis of CNS infections [16].

1.5. Toll-like receptors (TLRs)

The innate immune system is the major contributor to acute inflammation induced by microbial infection or tissue damage. Furthermore, innate immunity is also important for the activation of acquired immunity. Inflammatory response are initiated by the recognition of pathogen-associated molecular patterns (PAMPs), the molecules associated with groups of pathogens, which are recognized by of receptors belonging to multiple receptor families collectively called pattern recognition receptors (PRRs) [46-49].

The TLR family is one of the best-characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes. TLRs are conserved type I membrane glycoproteins characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain [50].

TLR expression has been detected in cells of the innate and adaptive immune system and in non-immune cells.

Currently, there are at least 11 TLRs identified in humans and 12 TLRs identified in mice [51].

Different TLRs recognize the different molecular patterns of microorganisms and self-components.

These TLRs can be separated into two broad categories based on their cellular location or their signal transduction machinery. TLR1, TLR2, TLR4, TLR5 and TLR6 are localized to the plasma membrane and were originally identified as receptors for bacterial. However, several studies have shown that TLR2 and TLR4 can contribute to anti-viral responses [52-56].

TLR3, TLR7, TLR8 and TLR9 are primarily located on intracellular membranes and are shuttled from the endoplasmic reticulum to the endolysosome where they recognize microbial nucleic acids.

Activation of these TLRs leads to the production of type I interferons (IFNs) in addition to proinflammatory cytokines.

TLR3 recognizes double stranded RNA (dsRNA) generated during replication of certain RNA viruses as well as polyinosinic:polycytidylic acid, termed poly (I:C). TLR9 recognizes bacterial and viral cytosine-phosphate-guanosine (CpG) DNA motifs (unmethylated DNA motifs containing CpG nucleotides flanked by 5’ purines and two 3’ pyrimidines) [57, 58].

TLR7 and TLR8, which are highly similar, recognize guanidine-uridine rich single stranded RNA (ssRNA) from certain RNA viruses [59-61].

TLR7 and TLR8 also recognize a number of synthetic molecules such as imidazoquinolines and nucleoside analogues that have been used for anti-viral treatment [62, 63].

In addition to recognizing viral RNA or DNA during virus entry and uncoating in the endosome, these endosomal TLRs also sense intracellular virus replication through autophagy of viral nucleic acids [64, 65].

Recognition of PAMPs by TLRs leads to transcriptional upregulation of distinct genes, TLRs initiate signaling through their cytoplasmic Toll/IL-1R (TIR) domains. These interact with other TIR domains such that upon activation, each TLR binds to a specific set of adapter proteins that also contain TIR domains. TRL7/9 recognize nucleic acids, and signal via Myeloid differentiation factor 88 (MyD88).

Emerging evidence suggests a role for TLRs in infectious and noninfectious CNS injury [66-69].

The expression of TLRs and related signaling proteins has been demonstrated in all major glial cell types, including microglia, astrocytes and oligodendrocytes, as well as a more limited repertoire in neurons. Both murine astrocytes and microglia express TLR7 and TLR9 mRNA [69, 70].

Furthermore, both astrocytes and microglia can respond to TLR7 or TLR9 agonist stimulation in vitro [71-73]. Thus, innate immune responses elicited by TLR stimulation may have an important role in inducing neuroinflammation during insult or injury to the CNS.

1.6. Expression of PRRs in the Central Nervous System

Most studies of PRRs have been directed at understanding their function in immune cells, but it is also important to understand the expression and function of these receptors in tissue specific environments. In the CNS, this includes not only neurons, but also microglia, astrocytes, oligodendrocytes, perivascular macrophages, ependymal, endothelial and epithelial cells. These cells can mediate the innate immune response to virus infection as well as regulate the trafficking of inflammatory cells to the CNS. Thus, there are a number of cell types in the CNS that may influence virus infection, but may differ in their response to PRR stimulation compared to bone marrow-derived immune cells [74].

Immunofluorescence studies have resulted in limited detection of TLRs in the CNS, with no detection of TLR1, TLR3, TLR4 or TLR9 in normal mouse brain [72].

However, RNA analysis and functional studies have demonstrated that multiple cell types in the CNS respond to TLR stimulation including glial cells (microglia, astrocytes and oligodendrocytes). Microglia express mRNA for all known TLRs [75-79].

Oligodendrocytes also express a number of TLRs and respond to LPS stimulation [73, 80, 81].

The expression of TLRs by astrocytes appears to vary depending on species and activation state. Murine astrocytes express mRNA for TLRs from 1-10, with the exception of TLR8 [82].

Human astrocytes express TLR3 mRNA at robust levels, TLR 1, 4, 5, and 9 at low-levels, and TLR 2, 6, 7, 8, and 10 mRNA at rare-to-undetectable levels by quantitative real-time PCR [83].

Ex vivo analyses of primary astrocyte and microglia cultures demonstrate that both cell types respond to multiple TLR agonists through the production of proinflammatory cytokines [70, 73, 78, 84, 85].

1.7. Use of the mouse model of virus infection to study innate immunity in the central nervous system

The activation of astrocytes and microglia, along with increased production of proinflammatory cytokines and chemokines is a common response in the CNS to virus infection. Similar neuroinflammatory responses are observed in animal models of virus infection. The mouse model of virus infection provides a valuable animal model to study the contribution of the innate immune response to viral neuropathogenesis because of the ability to use mice deficient in specific PRRs, cytokines or cytokine receptors [86-95].

To identify specific patterns of gene expression specific to astrocytes and microglia in the CNS following infection, we utilized the mouse model of virus infection. We utilized two different mouse models of virus infection; a retrovirus (MuLV) infection model, where the primary cells infected are microglia and macrophages, and a La Crosse virus (LACV) infection, where the primary infected cells are neurons. In both model systems, gliosis is associated with pathogenesis.

1.8. La Crosse virus LACV

La Crosse virus (LACV) is a mosquito-borne pathogen that infects up to 300,000 people in the United States and can cause encephalitis in children and young adults [96].

The virus is transmitted by hardwood forest dwelling mosquitoes, *Aedes triseriatus*, which breed in tree holes and outdoor containers. *Ae. triseriatus* feed on Eastern chipmunks (*Tamias striatus grinseus*) and Eastern gray squirrels (*Sciurus carolinensis*) which serve as amplifying hosts for LACV [97].

Interestingly, the virus can be maintained in the mosquito population in the absence of vertebrate hosts by transovarial (vertical) transmission, thus allowing the virus to over-winter in mosquito eggs [98].

The geographic distribution of LAC virus is associated with the distribution of hardwood forests and the distribution of the vector, *Aedes triseriatus.* The virus is transmitted to humans through a mosquito bite [99-101].

LACV belongs to the genus *Orthobunyavirus*, family *Bunyaviridae* [102]. Virions are spherical, 80 to 120 nm in diameter and have a host cell-derived bilipid-layer envelope The virions contain three major structural proteins: two envelope glycoproteins, G1 and G2, and a nucleocapsid protein N, plus minor quantities of a large or L protein believed to be the viral transcriptase, an RNA-dependent RNA polymerase. The LACV genome consists of three linear single-stranded, negative-sense RNA genome segments designated large (L), medium (M), and small (S) [103]. All three gene segments of a virus have the same complementary nucleotides at their 3' and 5' termini. Viral entry into its host cell is thought to occur by receptor-mediated endocytosis. All subsequent steps take place exclusively in the cytoplasm. After penetration of the host cell, the virion transcriptase is activated and transcribes subgenomic mRNAs from each of the three virion RNA segmets while still associated with their nucleocapsids. After translation of these mRNAs, replication of the RNA can occur and a second round of transcription begins. The L RNA segment codes a single open reading frame for the transcriptase (RNA-dependent RNA polymerase) [104] the M segment codes a single polyprotein (M polyprotein) that is post-translationally processed into two glycoproteins (G1 and G2), and a non-structural protein (NS_M) the S RNA segment codes for the nucleocapsid protein (N) and a non-structural protein (NS_S) [105-107].

A hallmark of neurological diseases caused by LACV and other encephalitic viruses is the induction of neuronal cell death. Innate immune responses have been implicated in neuronal damage. No specific therapies or vaccines are available for LACV.

1.9. Retrovirus

Murine leukemia viruses (MuLVs), are subfamily of the gamma retroviruses. The MuLV family can be further divided depending on the type of cellular receptor utilized by the virus, which also affects the host range of the virus. For example, ecotropic retroviruses, which primarily infect mouse cells, utilize the cationic amino acid transporter (solute carrier family 7 member 1, SLC7A1) protein as a receptor. Amphotropic retroviruses, which infect a broad range of species, utilize the phosphate transporter solute carrier family 20 member 2 (SLC20A2) protein as a receptor. Polytropic as well as xenotropic retroviruses infect cells through the xenotropic/polytropic receptor 1 (XPR1), a member of the G-protein coupled receptor protein signaling pathway family [108, 109]. Polymorphisms in XPR1 between mammalian species mediate the host range of xenotropic and polytropic viruses [110, 111].

