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# Innate Immune Responses in the Central Nervous System Following Toll-Like Receptor (TLR) 7 and TLR9 Activation

Niranjan Babu Butchi

*Louisiana State University and Agricultural and Mechanical College*

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INNATE IMMUNE RESPONSES IN THE CENTRAL NERVOUS SYSTEM FOLLOWING  
TOLL-LIKE RECEPTOR (TLR) 7 AND TLR9 ACTIVATION

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
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Doctor of Philosophy

In  
The Interdepartmental Program in  
Veterinary Medical Sciences through the  
Department of Pathobiological Sciences

By  
Niranjan Babu Butchi  
B.V.Sc.&A.H., A.N.G. R. Agricultural University, INDIA, 2001  
M.V.Sc., A. N.G. R. Agricultural University, INDIA, 2003  
M.S., Kansas State University, 2006  
December 2009

## **DEDICATION**

This dissertation is dedicated to my wife, Rajkumari, who believed in me and provided love and support without which I would have not been able to complete this degree.

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## ABSTRACT

Activation of astrocytes and microglia and the production of proinflammatory cytokines and chemokines are often associated with virus infection in the central nervous system (CNS) as well as a number of neurological diseases of unknown etiology. These inflammatory responses may be initiated by recognition of pathogen-associated molecular patterns that stimulate toll-like receptors (TLRs). Recent studies demonstrated that TLR9 family receptors play a role in neuropathogenesis and the agonists of these receptors may be used in therapeutics treatment of brain tumors and viral infections. However, we lack the basic understanding of how these receptors function in the CNS. In the present study, we examined the potential of TLR7 and TLR9 agonists to induce glial activation and neuroinflammation using an *in vivo* model of intracerebroventricular inoculation of these agonists in newborn mice and by *in vitro* stimulation of glial cells. TLR9 agonist induced a stronger neuroinflammatory response than TLR7 agonist administration in the CNS, with higher levels of proinflammatory cytokines, and the break down of the blood-brain barrier which resulted in influx of peripheral immune cells. Despite the differences in inflammation, analysis of cell types indicated involvement of similar cell types to respond to TLR7 and TLR9 agonists including ependymal cells, endothelial cells, microglia and astrocytes. This disparity in TLR7 and TLR9 activation *in vivo* was not observed at the *in vitro* level, where similar responses were observed to either agonist. The present study also determined the cross-regulatory capabilities of these receptors. Interactions between agonists were observed both *in vitro* and *in vivo* with the TLR7 agonist, imiquimod, inhibiting TLR9 agonist, CpG-ODN, or TLR9 itself, to inhibit CpG-ODN induced cytokine production. In addition to agonist interactions, an antagonistic relationship was also observed between the two receptors in microglia, with TLR7 deficiency resulting in enhanced cytokine responses to CpG-ODN stimulation. Overall, these studies demonstrate a complex interaction between TLR7 and

TLR9 in regulating the initiation of innate immune responses in the brain, with TLR9 stimulation inducing more damage in the CNS than TLR7 stimulation. However, TLR7 and its agonist appear to regulate TLR9 stimulation and can diminish TLR9 agonist induced neuroinflammation.

## **CHAPTER 1**

### **INTRODUCTION AND LITERATURE REVIEW**



## **1.1. INNATE IMMUNITY AND THE CENTRAL NERVOUS SYSTEM**

### **1.1.1. Neuroinflammation**

The inflammatory response that occurs in the central nervous system (CNS) following infection or injury is termed neuroinflammation. This often includes activation of astrocytes and microglia (innate immune cells of the brain), production of proinflammatory cytokines and chemokines, as well as the recruitment of peripheral immune cells including dendritic cells into the CNS.

Neuroinflammation in the CNS is commonly observed in a number of viral infections including cytomegalovirus, herpes simplex virus, flaviviruses, paramyxoviruses, rhabdoviruses, and retroviruses, as well as in bacterial infections, parasitic infections (Aravalli et al., 2007; Kirton et al., 2005; Kristensson, 2006; Mishra et al., 2006; Tardieu et al., 2000).