Differences between the subfamilies of MuLVs are also observed with virus infection and pathogenesis of the CNS. Ecotropic retroviruses can induce either intracerebral hemorrhages or widespread spongiform degeneration depending on determinants in the ecotropic envelope protein [112, 113]. In contrast, the brain pathology following infection with neurovirulent polytropic retroviruses is more limited and primarily consists of reactive astrogliosis, white matter microglial infection with microgliosis and microglial nodules. There is only minimal vacuolation and only occasional neuronal death. Polytropic retrovirus infection of the CNS has some similarities with HIV-encephalopathy as HIV infection of the CNS also induces reactive astrogliosis, microglia nodules without an extensive infiltration of inflammatory cells from outside the CNS .

In the brain, the polytropic viruses productively infect endothelia, microglia, and macrophages with only rare infection of oligodendrocytes. Polytropic retroviruses do not productively infect neurons or astrocytes indicating that effects on these cell types are indirect.

The most well-studied neurovirulent polytropic retrovirus is a molecular clone, Fr98. Infection with Fr98 induces neurologic disease in 100% of newborn Inbred Rocky Mountain White (IRW) or 129S6 mice within 2–4 weeks post-infection. Mice have initial clinical signs of tremors and hind limb weakness, followed by ataxia, seizures, and ultimately death [114, 115]. As all animals develop neurologic disease, infecting knockout mice with Fr98 provides a useful method to determine if individual inflammatory genes affect the disease process. In this study, we utilized neurovirulent chimeric viruses BE that differ by only a few amino acid residues in envelope regions. The construction of chimeric virus clones for BE has been previously published [116, 117].

Aim of the thesis

In the CNS, glial cells not only serve supportive and nutritive roles for neurons, but also respond to protracted stress and insults by up-regulating inflammatory processes. Reactive glial cells produce a wide array of pro-inflammatory molecules, including, cytokines and chemokines. The complexity of studying glial activation in vivo has led to the widespread adoption of in vitro approaches, for example the use of the TLRs as an experimental model of glial activation.

In the present study, we identify genes that are differentially expressed between astrocytes and microglia when they are activated by the innate immune response. Understanding how activation alters the normal function of microglia and astrocytes and how the expression of these genes influences virus infection and pathogenesis in the CNS is of great importance as it may reveal opportunities for novel anti-inflammatory strategies for therapeutic intervention to prevent or delay onset of chronic neurodegenerative disease. Furthermore, identifying common and unique pathways of gene expression during activation may provide new insight into the distinct roles these cells have in the CNS during infection and inflammation.

2. Materials and methods

2.1. TLR Agonists

The TLR7 agonist imiquimod (R837) and TLR9 agonist type B CpG-ODN 1826 [5'-TCC ATG ACG TTC CTG ACG TT-3'] were purchased from InvivoGen. All the agonists were suspended in endotoxin-free water, aliquoted, and stored at -20°C. Just before use, agonists were diluted in media specific for either astrocytes or microglia.

2.2. Media

Astrocyte cultures were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 4,500 mg glucose/L, 110 mg sodium pyruvate/L, 0.584 g L-glutamine/L, supplemented with 10% inactivated fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Gibco). Microglia-specific media contained 20% LADMAC culture supernatant (mouse bone marrow cells producing macrophage colony stimulating factor/M-CSF) in RNA Isolation and Quantitative Real-Time RT-PCR addition to the media used for astrocyte cultures.

2.3. Isolation and Culturing of Cortical Astrocytes and Microglia by Percoll Gradient Method

Astrocyte and microglia cultures were prepared from the brain cortex of 1-2 day old Inbred Rocky Mountain White (IRW) mice. Mice were anesthetized, brain tissue removed, and placed in ice cold phosphate buffered saline (PBS). Hind brains, mid brains, and meninges were dissected out. Cerebral cortices were transferred to a 15-mL conical tube containing 2% glucose in PBS and made into single cell suspension. Cells were pelleted by centrifugation at 500 g for 5 min. Cells from two brain cortices were suspended in 2 mL of 70% percoll and transferred to the bottom of 30% and 0% percoll gradient. The gradients were centrifuged at 500g for 20 min. Cells between 0% and 30% percoll layers were rich in astrocytes and were seeded at 2 x 10^5 cells in Primaria T-25 flasks (BD Bioscience) containing astrocyte specific media. The microglia cell populations collected between 30% and 70% percoll layers were seeded at 5 x 10^5 in T-25 flasks containing microglia-specific media. When cells became confluent, after 7–10 days of culture, flasks containing astrocyte rich cells (0/30 fraction) were orbitally shaken overnight at 250 rpm to remove contaminating microglia and oligodendrocytes. Astrocytes were then treated with 0.25% Trypsin-EDTA (Gibco), reseeded in Corning 12-well cell bind plates (ISC BioExpress). Microglia were removed from confluent T-25 flasks using a cell scrapper and reseeded in 12-well cell bind plates.

2.4. Stimulation of Astrocyte and Microglia Cultures with Imiquimod or CpG-ODN

Astrocytes and microglia were treated with either 5 µM imiquimod or 80 nM of CpG-ODN 1826 or both for 3, 6, 12, 24 or 48h.

2.5. RNA Isolation and Quantitative Real-Time RT-PCR

At specified time-points post stimulation astrocyte and microglia cells were lysed and processed for RNA extraction using a mini RNA isolation kit (Zymo Research) following manufacturer's instructions. RNA was treated with DNAse I (Ambion) for 30 min at 37°C to remove any genomic DNA contamination and purified using RNA cleanup columns (Zymo Research).

cDNA was generated using an iScript reverse transcription kit (Bio-Rad) following manufacturer's instructions and included DNA contamination controls that did not undergo reverse transcription. cDNA samples were diluted fivefold in RNase-free water after reverse transcription, prior to use in quantitative real-time PCR reaction. All the real-time PCR reactions were performed using a 7900 Applied Biosystems PRISM instrument. All the samples were run in triplicate on a 384-well plate. Each reaction contained iTaq SYBR green supermix with ROX (Bio-Rad), 0.5 µM forward and reverse primers, approximately 10 ng of cDNA template and nuclease-free water. Primers were confirmed to be specific for the gene of interest. No homology to other genes was detected by blast analysis of primers against the National Center for Biotechnology Information (NCBI) database. RNA that did not undergo reverse transcription and water were used as negative controls. Dissociation curves were used to confirm amplification of a single product for each primer pair per sample.

Data were calculated as the difference in CT value (log2) for Gapdh minus the CT value of the gene of interest for each sample ($\Delta C_T = C_T$ Gapdh $- C_T$ gene of interest) to control for variations in RNA amounts in each sample. The data are presented as the fold expression of the gene of interest relative to Gapdh. For example, a value of one indicates the same level of mRNA expression as Gapdh ($\Delta C_T = 0$), whereas a value of two indicates a two folds higher level of mRNA expression than GAPDH ($\Delta C_T = 1$).

2.6. Microarray analysis

Wells from six well plates of microglia or astrocytes were randomly assigned to either mock or imiquimod stimulation groups. Six replicates per group were used for microarray analysis.

Microarray analysis was performed by the Genomics Unit of the Research and Technologies branch, NIAID using GeneChip Mouse Gene 1.0 ST Array from Affymetrix.

2.7. Immunohistochemistry using fluorescent secondary antibodies

The half of the brain tissue that was formalin fixed was further divided into four coronal and sagittal sections, embedded in paraffin, and cut in 4 m sections. Slides were rehydrated, and antigen retrieval was performed by incubating slides in 0.018 M citric acid and 8.2 M sodium citrate dihydrate, pH 6.0, for 20 min at 120°C. Slides were then incubated in 0.2% fish skin gelatin (FSG) (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS). Sections were blocked for a minimum of 30 min with blocking solution consisting of 2% donkey serum (Sigma), 1% bovine serum albumin (Sigma), 0.05% FSG, 0.1% Triton X-100 (Sigma), and 0.05% Tween 20 (Bio-Rad) in PBS. Slides were incubated in primary antibodies at 4°C overnight. For the detection of virus-infected cells, a 500-fold dilution of goat polyclonal anti-gp70 was used (10). Primary antibodies were detected using Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa-Fluor488-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated chicken anti-goat IgG, biotin-labeled goat anti-rabbit IgG, and Alexa Fluor 594 conjugated to streptavidin (Invitrogen). All sections were counterstained with 100 ng/ml DAPI (4,6-diamidino-2-phenylindole; Molecular Probes) for 20 min. Slides were mounted with ProLong Gold antifade reagent (Molecular Probes). Additional slides were incubated with individual primary antibodies or no primary antibodies prior to incubation with secondary antibodies to confirm the lack of nonspecific staining or cross-reactivity. Slides from not infected mice were used as a negative control. Digital images were captured using NIS Elements software (Nikon) and compiled using Canvas XI software (ACD Systems). For counting positive cells in each field, images were captured, using NIS Elements software, of the appropriate regions of tissue in a blind fashion. The number of positive cells per image per 200field of view was recorded. Multiple images from at least three mice per group were used for analysis.

2.8. Immunocytochemistry using fluorescent secondary antibodies

Glia cultures in 8-chamber slides (BD Biocoat) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and 0.1% sodium citrate (Sigma- Aldrich) in 13 PBS for 30 min. Cells were then treated with 0.1M glycine for 30 min and incubated with blocking solution containing 2% donkey serum (Millipore), 1% BSA (Sigma-Aldrich), 0.1% cold fish skin gelatin (Sigma- Aldrich), 0.1% Triton X-100, 0.05% Tween 20 (Sigma- Aldrich) in PBS for 30 min. Cells were incubated with primary antibodies for 30 min at

room temperature. Cells were then incubated with goat anti-mouse Alexa Fluor 488 (Invitrogen) or goat anti-rabbit Alexa Fluor 555 (Invitrogen) for 30 min at room temperature. Finally, slides were mounted in Fluorogel II with DAPI (Electron Microscopy Sciences). All images were taken using a Nikon Eclipse 55i fluorescent microscope.

3. Results

3.1. Comparison of gene profile between microglia and astrocytes following TLR7-induced activation

To directly compare how TLR7 activation altered the gene expression profile in astrocytes and microglia, we used primary cultures of both cells types generated from neonatal mice. Purity of cultures was greater than 99% as determined by flow cytometry. A time point of 6 hr post stimulation was chosen as this time point correlates with high cytokine mRNA production. Analysis of microarray data indicated a total of 260 genes that were upregulated by TLR7 ligand stimulation in either microglia or astrocytes, and 124 genes that were down regulated. Most of these genes were up or down regulated only in microglia (Tables 1, Sup. Table 1), while a much smaller subset was found regulated in both cell types (Table 2). Only 9 genes were upregulated only in astrocytes (Table 3). This indicates that microglia generate a more robust gene response to TLR7 stimulation compared to astrocytes, both in the number of genes whose mRNA expression is induced as well as the overall level or mRNA upregulation.

Gene Symbol	Gene Description	Microglia	Astrocyte
Marco	macrophage receptor with collagenous structure	17,20	1,32
Ccl5	chemokine (C-C motif) ligand 5	14,66	1,01
Il1f9	interleukin 1 family, member 9	14,56	1,17
Saa3	serum amyloid A 3	13,03	1,10
Nos2	nitric oxide synthase 2, inducible	11,22	1,61
Gbp2	guanylate binding protein 2	10,00	1,86
Ptges	prostaglandin E synthase	9,32	1,39
H2-M2	histocompatibility 2, M region locus 2	9,05	1,26
Traf1	TNF receptor-associated factor 1	9,02	1,88
EG622976	predicted gene, EG622976	7,94	1,66
Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2	7,59	1,27
Cd38	CD38 antigen	7,23	1,41
Ccrl2	chemokine (C-C motif) receptor-like 2	5,60	1,64
Jak2	Janus kinase 2	5,48	1,74
Lox	lysyl oxidase	5,42	1,57
Gbp3	guanylate binding protein 3	5,12	1,40
Cfb	complement factor B	5,04	-1,01
Niacr1	niacin receptor 1	4,98	1,34
Il13ra2	interleukin 13 receptor, alpha 2	4,94	1,10
Rsad2	radical S-adenosyl methionine domain containing 2	4,92	1,58
Cd40	CD40 antigen	4,80	1,27
Gadd45b	growth arrest and DNA-damage-inducible 45 beta	4,77	1,24
Met	met proto-oncogene	4,71	1,04
Rab11fip1	RAB11 family interacting protein 1 (class I)	4,68	1,13
C3	complement component 3	4,20	1,15
Cd274	CD274 antigen	4,19	1,77
Il10	interleukin 10	4,09	1,14
Fabp3	fatty acid binding protein 3, muscle and heart	4,03	1,13
Itga8	integrin alpha 8	-3,13	-1,02
Gmnn	geminin	-3,20	-1,20
Sesn1	sestrin 1	-3,22	-1,10
Ivns1abp	influenza virus NS1A binding protein	-3,39	-1,03
Slc37a2	solute carrier family 37 (glycerol-3-phosphate transporter), memb	-3,44	-1,23
Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	-3,67	-1,28
Ccnd1	cyclin D1	-3,84	-1,04
Mcm6	minichromosome maintenance deficient 6 (MIS5 homolog, S. pon	-3,85	-1,06
Plau	plasminogen activator, urokinase	-4,12	-1,50
Tmem154	transmembrane protein 154	-7,64	-1,38
Rgs2	regulator of G-protein signaling 2	-8,90	-1,93

Table 1. Top 40 genes upregulated or down-regulated in microglia only.

Gene Symbol	Gene Description	Astrocyte	Microglia
Marco	macrophage receptor with collagenous structure	1,32	17,20
Ccl5	chemokine (C-C motif) ligand 5	1,01	14,66
Il1f9	interleukin 1 family, member 9	1,17	14,56
Saa3	serum amyloid A 3	1,10	13,03
Nos2	nitric oxide synthase 2, inducible	1,61	11,22
Gbp2	guanylate binding protein 2	1,86	10,00
Ptges	prostaglandin E synthase	1,39	9,32
H2-M2	histocompatibility 2, M region locus 2	1,26	9,05
Traf1	TNF receptor-associated factor 1	1,88	9,02
EG622976	predicted gene, EG622976	1,66	7,94
Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2	1,27	7,59
Cd38	CD38 antigen	1,41	7,23
Ccrl2	chemokine (C-C motif) receptor-like 2	1,64	5,60
Jak2	Janus kinase 2	1,74	5,48
Lox	lysyl oxidase	1,57	5,42
Gbp3	guanylate binding protein 3	1,40	5,12
Cfb	complement factor B	-1,01	5,04
Niacr1	niacin receptor 1	1,34	4,98
Il13ra2	interleukin 13 receptor, alpha 2	1,10	4,94
Rsad2	radical S-adenosyl methionine domain containing 2	1,58	4,92
Cd40	CD40 antigen	1,27	4,80
Gadd45b	growth arrest and DNA-damage-inducible 45 beta	1,24	4,77
Met	met proto-oncogene	1,04	4,71
Rab11fip1	RAB11 family interacting protein 1 (class I)	1,13	4,68
C3	complement component 3	1,15	4,20
Cd274	CD274 antigen	1,77	4,19
Il10	interleukin 10	1,14	4,09
Fabp3	fatty acid binding protein 3, muscle and heart	1,13	4,03
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	1,13	3,98
Nlrp3	NLR family, pyrin domain containing 3	1,86	3,95
Hp	haptoglobin	1,31	3,94
Fabp3	fatty acid binding protein 3, muscle and heart	1,16	3,93
Edn1	endothelin 1	1,20	3,86
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	1,31	3,77
Ccl22	chemokine (C-C motif) ligand 22	1,23	3,76
Hck	hemopoietic cell kinase	1,41	3,75
Il18	interleukin 18	1,25	3,67
D16Ertd472e	DNA segment, Chr 16, ERATO Doi 472, expressed	1,23	3,65
Ak3l1	adenylate kinase 3-like 1	1,16	3,64
Lztfl1	leucine zipper transcription factor-like 1	1,12	3,60
Zfp558	zinc finger protein 558	-1,04	3,58
Mpa2l	macrophage activation 2 like	1,74	3,56
Socs3	suppressor of cytokine signaling 3	1,23	3,55
Zc3h12c	zinc finger CCCH type containing 12C	1,73	3,54
Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), me	1,65	3,51
Slco3a1	solute carrier organic anion transporter family, member 3a1	1,18	3,49
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	1,00	3,49
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	1,05	3,46
Mmp13	matrix metallopeptidase 13	1,24	3,39

Gene Symbol	Gene Description	Astrocyte	Microglia
Dcbld2	discoidin, CUB and LCCL domain containing 2	1,18	3,32
Dusp1	dual specificity phosphatase 1	-1,03	3,31
Ifi202b	interferon activated gene 202B	1,13	3,30
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1,06	3,27
Pstpip2	proline-serine-threonine phosphatase-interacting protein 2	1,47	3,16
Pde4b	phosphodiesterase 4B, cAMP specific	1,52	3,13
AA467197	expressed sequence AA467197	1,52	3,10
Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial	-1,04	3,07
Gm129	gene model 129, (NCBI)	-1,12	3,07
Phlda1	pleckstrin homology-like domain, family A, member 1	1,46	3,07
Cd300e	CD300e antigen	1,10	3,05
Upp1	uridine phosphorylase 1	1,20	3,03
Mx2	myxovirus (influenza virus) resistance 2	1,07	3,02
Ebi3	Epstein-Barr virus induced gene 3	1,30	2,99
Mmp9	matrix metallopeptidase 9	1,43	2,98
Pilrb1	paired immunoglobin-like type 2 receptor beta 1	1,20	2,95
Abcc5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	1,13	2,95
Egln3	EGL nine homolog 3 (C. elegans)	1,05	2,90
Slc6a12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA),	1,07	2,90
Isg15	ISG15 ubiquitin-like modifier	1,07	2,88
Mmp3	matrix metallopeptidase 3	1,14	2,88
Zc3h12c	zinc finger CCCH type containing 12C	1,43	2,87
Adm	adrenomedullin	1,17	2,86
Dusp16	dual specificity phosphatase 16	1,20	2,86
Ptx3	pentraxin related gene	1,19	2,82
Lif	leukemia inhibitory factor	1,36	2,81
Lztfl1	leucine zipper transcription factor-like 1	1,20	2,78
Ptafr	platelet-activating factor receptor	-1,04	2,77
Tet2	tet oncogene family member 2	1,02	2,75
Rilpl2	Rab interacting lysosomal protein-like 2	1,31	2,75
Bnip3	BCL2/adenovirus E1B interacting protein 3	1,14	2,74
Adora2b	adenosine A2b receptor	1,25	2,73
Rapgef2	Rap guanine nucleotide exchange factor (GEF) 2	1,02	2,68
Inhba	inhibin beta-A	1,15	2,67
Thbs1	thrombospondin 1	-1,06	2,67
Fam102b	family with sequence similarity 102, member B	-1,06	2,66
Slfn8	schlafen 8	1,27	2,66
Robo2	roundabout homolog 2 (Drosophila)	1,01	2,65
Lix1	limb expression 1 homolog (chicken)	1,01	2,63
F10	coagulation factor X	1,02	2,58
Epha4	Eph receptor A4	-1,07	2,53
Has1	hyaluronan synthase1	-1,01	2,52
Ift57	intraflagellar transport 57 homolog (Chlamydomonas)	1,06	2,51
Csf3	colony stimulating factor 3 (granulocyte)	1,08	2,50
Il1rn	interleukin 1 receptor antagonist	1,16	2,49
Peg10	paternally expressed 10	-1,17	2,49
Ak3l1	adenylate kinase 3-like 1	1,03	2,47
Acp5	acid phosphatase 5, tartrate resistant	1,11	2,45
Socs3	suppressor of cytokine signaling 3	1,08	2,41
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	1,17	2,41