Neuroinflammatory responses are also observed in autism, Alzheimer's disease, multiple sclerosis, and other neurological diseases of unknown etiology (Eikelenboom et al., 2002; Vargas et al., 2005). The cytokines and chemokines produced during neuroinflammation can regulate the blood-brain barrier (BBB) permeability, cause infiltration and accumulation of immune cells from the periphery into the brain depending on the type of infection or injury (Eikelenboom et al., 2002; Garden, 2002). Only subtle responses in the CNS innate immune system are observed in the absence of BBB breakdown. These are composed largely of activation of glia cells without infiltration of leukocytes from the periphery (Eikelenboom et al., 2002; Garden, 2002). A lack of leukocyte infiltration is often observed in neuroinflammation of the developing brain. However, the neuroinflammatory response in neonates may have detrimental effects in terms of neural progenitor cell migration and neuronal apoptosis which may lead to long term neurological disorders (Bagri et al., 2002; Hornig and Lipkin, 2001; Hornig et al., 2002; Rezaie et al., 2002; Tran et al., 2004).

### **1.1.2. Cell Types in the CNS**

The CNS consists of two broad categories of cells; (A) glial cells, which include microglia, astrocytes, and oligodendrocytes, and (B) non-glial cells, which include neurons, macrophages, brain capillary endothelial cells, ependymal cells, and meningeal cells.

#### **• Glial Cells:**

The term glia originated from Greek for glue, as glial cells were originally known to be support cells for the neurons in the CNS. There are three types of glial cells in the CNS: microglia, astrocytes and oligodendrocytes. Glial cells constitute up to 90% of the total cells in the CNS.

#### **1.1.2.1. Microglia**

Microglia are the smallest of the glial cells in the CNS with a small cell soma, little perinuclear cytoplasm and a number of fine branched processes. Microglia constitute approximately 15% of total cells in the CNS. These are the resident macrophages of the CNS and are myeloid in origin, which are derived from monocytes that entered into the brain from circulation during embryonal development (Ransohoff and Perry, 2009). Microglia are embryologically and physiologically unrelated to other glial cells. Following entry into the brain, microglia undergo morphological changes including extension of processes and develop into resting ramified microglia. The resident microglia in the adult do not require significant turnover rate from circulating monocytes from peripheral circulation, as microglia are actually populated in the CNS during embryonic development well before birth and can be replenished intrinsically (Chan et al., 2007).

Resting/ramified microglia have low phagocytic and endocytic activity and contribute a supportive role for neurons along with astrocytes. Microglia also have developmental functions during embryonic development. Ramified microglia have a scavenger function in the developing

brain by removing the cells in the neocortex that die in the remodeling of the fetal brain (Voyvodic, 1996). Microglia also participate in a process called synaptic pruning, an activity by which excessively formed neuronal synapses in the brain are removed and thus help in brain maturation after birth (Ransohoff and Perry, 2009).

Microglia are usually the first line of defense in the CNS, and may be stimulated either by non-self pathogens (stranger signal) or by injured self components (danger signal). In response to pathogen or CNS insult, resting microglia become activated, retract their processes and shift into amoeboid or macrophage-like morphology (Milner and Campbell, 2003). In addition to change in morphology, microglia rapidly upregulate a large number of receptors and secrete multiple cytokines and chemokines (Albright and Gonzalez-Scarano, 2004; Baker and Manuelidis, 2003). Some of the important receptors expressed by microglia include scavenger receptors, receptors involved in recognition of apoptotic blebs, receptors important for pathogen recognition, complement receptors, and major histocompatibility protein class I and II. Microglia cells also express a receptor for the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) which can induce microglial proliferation *in vitro* (Rock et al., 2004). Cytokine and/or chemokine production by microglia following activation may play important roles both in protection against pathogens, as well as in contributing to neuronal damage (Giese et al., 1998). Multiple cell surface markers, including F4/80, Iba-1 and CD45 are expressed on the microglial cell surface and are useful in molecular phenotyping of these cells (Table 1.1). F4/80 is used to determine activation state of the microglia, as F4/80 is upregulated following activation (Ford et al., 1995). Additionally, ionized calcium binding adaptor molecule-1 (Iba-1) has been used as a general marker for immunohistochemical identification (Babcock et al., 2003). Microglia also express CD45, albeit at lower level compared to macrophages, allowing them to differentiate between CD45<sup>hi</sup> macrophages and CD45<sup>lo</sup> microglia by flow cytometry (Stevens et al., 2002).