Gene Symbol	Gene Description	Astrocyte	Microglia
Nampt	nicotinamide phosphoribosyltransferase	1,06	2,40
Bnip3	BCL2/adenovirus E1B interacting protein 3	1,08	2,39
Mx1	myxovirus (influenza virus) resistance 1	1,10	2,39
Flnb	filamin, beta	-1,01	2,38
Ext1	exostoses (multiple) 1	1,14	2,37
Cav1	caveolin 1, caveolae protein	-1,08	2,37
Prelid2	PRELI domain containing 2	1,12	2,34
Ifi204	interferon activated gene 204	1,15	2,34
Apbb3	amyloid beta (A4) precursor protein-binding, family B, member 3	1,01	2,34
Cdc42ep2	CDC42 effector protein (Rho GTPase binding) 2	1,00	2,33
Gyk	glycerol kinase	1,11	2,31
Gys1	glycogen synthase 1, muscle	1,08	2,28
Agtrap	angiotensin II, type I receptor-associated protein	1,06	2,27
Ppap2a	phosphatidic acid phosphatase type 2A	1,06	2,25
Ass1	argininosuccinate synthetase 1	1,10	2,24
Vcan	versican	1,05	2,23
Procr	protein C receptor, endothelial	1,07	2,22
Ube2e2	ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	-1,03	2,18
Treml4	triggering receptor expressed on myeloid cells-like 4	1,01	2,10
Tmem2	transmembrane protein 2	1,00	2,07
Eif2c3	eukaryotic translation initiation factor 2C, 3	1,01	2,05
Camp	cathelicidin antimicrobial peptide	1,02	2,04
Nav2	neuron navigator 2	-1,01	-2,01
Nt5e	5' nucleotidase, ecto	1,02	-2,05
Tec	tec protein tyrosine kinase	-1,03	-2,08
Etv5	ets variant gene 5	-1,04	-2,09
Cenph	centromere protein H	-1,01	-2,09
Slc36a1	solute carrier family 36 (proton/amino acid symporter), member 1	-1,01	-2,09
Dkc1	dyskeratosis congenita 1, dyskerin homolog (human)	1,00	-2,10
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	-1,02	-2,12
Dnajc9	DnaJ (Hsp40) homolog, subfamily C, member 9	1,01	-2,12
Lrrc33	leucine rich repeat containing 33	-1,05	-2,16
Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)	1,08	-2,17
Epas1	endothelial PAS domain protein 1	-1,06	-2,17
EG574403	predicted gene, EG574403	-1,05	-2,17
Arhgef7	Rho guanine nucleotide exchange factor (GEF7)	-1,06	-2,19
Enc1	ectodermal-neural cortex 1	-1,09	-2,20
Kctd12b	potassium channel tetramerisation domain containing 12b	-1,04	-2,21
Tmem173	transmembrane protein 173	1,03	-2,23
Rbl1	retinoblastoma-like 1 (p107)	-1,10	-2,23
Lrp12	low density lipoprotein-related protein 12	-1,09	-2,24
Rmi1	RMI1, RecQ mediated genome instability 1, homolog (S. cerevisiae)	-1,10	-2,26
Cugbp2	CUG triplet repeat, RNA binding protein 2	-1,13	-2,27
Tnfrsf23	tumor necrosis factor receptor superfamily, member 23	1,00	-2,27
Chek1	checkpoint kinase 1 homolog (S. pombe)	-1,04	-2,28
Cdca7l	cell division cycle associated 7 like	1,04	-2,28
Hyal1	hyaluronoglucosaminidase 1	-1,04	-2,33
Xylt1	xylosyltransferase 1	-1,07	-2,34
Prim1	DNA primase, p49 subunit	-1,09	-2,34
Cenpk	centromere protein K	-1,11	-2,35

Gene Symbol	Gene Description	Astrocyte	Microglia
Wdhd1	WD repeat and HMG-box DNA binding protein 1	-1,05	-2,39
Apobec1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	-1,14	-2,42
Arhgap18	Rho GTPase activating protein 18	-1,13	-2,42
Msh2	mutS homolog 2 (E. coli)	1,01	-2,48
Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	-1,05	-2,56
Fat3	FAT tumor suppressor homolog 3 (Drosophila)	1,04	-2,57
Fat3	FAT tumor suppressor homolog 3 (Drosophila)	-1,20	-2,57
Gins1	GINS complex subunit 1 (Psf1 homolog)	-1,09	-2,60
Cd180	CD180 antigen	-1,06	-2,60
Mcm4	minichromosome maintenance deficient 4 homolog (S. cerevisiae)	-1,06	-2,64
Fat3	FAT tumor suppressor homolog 3 (Drosophila)	-1,10	-2,64
Mtss1	metastasis suppressor 1	-1,23	-2,65
Tcf7l2	transcription factor 7-like 2, T-cell specific, HMG-box	-1,14	-2,67
Rcbtb2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain	-1,17	-2,71
Zdhhc14	zinc finger, DHHC domain containing 14	-1,16	-2,72
Pola1	polymerase (DNA directed), alpha 1	1,01	-2,75
Vps13c	vacuolar protein sorting 13C (yeast)	-1,23	-2,80
Ptgs1	prostaglandin-endoperoxide synthase 1	-1,14	-2,81
Adrb2	adrenergic receptor, beta 2	-1,04	-2,82
Pdgfc	platelet-derived growth factor, C polypeptide	-1,28	-2,85
Fgd4	FYVE, RhoGEF and PH domain containing 4	-1,07	-2,86
Arap2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	1,03	-2,92
Mfsd6	major facilitator superfamily domain containing 6	-1,10	-2,95
Hells	helicase, lymphoid specific	-1,03	-2,96
Fat3	FAT tumor suppressor homolog 3 (Drosophila)	-1,18	-3,07
Itga8	integrin alpha 8	-1,02	-3,13
Gmnn	geminin	-1,20	-3,20
Sesn1	sestrin 1	-1,10	-3,22
Ivns1abp	influenza virus NS1A binding protein	-1,03	-3,39
Slc37a2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	-1,23	-3,44
Abcd2	ATP-binding cassette, sub-family D (ALD), member 2	-1,28	-3,67
Ccnd1	cyclin D1	-1,04	-3,84
Mcm6	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe)	-1,06	-3,85
Plau	plasminogen activator, urokinase	-1,50	-4,12
Tmem154	transmembrane protein 154	-1,38	-7,64
Rgs2	regulator of G-protein signaling 2	-1,93	-8,90

Sup. Table 1: Genes upregulated or down-regulated in microglia only.