---

**Table 1.1. Markers to Identify Glial Cells in the CNS.**

**Microglia:**

**IBA1 / Aif1** (ionized calcium binding adaptor molecule 1 / allograft inflammatory factor 1). Induced in microglia following activation and is a marker for immunohistochemical analysis.

**F4/80/ Emr1** (EGF-like module containing, mucin-like, hormone receptor-like sequence 1). Upregulated following activation of microglia, thus helps in identification of activation state of microglia.

**CD45<sup>lo</sup>** Microglia express CD45 at low levels compared to macrophages. This helps in differentiating between microglia and macrophages by flow cytometry.

**GM-CSFR** (Granulocyte-macrophage colony stimulating factor receptor low-affinity subunit). Receptor for the cytokine, colony stimulating factor 2 (GM-CSF), a cytokine which controls the production, differentiation, and function of granulocytes, macrophages and microglia.

**Astrocytes:**

**GFAP** (Glial fibrillary acidic protein). Major intermediate filament protein of mature astrocytes, upregulated during astrocyte activation.

**S100B** (S100 calcium binding protein B). Expressed and secreted by astrocytes, function in neurite extension, proliferation of astrocytes and axons.

**SLC1A2/GLT1** (Solute carrier family 1, member 2/glial high affinity glutamate transporter1). Membrane-bound transporter protein that clears the excitatory neurotransmitter glutamate from the extracellular space at synapses in the central nervous system.

**SLC1A3 / GLAST** (Solute carrier family 1, member 3 / glutamate/aspartate transporter). High-affinity sodium-dependent transporter that regulate neurotransmitters glutamate and aspartate concentrations at the excitatory glutamatergic synapses of the central nervous system.

**Oligodendrocytes:**

**OLIG2** (Oligodendrocyte lineage transcription factor 2) Expressed in oligodendroglia in the brain. This is an essential regulator of ventral neuroectodermal progenitor cell fate.

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### **1.1.2.2. Astrocytes**

Astrocytes, along with other major cell types in the brain including neurons, and oligodendrocytes, are neuroectodermal in origin. These cells are derived from a single layer of proliferating neuroepithelial cells, called neuroprogenitor cells (NPC), during development. These NPCs line the ventricles in the brain and form the ventricular zone (VZ). NPCs in the VZ elongate and give rise to radial glia. Radial glia divide asymmetrically to generate some early neurons directly or intermediate progenitor cells (IPC). These IPCs can be subdivided into neurogenic IPCs, astrocytic IPCs and oligodendrocytic IPCs that generate neurons, astrocytes and oligodendrocytes respectively (Kriegstein and varez-Buylla, 2009).

Astrocytes are the most abundant glial cells that constitute nearly 35% of the total CNS cell population. Astrocytes play an important role in CNS support and homeostasis. They interact extensively with neurons and provide a supportive role both structurally and metabolically. Astrocytes have irregular star shaped cell bodies with broad end feet on their processes. The foot processes (end feet) of astrocytes are in close opposition to the micro vascular endothelium and cover >90% of the endothelium, preventing entry of toxic substances and cells from the peripheral circulation into the CNS and thus contribute to both structural and functional integrity of the blood-brain barrier (Wolburg and Lippoldt, 2002).

Astrocytes can be visualized by immunolabeling with antisera specific for glial fibrillary acidic protein (GFAP), S100b or the astrocyte specific glutamate transporters GLT1 and GLAST (Table 1.1) (Walz, 2000). GFAP is expressed mainly on the intermediate filament proteins of mature astrocytes and is used as a marker to distinguish astrocytes from other glial cells. During development, intermediate filaments of astrocyte precursors express vimentin, which is replaced by GFAP during maturation of astrocytes. S100B is a calcium-binding protein that is

predominantly expressed and secreted by astrocytes, which function in neurite outgrowth, astrocytes and axonal proliferation.