Gene Symbol	Gene Description	Microglia	Astrocyte
Il6	interleukin 6	52,46	4,36
Il1a	interleukin 1 alpha	26,11	12,58
Il1b	interleukin 1 beta	29,52	5,26
Cd69	CD69 antigen	22,14	10,43
Il12b	interleukin 12b	22,57	3,65
AW112010	expressed sequence AW112010	20,74	2,49
Cxcl3	chemokine (C-X-C motif) ligand 3	18,97	3,00
Ptgs2	prostaglandin-endoperoxide synthase 2	19,11	2,43
Irg1	immunoresponsive gene 1	15,91	5,21
Cxcl2	chemokine (C-X-C motif) ligand 2	10,86	9,59
Acpp	acid phosphatase, prostate	16,60	2,30
Zfp811	zinc finger protein 811	11,46	4,70
Fpr1	formyl peptide receptor 1	9,44	6,10
Gbp5	guanylate binding protein 5	10,54	4,20
Ccl3	chemokine (C-C motif) ligand 3	4,25	9,42
Tnf	tumor necrosis factor	5,56	4,44
Clec4a1	C-type lectin domain family 4, member a1	7,38	2,40
Cxcl10	chemokine (C-X-C motif) ligand 10	4,63	4,83
Cxcl1	chemokine (C-X-C motif) ligand 1	7,16	2,27
Clec4e	C-type lectin domain family 4, member e	3,54	5,25
Slamf7	SLAM family member 7	5,45	3,06
Tnfrsf1b	tumor necrosis factor receptor superfamily, member 1b	5,50	2,99
Irak3	interleukin-1 receptor-associated kinase 3	5,30	3,04
Tlr1	toll-like receptor 1	5,24	3,07
Malt1	mucosa associated lymphoid tissue lymphoma translocation gene 1	5,45	2,65
Sla	src-like adaptor	5,27	2,41
Gpr84	G protein-coupled receptor 84	4,36	3,11
Dgkh	diacylglycerol kinase, eta	4,92	2,23
Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	4,53	2,43
Siglece	sialic acid binding Ig-like lectin E	4,28	2,38
Batf	basic leucine zipper transcription factor, ATF-like	3,66	2,72
Tnfaip2	tumor necrosis factor, alpha-induced protein 2	2,95	3,35
Ppfibp2	protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein	4,17	2,09
Acsl1	acyl-CoA synthetase long-chain family member 1	3,87	2,37
Casp4	caspase 4, apoptosis-related cysteine peptidase	3,74	2,23
Birc3	baculoviral IAP repeat-containing 3	3,48	2,18
Ccl4	chemokine (C-C motif) ligand 4	3,08	2,26
AI504432	expressed sequence AI504432	2,61	2,50
Tlr2	toll-like receptor 2	2,18	2,89
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2,72	2,23
Gch1	GTP cyclohydrolase 1	2,65	2,07
Pik3ap1	phosphoinositide-3-kinase adaptor protein 1	2,59	2,09
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	2,04	2,52
Pilra	paired immunoglobin-like type 2 receptor alpha	2,23	2,01
Havcr2	hepatitis A virus cellular receptor 2	-2,41	-2,07
Cx3cr1	chemokine (C-X3-C) receptor 1	-3,93	-2,02
Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	-3,40	-2,69
Gcnt1	glucosaminyl (N-acetyl) transferase 1, core 2	-7,19	-2,61
Rasgrp3	RAS, guanyl releasing protein 3	-8,58	-3,01

Tab 2: Genes induced in both microglia and astrocytes.

Gene Symbol	Gene Description	Microglia	Astrocyte
Ccl2	chemokine (C-C motif) ligand 2	1,20	4,55
Stap1	signal transducing adaptor family member 1	1,56	3,06
Rapgef5	Rap guanine nucleotide exchange factor (GEF) 5	1,13	2,51
BC023105	cDNA sequence BC023105 // cDNA sequence BC023105	1,14	2,22
Clec12a	C-type lectin domain family 12, member a	1,53	2,17
Ccl7	chemokine (C-C motif) ligand 7	1,29	2,14
Elovl7	ELOVL family member 7, elongation of long chain fatty acids (yeast)	1,49	2,13
Cxcl11	chemokine (C-X-C motif) ligand 11	1,30	2,11
Plek	pleckstrin	1,50	2,08

Tab 3: Genes upregulated or down-regulated in astrocytes only.

3.2. Analysis of gene expression by real-time PCR analysis

Many of the genes induced TLR7 stimulation of microglia and astrocytes have already been described as being produced by these cells following TLR, bacterial or viral activation. One of the focuses of the current microarray study was to identify new markers of microglia and astrocyte activation that could be used for future studies. Therefore, we selected several genes that were either induced only by microglia, only by astrocytes or induced by both for further analysis (Table 4). Real-time PCR detection of gene expression was more sensitive than that observed with microarray when calculated as fold change relative to mock stimulated controls. Interestingly, this lead to fold changes being detected in both microglia and astrocyte populations. Of the eight genes analyzed that were detected only in stimulated microglia, seven were found induced by greater than two fold by TLR7 stimulation of astrocytes and only five of these were upregulated at a much higher level in microglia than in astrocytes (Table 4). Two of the four genes (*Cxc1ll* and BC023105) upregulated in astrocytes were also found to be upregulated in microglia, while the other two genes (*Elovl7* and *Rapgef5*) remained upregulated greater than 2 fold in astrocytes only. Thus, real-time PCR analysis of gene expression was more sensitive in detecting gene upregulation than microarray analysis, particularly for astrocytes. Furthermore, the ratio of fold gene expression relative to mock-stimulated controls was generally higher as detected by real-time PCR analysis. However, the genes found upregulated by microarray analysis were also upregulated as determined by real-time PCR indicating that these genes may be useful to study microglia and astrocyte activation.

		microarray imiquimod			real time imiquimod (fold)		
	Gene	micro	astro	Difference	micro	astro	Difference
Microglia	***Marco***	17,2	1,3	13,0	562,0	68,9	8,16
	Saa3	13,0	1,1	11,8	3.075,7	186,9	16,46
	Gbp2	10,0	1,9	5,4	31,7	8,2	3,88
	Traf1	9,0	1,9	4,8	158,8	6,8	23,29
	Ifi202b	3,3	1,1	2,9	4,2	1,8	2,35
	Ifit1	3,8	1,3	2,9	5,0	5,6	0,88
	Nlrp3	3,9	1,9	2,1	7,4	19,9	0,37
	Zc3h12c	3,5	1,7	2,0	5,0	6,7	0,75
Both	***AW112010***	20,7	2,5	8,3	106,2	55,5	1,91
	Ptgs2 (Cox 2)	19,1	2,4	7,9	30,6	4,4	6,95
	Irg1	15,9	5,2	3,1	193,9	63,2	3,07
	Gbp5	10,5	4,2	2,5	443,3	28,2	15,74
	Cd69	22,1	10,4	2,1	37,7	205,0	0,18
	Nfkbiz	4,5	2,4	1,9	28,2	14,2	1,98
	Tnfrsf1b	5,5	3,0	1,8	4,7	10,3	0,46
	Malt1	5,5	3,1	1,8	11,4	8,5	1,34
	Irak3	5,3	3,0	1,7	7,1	16,0	0,44
	Casp4	3,7	2,2	1,7	7,3	4,7	1,55
	Birc3	3,5	2,2	1,6	4,4	6,4	0,69
	Fpr1	9,4	6,1	1,5	79,2	40,2	1,97
	Gpr84	4,4	3,1	1,4	21,2	14,9	1,43
	Tnfaip2	2,9	3,3	0,9	2,9	13,6	0,22
Astrocytes	***Elovl7***	1,5	2,1	0,7	1,9	4,4	0,43
	Cxcl11	1,3	2,1	0,6	61,7	20,2	3,06
	BC023105	1,1	2,2	0,5	31,9	6,6	4,84
	Rapgef5	1,1	2,5	0,4	0,9	3,1	0,29

Tab 4: Comparison of gene expression between microarray and real-time PCR.