Astrocytes modulate the levels of excitatory neurotransmitters such as glutamate and aspartate in the extracellular space. Astrocytes express high affinity glutamate transporters, SLC1A2 (solute carrier family 1, member 2/ also called GLT-1 glutamate transporter-1/EAAT2) and SLC1A3 (solute carrier family 1, member 3, also called GLAST-glia high affinity glutamate transporter/EAAT-1), which rapidly remove the glutamate released into the neuronal synapse from the presynaptic neuro-terminal. Thus, astrocytes contribute to functional neuronal synapse and prevent glutamate induced neurotoxicity, which can induce neuronal damage and death if accumulated at high concentrations (Parpura et al., 2004). Astrocytes are also associated with synaptogenesis, as co-cultures of purified neurons with astrocytes can greatly increase the number of structurally mature and functional synapses (Allen and Barres, 2005; He and Sun, 2007). In the event of neuronal damage, astrocytes produce nerve growth factors and neurotrophins, including nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) which can promote axon growth and survival of neurons (Dong and Benveniste, 2001).

In addition to the neuronal support functions, astrocytes also respond to CNS insult or infection. Activation of astrocytes can result in proliferation, morphological changes, process extension and interdigitation, enhanced expression of GFAP and vimentin in the intermediate filaments. This process is called astrogliosis (Hatten et al., 1991). Activated astrocytes release multiple proinflammatory cytokines and chemokines that act on and engender responses in target cells analogous to the responses of activated immune cells in the periphery. The process by which astrocytes and microglia undergo activation during infection or injury is not known and the subsequent downstream effects are still under investigation.

### **1.1.2.3. Oligodendrocytes**

Oligodendrocytes are the specialized cells in the brain that form the myelin sheath, wrapped in layers surrounding the neurons in the CNS and insulate them for impulse conduction. These cells have a relatively small amount of cytoplasm around the nucleus and have several long processes that wrap around the neurons to form the myelin sheath. These cells are associated with pathologic conditions such as multiple sclerosis, which is characterized by formation of multiple lesions in the brain, demyelination and death of oligodendrocytes.

#### **• Non-Glial Cells :**

### **1.1.2.4. Neurons**

Neurons are highly specialized cells in the brain, consisting of a cell body or soma with small dendrites that receive signals and a long axon that conducts the nerve signal through neurotransmitters. The gap between the axon terminals of one neuron and the receiving neurons is called neuronal synapse. The neuronal synapses are also covered by astrocytic foot processes, which modulate the levels of excitatory neurotransmitters in the neuronal synapse and prevent neurotoxicity. Neurons can be identified *in vivo* or *in vitro* by expression of  $\beta$ -III-tubulin, MAP2 or NeuN (Table. 1.2.).

Recent studies indicate that the neurons are capable of responding to infections or injury and induce immune responses. Several studies reported that neurons express NF-kB in response to mechanical injury, synthesize tumor necrosis factor (TNF), and induce expression of inducible nitric oxide synthase (iNOS) and superoxide dismutase (SOD) (Carson et al., 2006).

Neurons also have the potential to directly regulate the effector functions of the immune system. Systemic exposure of the host to proinflammatory stimuli can induce multiple neuronal pathways resulting in release of neurotransmitters from the sympathetic nerve endings in the immune organs. This inhibits proliferation and secretion of proinflammatory cytokines from

macrophages and natural killer (NK) cells resulting in restoration of homeostasis (Sternberg, 2006). Neurons also synthesize and secrete a protein called neuropeptide Y (NPY) which is associated with a number of physiologic processes in the brain including regulation of energy balance and epilepsy. NPY is the most abundant neuropeptide secreted in the brain. The NPY knockout mice are more susceptible to develop seizures (Bannon et al., 2000).

Neurons can get infected following viral (flavi, paramyxo, rhabdo, polio, herpes), bacterial (*Streptococcus pneumoniae*, *Neisseria meningitidis*, *Listeria monocytogenes*) and parasitic infections (toxoplasmosis, cerebral cysticercosis, trichinellosis), which can induce neuronal death and lead to severe neurological disease. Neuronal death can also be induced by non-neurotropic viruses (Retroviruses) and bacteria through infection of other brain cells, and the release of TNF and other neurotoxins. A better understanding of how neurons and glial cells communicate with each other and induce immune responses in the brain is necessary in combating the neurologic diseases and in developing new treatments.