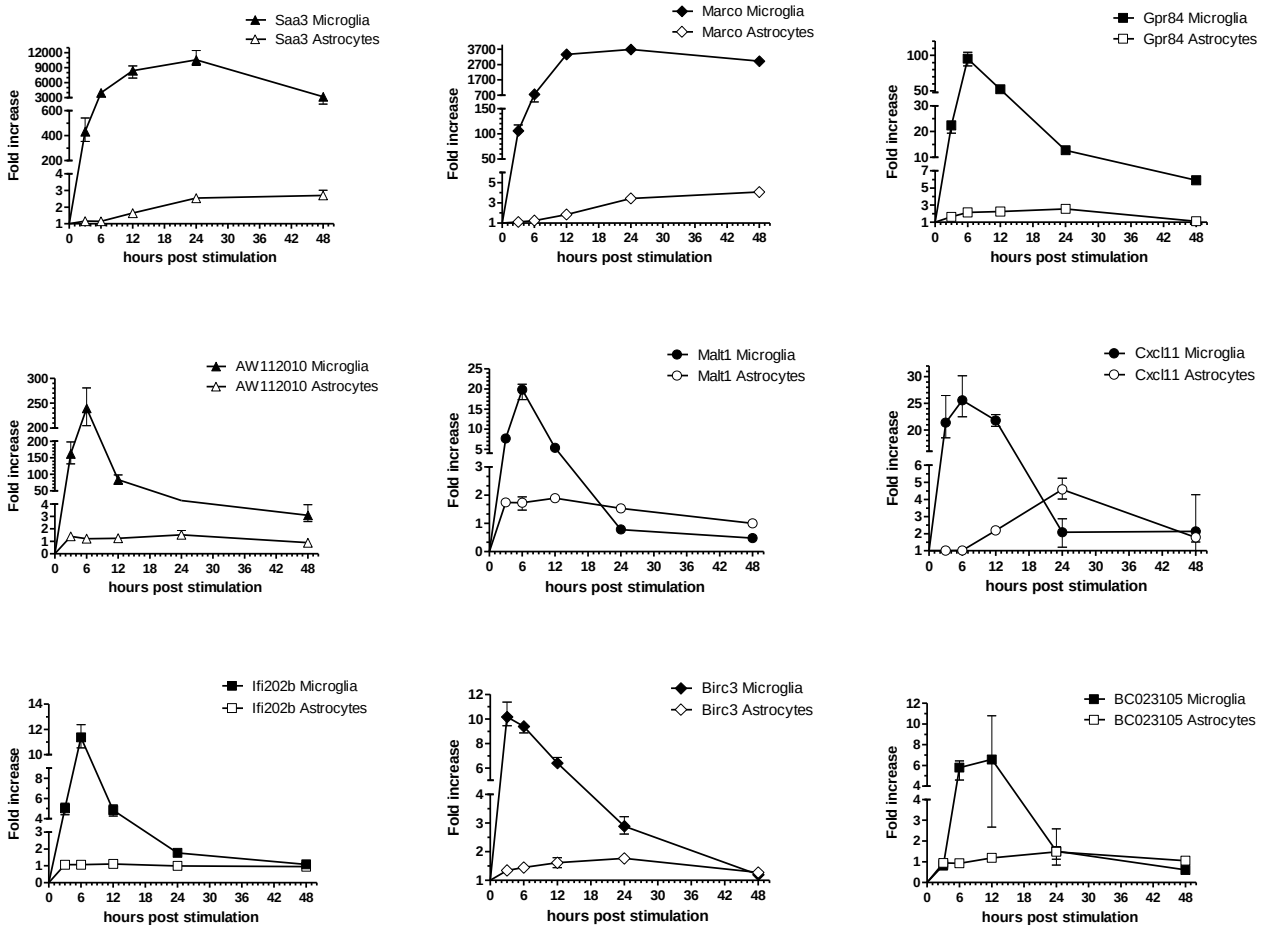

Fig. 1: Comparison of TLR7 stimulation in microglia and astrocytes. Primary cortical cultures were generated as described in materials and methods. Glial cells were stimulated with 5 µM imiquimod. RNA was isolated from cells at indicated times post stimulation. RNA samples were processed for real-time quantitative RT-PCR analysis. Data were calculated relative to the expression of Gapdh mRNA and then compared as fold change relative to the average of mock infected controls.

3.3. Gene expression pattern similar following stimulation of another endosomal receptor, TLR9

To determine if the above genes were a common response to TLR-induced activation, we analyzed microglia and astrocytes stimulated with the TLR9 ligand, unmethylated CpG-ODN. TLR9 can be stimulated by both bacterial or DNA viral infections, both of which can infect the CNS and induce meningitis. TLR9-induced activation of microglia and astrocytes induced a very similar response to TLR7-induced activation, with the upregulation of mRNA expression for all analyzed genes except *Ifi202b* mRNA in astrocytes and *Elvovl7* and *Rapgef5* mRNA in microglia (Fig. 2, Table 5). Comparison between TLR7 vs TLR9 stimulation indicated two genes in microglia that were

induced at a two-fold or more greater level by TLR7 stimulation (*Saa3* and *Traf1*) and four genes induced at a greater level by TLR9 stimulation (*Ifit1*, *Nlrp3*, *Cd69*, BC023105). TLR7 stimulation induced stronger responses than TLR9 stimulation in astrocytes with 12 genes induced at a two-fold greater level (Table 6). Thus, TLR9-induced activation of microglia and astrocytes induced a similar gene profile compared to TLR7-induced activation, although, the fold induction varied between the two stimuli.

		CpG-ODN (fold)			Imiquimod/CpG-ODN	
	Gene	micro	astro	Difference	microglia	astrocytes
Microglia	***Marco***	620,0	26,3	23,6	0,91	2,62
	Saa3	692,9	45,8	15,1	4,44	4,08
	Gbp2	25,5	3,8	6,7	1,24	2,13
	Traf1	74,8	2,7	27,4	2,12	2,50
	Ifi202b	6,1	1,2	5,0	0,69	1,48
	Ifit1	20,3	2,5	8,1	0,25	2,23
	Nlrp3	15,4	10,0	1,6	0,48	1,99
	Zc3h12c	3,1	3,8	0,8	1,65	1,78
Both	***AW112010***	110,3	14,3	7,7	0,96	3,89
	Ptgs2 (Cox 2)	18,8	2,2	8,7	1,62	2,03
	Irg1	220,8	27,7	8,0	0,88	2,28
	Gbp5	268,8	10,8	24,9	1,65	2,61
	Cd69	126,1	89,9	1,4	0,30	2,28
	Nfkbiz	39,6	9,0	4,4	0,71	1,58
	Tnfrsf1b	3,9	5,2	0,7	1,22	1,99
	Malt1	7,1	3,0	2,3	1,61	2,79
	Irak3	4,9	7,2	0,7	1,42	2,22
	Casp4	7,3	3,0	2,4	1,01	1,60
	Birc3	5,8	4,4	1,3	0,76	1,47
	Fpr1	98,5	22,0	4,5	0,80	1,83
	Gpr84	27,0	8,1	3,3	0,79	1,84
	Tnfaip2	5,9	8,9	0,7	0,50	1,53
Astrocytes	***Elovl7***	1,9	2,9	0,7	0,97	1,52
	Cxcl11	95,4	10,6	9,0	0,65	1,90
	BC023105	78,0	3,4	23,1	0,41	1,95
	Rapgef5	1,4	2,6	0,5	0,62	1,17

Tab. 5: Gene expression following TLR9 ligand stimulation. Comparison of gene expression between Imiquimod and CpG-ODN stimulation.

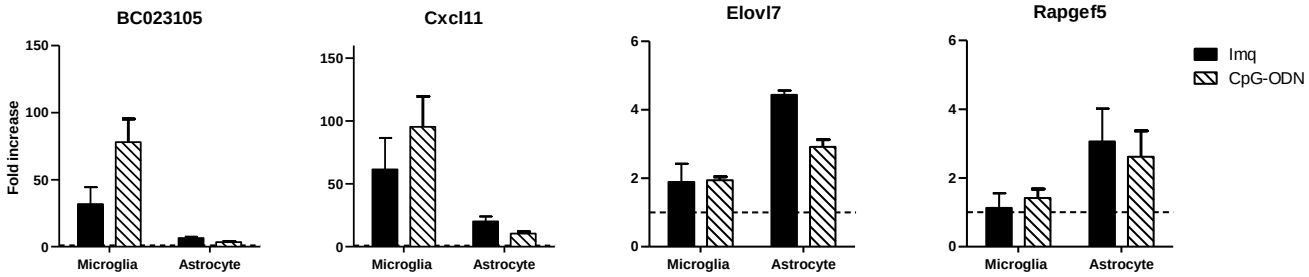

Fig. 2: Analysis of mRNA expression in TLR7/9 stimulated microglia and astrocyte. Comparison of mRNA expression of BC023105, *Cxcl11*, *Elovl7* and *Rapgef5* by astrocytes and microglia. Primary cortical cultures were generated as described in materials and methods. Glial cells were stimulated with 5 µM imiquimod or 80 nM CpGODN 1826. RNA was isolated from cells at 6 hps. RNA samples were processed for real-time quantitative RT-PCR analysis. Data were calculated relative to the expression of *Gapdh* mRNA and then compared as fold change relative to the average of mock infected controls.

3.4. Induction of gene expression by viral infection in the CNS

The above studies indicate that the identified genes may be useful markers of microglia and astrocyte activation *in vitro*. We next determined if any of these identified genes were induced in the brain following virus infection. We utilized two different mouse models of virus infection; a retrovirus (MuLV) infection model, where the primary cells infected are microglia and macrophages, and a La Crosse virus (LACV) infection, where the primary infected cells are neurons. In both model systems, gliosis is associated with pathogenesis. Of the ten genes examined, six were upregulated in the CNS in at least one model of virus infection (Fig. 3 and 4). Ifi202b mRNA was induced at higher levels with MuLV infection, while *Traf1*, AW11202010, *Birc3*, *Gpr84*,*Cxcl11* and BC023105 were at higher levels in LACV infected mice. Interestingly, the two genes whose mRNAs were only upregulated in astrocytes, *Elovl7* and *Rapgef5*, were not elevated in brain tissue following either virus infection. The increased gene expression of multiple mRNAs following LACV infection may be due the stronger neuroinflammatory response and greater inflammatory infiltrated observed in brain tissue from LACV-infected mice compared to MuLV-infected mice. However, there was no clear association with any gene and the induction of activation of astrocytes and microglia, which is observed in both virus models.