#### **1.1.2.5. Macrophages**

In addition to microglia, the CNS also contains macrophage populations located in the perivascular space (perivascular macrophages), circumventricular organs, the choroid plexus and the meninges. These are considered to be the primary resident immune cells along with microglia. Perivascular macrophages can be easily distinguished from microglia as they are elongated and are located in close proximity to capillary endothelial cells. Macrophages are more phagocytic than microglia and express slightly different cell surface markers including higher levels of CD45 (Table. 1.2.). Macrophages have a higher turnover rate than microglia (Hickey and Kimura, 1988; Lassmann and Hickey, 1993). Both macrophages and microglia produce proinflammatory cytokines and chemokines in response to infections in the CNS (Dickson et al., 1993; Minagar et al., 2002).



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**Table 1.2. Markers to Identify Non-Glial Cells in the CNS**

**Neurons**

**B-III- tubulin** (*Tubulin, beta 3*). Microtubule protein expressed exclusively in neurons.

**MAP2** (*Microtubule-associated protein 2*). Neuron-specific cytoskeletal protein enriched in dendrites.

**NeuN** (Neural specific nuclear protein). Expressed in most neuronal cell types in the CNS of adult mice.

**Ependymal Cells**

**CD24** Expressed by ependymal cells.

**S100b** Expressed by ependymal progenitor cells.

**Meningeal Cells**

**Fibronectin** A glycoprotein involved in cell adhesion and migration processes.

**Laminin** Extracellular matrix glycoprotein, implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, and neurite outgrowth.

**Versican** Chondroitin sulfate proteoglycan 2 expressed and secreted by meninges.

**Perivascular Macrophages**

**IBA1 / Aif1** (ionized calcium binding adaptor molecule 1 / allograft inflammatory factor 1). Induced in microglia following activation and a marker for immunohistochemical analysis.

**F4/80/ Emr1** (*EGF-like module containing, mucin-like, hormone receptor-like sequence 1*). Upregulated following activation of microglia, thus helps in identification of activation state of microglia.

**CD45<sup>hi</sup>** Express CD45 at higher levels compared to microglia.

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#### **1.1.2.6. Brain Capillary Endothelial Cells**

Endothelial cells line the blood vessels in the brain and serve important barrier functions in the brain. Endothelial cells form tight junctions between each other and thus form the blood-brain barrier (BBB) preventing the entry of harmful pathogens into the CNS microenvironment. Endothelial cells also associate with astrocytic end feet, which cover the basement membrane of the blood vessels and further potentiates the BBB formed by tight junctions of the capillary endothelial cells (Saunders et al., 2000).

#### **1.1.2.7. Ependymal Cells**

Ependymal cells are a single layer of uninterrupted cells, consisting of ciliated squamous to columnar epithelial cells lining the ventricles of the brain and spinal cord. These cells secrete cerebrospinal fluid (CSF) and the apical cilia on the ependymal cells help in circulating the CSF in the ventricles of the brain. Ependymal cells also regulate the transport of water, ions and small molecules between CSF and neuropil, a region between neuronal cell bodies in the grey matter and thus serve an important barrier function. Ependymal cells express multiple pattern recognition receptors that might be critical in recognizing bacterial and viral infections (Hauwel et al., 2005).

#### **1.1.2.8. Meningeal Cells**

Meninges are comprised of mesodermal cells which envelop the brain parenchyma and spinal cord. Meninges play a physical role at the cerebrospinal fluid-blood barrier (Tanno et al., 1993) and also synthesize and secrete many proteins that constitute cerebrospinal fluid (Ohe et al., 1996). Meninges modulate survival of neurons and glial cells by secretion of growth factors, including insulin-like growth factor (IGF)-II and IGF binding protein-2 (Ishikawa et al., 1995a; Ishikawa et al., 1995b; Ohe et al., 1996). Meninges of the cerebral cortex were shown to secrete

proinflammatory cytokines including TNF, interleukin(IL)-1 $\beta$  and IL6 during systemic inflammation and activate astrocytes and microglia (Wu et al., 2005).