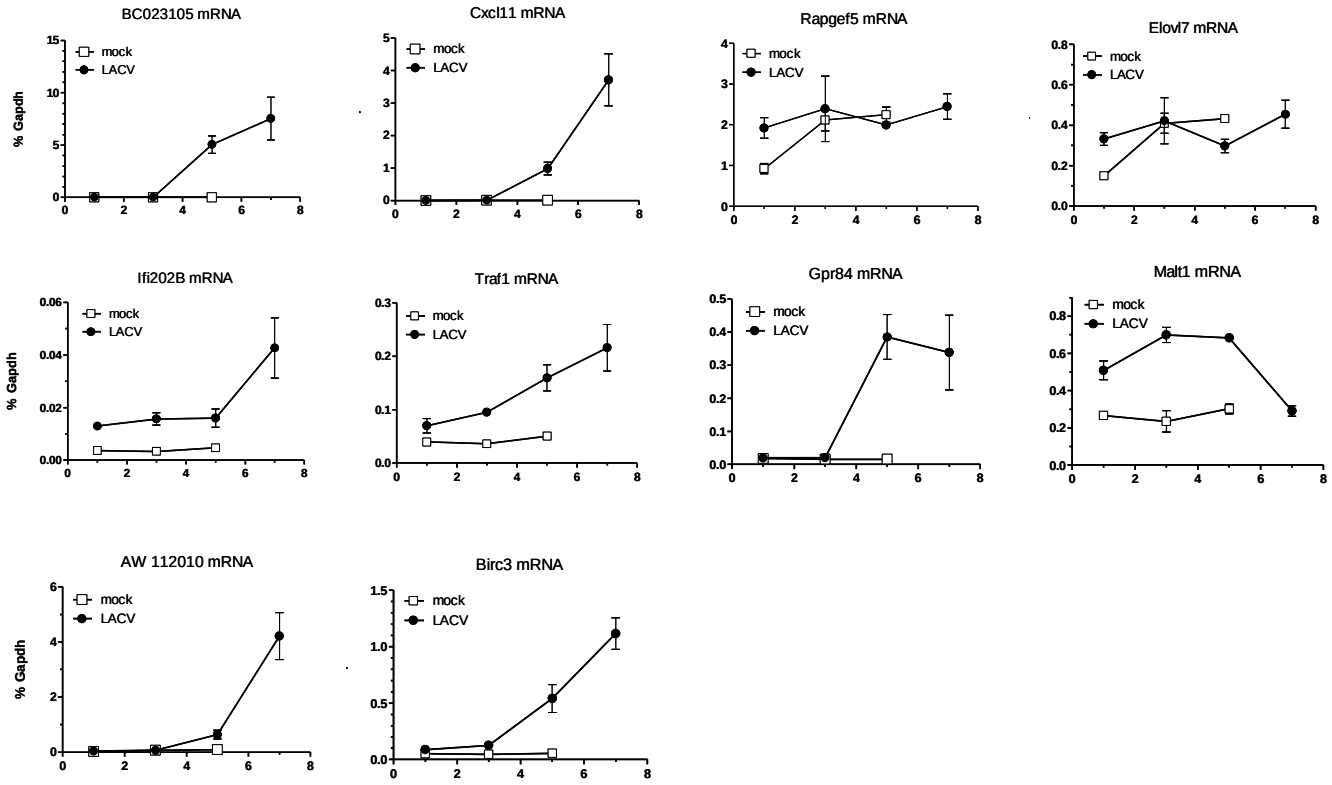

Fig. 3: Analysis of mRNA expression in LACV-infected mice. Brain tissues from mock- and LACV-infected mice were removed at the time points indicated, divided into two sagittal sections and snap frozen in liquid nitrogen and processed for RNA. Real-time quantitative RT-PCR analysis was performed using primers specific for *Rapgef5*, *Elovl7*, *Cxcl11*, BC023105, *Ifi202B*, *Birc3*, *Traf1*, *Malt1*, *Gpr84*, AW112010 and *Gapdh*. Data were calculated as gene expression relative to *Gapdh* expression (% of *Gapdh* expression) for each sample.

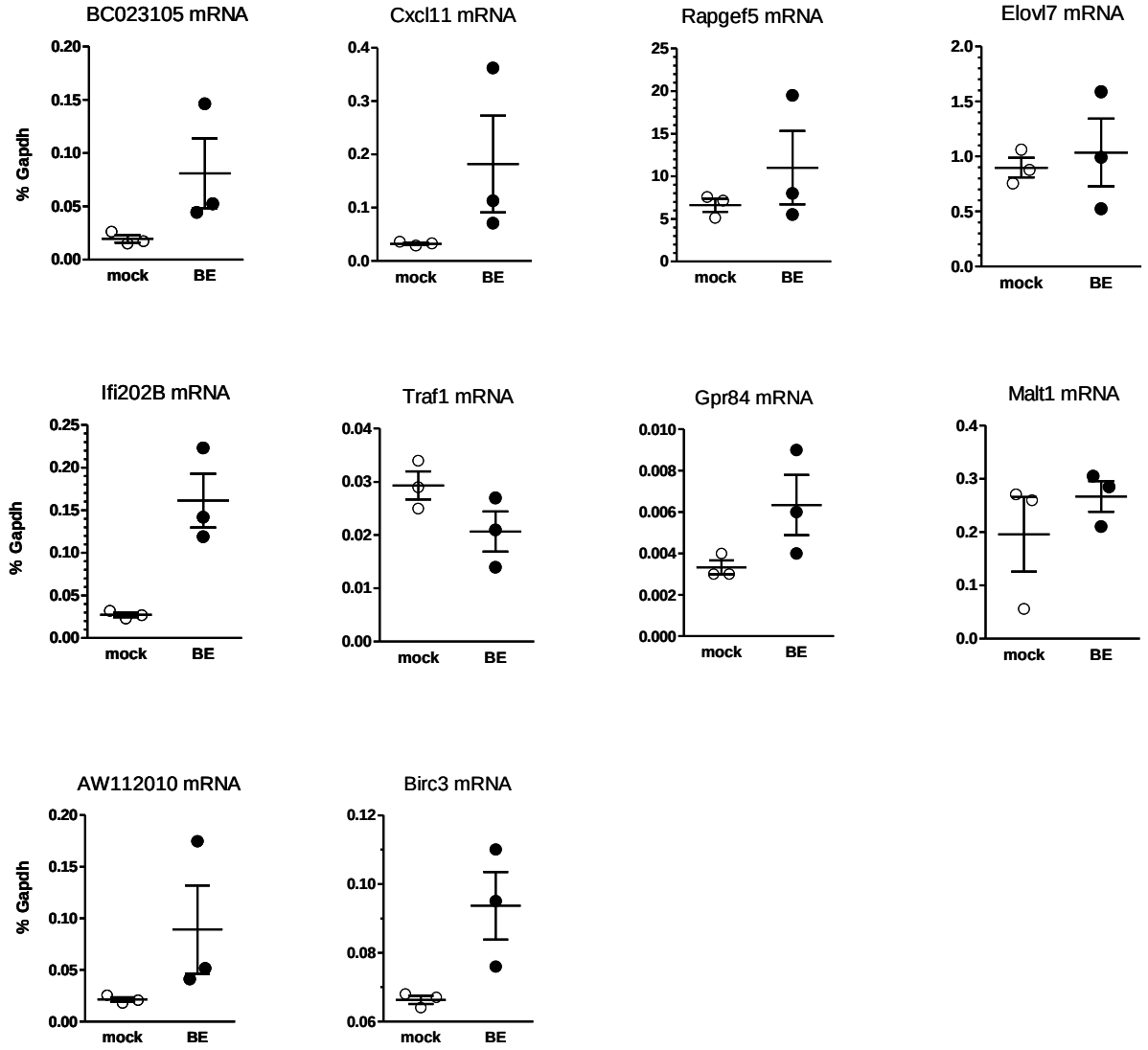

Fig. 4: Analysis of mRNA expression in BE-infected mice. Brain tissues from mock- and BE-infected mice were removed at the time of apparent clinical disease (tremors, ataxia, seizures), divided into two sagittal sections and snap frozen in liquid nitrogen and processed for RNA. Real-time quantitative RT-PCR analysis was performed using primers specific for *Rapgef5*, *Elovl7*, *Cxcl11*, BC023105, *Ifi202B*, *Birc3*, *Traf1*, *Malt1*, *Gpr84*, AW112010 and *Gapdh*. Data were calculated as gene expression relative to *Gapdh* expression (% of *Gapdh* expression) for each sample.

4. Discussion

In the current study, we identified patterns of gene expression specific to astrocytes and microglia in the CNS following infection and/or inflammation. Astrocytes and microglia were purified from neonatal mice (1-2 day old) and these primary cultures were stimulated with TLR7 agonist, imiquimod. Gene expression was analyzed by microarray followed by quantitative PCR. The expression of genes involved in TLR7 signalling and production of proinflammatory cytokines and chemokines by astrocytes and microglia were compared. Quantitative comparison of astrocyte and microglia gene expression allowed for identification of all genes that are differentially overexpressed in each cell type. Based on microarray results we identified genes that were differentially up- and down-regulated in microglia only, in astrocytes only and in both cell type (Tab 5).

One of the focuses of the current microarray study was to identify new markers of microglia and astrocyte activation that could be used for future studies. Therefore, we selected several genes and analyzed their expression by real-time PCR. For selection, we chose several genes from each category (microglia only, astrocyte only or both cells) including those with high expression as well as some of the genes that were on the lower spectrum of upregulation. We excluded the analysis of genes that were previously well characterized for their expression by microglia or astrocytes (e.g., Ccl5 and Nos2 for microglia as well as Ccl2 for astrocytes).