### **1.1.3. Immune Privilege in the CNS**

The CNS is considered immune privileged because of the presence of the blood-brain barrier mechanisms that control the exchange and transportation of molecules between peripheral circulation and central nervous system. There is no lymphatic system within the brain, limiting the entry of lymphocytes. There are also multiple barrier mechanisms involved in the brain. These include the tight junctions between blood capillary endothelial cells (Blood-brain barrier), tight junctions at apices of epithelial cells of choroid plexus (Blood-CSF barrier), intercellular junctions between radial glia end-feet and basement membrane (Outer CSF-brain barrier), and the junctions between neuroepithelial cells of the neuroependyma (Inner CSF-brain barrier) (Saunders et al., 2000).

The barriers in the developing brain are considered immature compared to the adult brain. In rodents, the first blood vessels in the developing brain appear at 10-11 day gestation. However, glial cell development starts from 17 days of gestation and continues to develop even after birth. Thus, the initial blood vessels in the fetal brain and immediately after birth are not covered by astrocytic end feet and are not completely protected from entry of cells or pathogens from the peripheral circulation (Saunders et al., 2000).

The presence of these blood-brain barriers and the absence of a lymphatic system restrict entry of immune cells into the brain from peripheral circulation. Because of these barrier mechanisms, the majority of the professional immune cells including dendritic cells, granulocytes and lymphocytes are absent in the CNS. Thus, the innate immune system of the brain mainly involves the resident cells of the brain called glia cells, primarily astrocytes and microglia, which are key cellular mediators of neuroinflammatory processes.

## **1.2. RECOGNITION OF PATHOGENS**

### **1.2.1. History of Pattern Recognition Receptors (PRRs)**

The discovery of pattern recognition receptors and their key role in sensing and recognition of microbes has established innate immunity as the missing link between detection and recognition of microbial pathogens and activation of the adaptive immune system (Fleer and Krediet, 2007). The role of PRRs in innate immunity was first described in *Drosophila*. *Drosophila* 'toll' was originally identified, through a mutagenesis screen, for its requirement in the establishment of dorso-ventral patterning of the developing embryo (1985) (Anderson et al., 1985; Anderson et al., 1992). Later, this gene was cloned and characterized as a transmembrane receptor with an intracytoplasmic domain having striking similarities to that of interleukin-1 receptor (IL-1R) and thus referred as TIR (Toll/IL-1R) domain (Hashimoto et al., 1988). However, the ectodomain of toll is composed of leucine rich repeats (LRRs) flanked by cysteine rich motifs that differ from the IL-1R ectodomain which is composed of immunoglobulin like motifs.

In 1996, the toll receptor was shown to control antifungal responses in adult *Drosophila* showing for the first time the involvement of *Drosophila* toll in adult immunity (Lemaitre et al., 1996). A role for toll receptor in resistance to gram-positive bacterial infections was also established (Rutschmann et al., 2002). In 1997, a mammalian homologue of *Drosophila* toll was identified that recognizes a bacterial cell wall component, lipopolysaccharide (LPS) (Medzhitov et al., 1997). This was later designated toll-like receptor 4 (Rock et al., 1998a). Subsequently, a family of proteins structurally related to *Drosophila* toll were identified and collectively referred to as toll-like receptors (TLRs) (Rock et al., 1998a). Further studies lead to identification of several classes of microbe-sensing receptors, collectively referred to as PRRs.

### 1.2.2. Pattern Recognition Receptors

The initiation of inflammation is often associated with the recognition of pathogen-associated molecular patterns (PAMPs), the repeated structural motifs that are unique to microorganisms (Akira et al., 2001; Janeway, Jr., 1992; Medzhitov and Janeway, Jr., 1997). This recognition of microorganisms is mediated by several families of germline-encoded receptors, called pattern recognition receptors (PRRs). These receptors collectively survey the extracellular space, intracellular compartments and cytoplasm for signs of infection or tissue damage.

#### 1.2.2.1. Extracellular PRRs

Extracellular PRRs are a group of pattern recognition receptors that are secreted into the body fluids and opsonize the pathogens and PAMPs in the body fluids. For example, bacterial

**Table 1.3. Types of Pattern Recognition Receptors.** Taken from Jayalakshmi Krishnan et al, Experimental and molecular medicine, 2007, 39 (4): 421-438.

Extracellular Receptors	Membrane-Associated Receptors	Intracellular Receptors	Endogenous Receptors
LPS binding protein (LBP) MBL, C1q, C3, SAP, CRP, PTX3	<u>Plasma membrane</u> TLR1 TLR2 TLR4 TLR5 TLR6 <u>Endosomal membranes</u> TLR3 TLR7 TLR8 TLR9	<u>Antibacterial</u> NOD-like receptors <u>Antiviral</u> RIG1 MDA5 PKR OAS	Scavenger receptor CD36 NALP3

**Abbreviations:** **LBP**-LPS binding protein, **MBL**-Mannose binding lectin, **C1q** and **C3**-complement components, **SAP**-serum amyloid P-component, **CRP**-C reactive protein, **PTX3**-pentraxin-related gene, rapidly induced by IL-1 beta, **TLR**-Toll like receptor, **NOD** (Nucleotide binding oligomerisation domain)-like receptors (NLRs), **RIG1**-Retinoic acid inducible gene 1, **MDA5**-Melanoma differentiation associated gene 5, **PKR**-Protein Kinase R, **OAS**-2,5 oligo adenylate synthetase 1A, **CD36**-Cluster of differentiation 36, **NALP3**- NLR family, pyrin domain containing 3.

lipopolysaccharides (LPS) can be captured by LPS binding protein (LBP) in the blood and these LPS-LBP complexes are recognized by toll-like receptor 4 (Jack et al., 1997; Wurfel et al., 1997). Complement pathways are activated by pentraxin family members which include serum amyloid protein, C reactive protein (CRP), pentraxin 3 (PTX3), mannose binding lectins (MBLs), and complement components C3 and C1q (Garlanda et al., 2005; Gasque, 2004).

#### **1.2.2.2. Intracellular/Cytoplasmic PRRs**

Intracellular PRRs are a group of pattern recognition receptors that are located in the cytoplasm and survey the cytoplasm for signs of infection. These include NOD-like receptors (NLRs) and RIG-like receptors (RLRs).

##### **• NOD-Like Receptors**

NOD (Nucleotide binding oligomerisation domain)-like receptors (NLRs) are a family of intracellular PRRs, located in the cytoplasm, that recognize the bacterial peptidoglycans (Chamaillard et al., 2003a; Chamaillard et al., 2003b; Inohara and Nunez, 2003; Ulevitch, 2004). NODs consist of more than 20 members including NOD1 and NOD2 (Chamaillard et al., 2003b; Inohara and Nunez, 2003; Ulevitch, 2004). NOD proteins have three functional domains: a ligand recognition domain (LRD) containing leucine rich repeats for ligand recognition, a centrally located NOD and an effector binding domain (EBD) that contain caspase recruitment domains (CARD) or pyrin domains. The signaling from EBD leads to activation of caspases and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Chamaillard et al., 2003b; Inohara and Nunez, 2003; Ulevitch, 2004).

##### **• RIG-Like Receptors**

Viral components in the cytoplasm can be recognized by multiple sensors including retinoic acid inducible gene 1 (RIG-1), melanoma differentiation associated gene 5 (MDA 5, also called Helicard) and LGP2, collectively called RIG-like receptors (RLRs). RLRs recognize RNA

structures including double stranded RNA and 5'-triphospho RNA, which is normally not present in the cytoplasm due to the 5'-CAP of cellular RNA. RIG-1 is a RNA helicase containing two caspase recruiting domain (CARD)-like domains. Helicase domain interacts with double stranded (ds) RNA, while CARD-like domains are important for downstream signaling to IRF3, NF- $\kappa$ B and MAP kinases (Kato et al., 2005; Yoneyama et al., 2004). MDA5 is structurally similar to RIG-1 which also contains two CARD-like domains and a single helicase domain. RIG-1 is suggested to mediate antiviral response through recognition of dsRNA (Andrejeva et al., 2004; Kang et al., 2002; Kovacsovics et al., 2002; Yoneyama et al., 2005). Other cytoplasmic viral sensors include protein kinase R (PKR) and 2,5 oligo adenylate synthetase 1A (OAS). PKR and OAS will be activated in the presence of dsRNA in the cytoplasm leading to RNA degradation and cellular apoptosis (Stark et al., 1998).