Real-time PCR detection of gene expression was more sensitive than that observed with microarray when calculated as fold change relative to mock stimulated controls. This is most likely due to the amplification component of real-time PCR compared to the direct hybridization used for microarray. Genes expressed at very low levels may be at the lower limits of detection by hybridization, even after cellular activation. In contrast, the amplification cycles in real-time PCR allows the detection of genes that are expressed at very low levels. This may explain the detection of fold-increases of genes by real-time PCR, when these genes were not detected in that cell population by microarray analysis. Quantitative real-time PCR is the ideal tool for analyzing the expression of a focused panel of genes. Its high degree of reproducibility makes real-time RT-PCR the preferred choice for the simultaneous quantification of both rare and abundant genes in the same sample. For some differentially expressed genes, the PCR-array confirmed the microarray prediction, though not for all. Real-time PCR has often been used as a validation of microarray studies. However, there are variables between real-time PCR and microarray analysis, besides the amplification step, that could lead to disparate results. For example, the probes used in microarray and the primers used for real-time PCR can vary in their affinity for the gene of interest. This could produce differences in the level of amplification observed in these samples.

Variability in both biological and technical procedures can have a great impact on both microarray and real-time PCR results, the quality of RNA is essential to accurate results, as gene expression can be affected by carry-over of contaminating factors which can affect the reverse transcriptase used in both real-time PCR and RNA amplification procedures for microarray labelling.

Gene expression data obtained from microarrays result were all of low intensity, overall, real-time PCR confirmed microarray results in the majority of genes analyzed. The relative change in expression for several genes as determined by microarray and real-time PCR are presented in Table 5. Of the eight genes analyzed that were detected only in stimulated microglia, seven were found induced by greater than two fold by TLR7 stimulation of astrocytes and only five of these were upregulated at a much higher level in microglia than in astrocytes. This indicates that microglia generate a more robust gene response to TLR7 stimulation, in the overall level of mRNA upregulation, compared to astrocytes. One explanation for the differences in the responses to microglia and astrocytes following TLR7 activation could be microglia are activated earlier than astrocytes and subsequently activated microglia promote astrocytes activation with up-regulation of different genes.

The current study analyzed different time point, at 3, 6, 12, 24, and 48h post stimulation, and found that there are fluctuations in genes expression at earlier and later time points. The ratio of fold gene expression relative to mock-stimulated controls was higher also at different time point with up-regulation of subset of genes that peaks between 6-12 hours post-infection in microglia (Fig. 1). Low level induction of genes by TLR7 stimulation of astrocytes was observed. It is possible that the expressions of this genes begin early, immediately upon stimulation and indicates that microglia generate a more robust gene response to TLR7 stimulation, in the overall level of mRNA upregulation, compared to astrocytes.

The gene expression of TLR7 activated astrocytes and microglia was also compared to cells stimulated with the TLR9 agonist, CpG-ODN 1826. Interestingly, the gene profile produced by agonist stimulation was similar between the type of TLR stimulation. A subset of genes were differentially regulated between TLR7 and TLR9 stimulation in microglia, including increased expression of *Ifit1*, *Nlpr*, *Cd69*, *Tnfaip2* and BCO23105. The reasons for these differences are not clear, but it is possible that TLR9 stimulation results in distinct induction of different levels of transcription factors than TLR7 stimulation, resulting in some distinctions in gene expression.

Upon TLR7/9 stimulation, we found that two of the four genes (*Cxc1ll* and BC023105) upregulated in astrocytes were also found to be upregulated in microglia, while the other two genes (*Elovl7* and *Rapgef5*) remained upregulated greater than 2 fold in astrocytes only (Fig. 2). Thus, *Elovl7* and *Rapgef5* appear not to be induced by microglia and may be reliable markers to look at specific

astrocyte activation. In contrast, *Cxcl11* and BC023105 are induced by activation in both cell types, but are most likely expressed at higher levels overall in astrocytes compared to microglia.

Of the genes analysed for astrocytes, *Cxcl11* generated the strongest upregulation as detected by real-time PCR (Table 4). *Cxcl11,* chemokine (C-X-C motif) ligand 11, also known as I-TAC (interferon-inducible T cell alpha chemoattractant) was originally identified in IFN-β-treated astrocytes [118-120], which correlates with the high detectability in our cultures. However, immunohistochemistry analysis of brain tissues from virus infected mice showed only limited expression of Cxcl11 by astrocytes and high levels of expression by brain capillary endothelia cells (data not shown). Thus, endothelia and astrocytes may both be important sources of *Cxcl11* during virus infection in the brain, similar to the expression of CCL2 by both astrocytes and endothelia (data not shown). *Cxcl11* is an important cytokine for the recruitment of T cells to the CNS. The production of *Cxcl11* by both astrocytes and endothelia in the CNS may allow for greater control of T cell recruitment to the CNS.

Only two genes (*Elovl7* and *Rapgef5)* remained upregulated greater than 2 fold in astrocytes only. Elongation of very-long-chain fatty acids (Elovl7) is a member of the gene family that catalyze the elongation reaction of very-long-chain fatty acids (VLCFAs) [121]. The ELOVLs catalyze the first, rate-limiting step of the VLCFA elongation cycle: condensation between the fatty acyl-CoA and malonyl-CoA to yield β-ketoacyl-CoA[122, 123]. It has been shown that VLCFAs, docosanoic (C22:0, behenic acid)-, tetracosanoic (C24:0, lignoceric acid) and hexacosanoic acids (C26:0,cerotinic acid), exert a strong cytotoxic activity in oligodendrocytes and astrocytes derived from rat brain. VLCFAs also cause cell death of the myelin-producing oligodendrocytes and astrocytes [124]. Increased *Elovl7* could lead to VLCFA accumulation, which results in demyelination and oxidative damage. Thus, the upregulation of *Elovl7* by TLR stimulation of astrocytes could be a contributing factor to damage associated with astrogliosis and neurological damage.

Ras-related GTP-binding proteins are molecular switch proteins which cycle between an inactive GDP-bound state and an active GTP-bound state. Activation is achieved by the exchange of tightly bound GDP for GTP. This reaction is very slow and is activated by guanine-nucleotide exchange factors (GEFs) or *Rapgef5* [125]. *Rapgef5* mediates the activation of Rap1 which is a member of the Ras GTPase family and is converted from inactive GDP-bound states to active GTP-bound [126, 127].

Although we were able to detect *Rapgef5* by real-time PCR in astrocyte cultures, we were unable to detect increased *Rapgef5* in brain tissue from virus-infected mice. It is possible that Rapgef5 is more tightly controlled in vivo than it is in vitro. Alternatively, *Rapgef5* may be expressed by several cell types and therefore, increased expression of *Rapgef5* in astrocytes may be masked by unchanged expression in other cell types.

Interestingly, *Elovl7* and *Rapgef5* were among the genes identified as being highly expressed in freshly-purified glia, but subsequent work revealed very few *Elovl7* and *Rapgef5* -expressing cells in the mouse models of virus infection. This could be due to many different reasons since the RNA analysed for virus infection was measured from whole brain samples which contain several other cell types besides astroglia. Furthermore, expression levels in vivo may differ from that in vitro. However, we can conclude from these data that neither Elovl7 or Rapgef5 are strong candidates for use as activation markers in astrocytes.

Of the ten genes examined, six were upregulated in the CNS in at least one model of virus infection. *Ifi202b* mRNA was induced at higher levels with MuLV infection (Fig. 4), although this increase was not statistically significant, while *Traf1*, AW11202010, *Birc3*, *Gpr84, Cxcl11* and BC023105 were at higher levels in LACV infected mice (Fig 3). Increased levels mRNA of *Cxcl11*, BC023105, *Traf1, Birc3* and *Gpr84* were first observed after 3 days post infection and remained high throughout the onset of neurological disease. The upregulation of these genes are indicative of the neuroinflammatory process associated with LACV infection, suggesting that these genes may be useful for the study of LACV-induced neurological disease.

The increased gene expression of multiple mRNAs following LACV infection may be due the stronger neuroinflammatory response and greater inflammatory infiltrated observed in brain tissue from LACV-infected mice compared to MuLV-infected mice. However, there was no clear association with any gene and the induction of activation of astrocytes and microglia, which is observed in both virus models.

Brain tissue from BE- and LACV-infected mice was analyzed for expression of some genes by immunohistochemistry. Tissue sections were analyzed for *Gpr84*, *Malt1*, *Marco*, *Rapgef5*, *Cxcl11* and *Elovl7,* but not positive cells were found (Data not shown). Interestingly, immunocytochemistry revealed expressions of these genes between 24 and 48 hours post stimulations with 5 µM imiquimod or 80 nM CpG-ODN 1826 (Data not shown) as detected by real-time PCR analysis. It is probable that inflammatory gene expression induced by TLR depends on an interplay between microglia and astrocytes, indeed astroglial-microglial cross talk is extremely important in glial cell activation.

Under our experimental conditions, we demonstrated that microglia generate a much stronger response to TLR7 activation than astrocytes, with the upregulation and downregulation of substantially more genes in microglia than that observed in astrocytes. Subsequently, we identified genes that were uniquely upregulated by each cell type and examined these genes for upregulation by other factors including virus infection. These data provide a useful starting point to elucidate the

activation of astrocytes and microglia that will be useful in characterizing the cellular responses of these genes during infection or insult of the CNS, especially where TLR activation plays a role.

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