### **1.2.2.3. Membrane Associated PRRs**

Membrane associated receptors mainly include toll-like receptors (TLRs).

### **1.2.3. Toll-Like Receptors**

There are at least 11 identified TLRs in humans and 12 TLRs in mice (Kaisho and Akira, 2006). TLRs are either located on the plasma membranes or intracellular membranes. TLR4, TLR5 and the heterodimers TLR1-TLR2 and TLR2-TLR6 are localized to the plasma membrane and recognize bacterial and fungal cell wall components. In contrast, TLR3, TLR7, TLR8 and TLR9 are primarily or completely located in intracellular membranes (endoplasmic reticulum, endolysosomes) and recognize nucleic acids.

A series of genetic studies revealed that TLRs recognize a wide range of PAMPs. TLR2 in concert with TLR1 or TLR6 recognizes various gram-positive cell wall components (Takeuchi et al., 1999; Takeuchi et al., 2001; Takeuchi et al., 2002). TLR4 recognizes LPS of gram-negative bacteria (Hoshino et al., 1999; Takeda and Akira, 2005). TLR3 recognizes dsRNA

produced during replication of many viruses (Alexopoulou et al., 2001). TLR5 recognizes bacterial flagellin (Hayashi et al., 2001). TLR7 and TLR8 recognize guanidine-uridine rich single stranded (ss) RNA from multiple viruses (Barchet et al., 2005; Diebold et al., 2004; Heil et al., 2004; Hornung et al., 2004), whereas TLR9 recognizes bacterial and viral CpG DNA motifs (unmethylated DNA motifs containing CpG nucleotides flanked by 5' purines and two 3' pyrimidines) (Hemmi et al., 2000).

### **1.2.3.1. Structure of Toll-Like Receptors**

All of the TLRs are structurally similar and are type-I transmembrane proteins (Rock et al., 1998a; Rock et al., 1998b). TLRs belong to a super family called the Toll/IL-1 receptor family and all TLRs contain three domains: an extracellular domain, a transmembrane domain and an intracellular domain (Rock et al., 1998a). The extracellular domain of TLRs has leucine rich repeats. The extracellular leucine rich repeat structure has 24-29 amino acid repeats and has an important role in recognizing PAMPs. The cytoplasmic portion of TLRs shows high similarity to that of interleukin 1 (IL-1) receptor family and is called Toll/IL-1 receptor domain (TIR). The TIR domain has approximately 200 amino acids and is important for downstream cellular signaling and contains three regions of importance, termed Box1, Box2 and Box3. Box1 is the signature sequence of TIR domain, Box2 forms a loop in the structure which probably engages with other TLRs or down stream adaptor molecules. The function of Box3 is not well known although it contains signaling residues (Slack et al., 2000).

### **1.2.3.2. Toll-Like Receptor Signaling**

The initiation of signaling pathways originates from cytoplasmic TIR domains upon binding of specific ligands or PAMPs to the extracellular leucine rich repeat (LRR) domain. Ligand binding initiates recruitment of one or more adaptor proteins that include MyD88 (Myeloid differentiation primary response protein 88), TIRAP (TIR domain containing adaptor



protein), TRIF (TIR domain containing adaptor protein inducing IFN $\beta$ , or TICAM1) or TRAM (TRIF related adaptor molecule or TICAM2). All TLRs use MyD88 as an adaptor protein (MyD88 dependent pathway), except TLR3 which utilizes the adaptor protein TRIF (MyD88 independent pathway). TLR1/2 and TLR2/6 utilize both MyD88 and TIRAP, whereas TLR4 utilizes all four adaptors forming TLR4-TIRAP-MyD88 complex or TLR4-TRAM-TRIF complex. Signaling events downstream of MyD88 or TRIF differ, although the outcome of these signaling pathways is conceptually similar with activation of transcriptional factors including NF- $\kappa$ B, Interferon-regulatory factors (IRFs) and other general transcriptional factors.

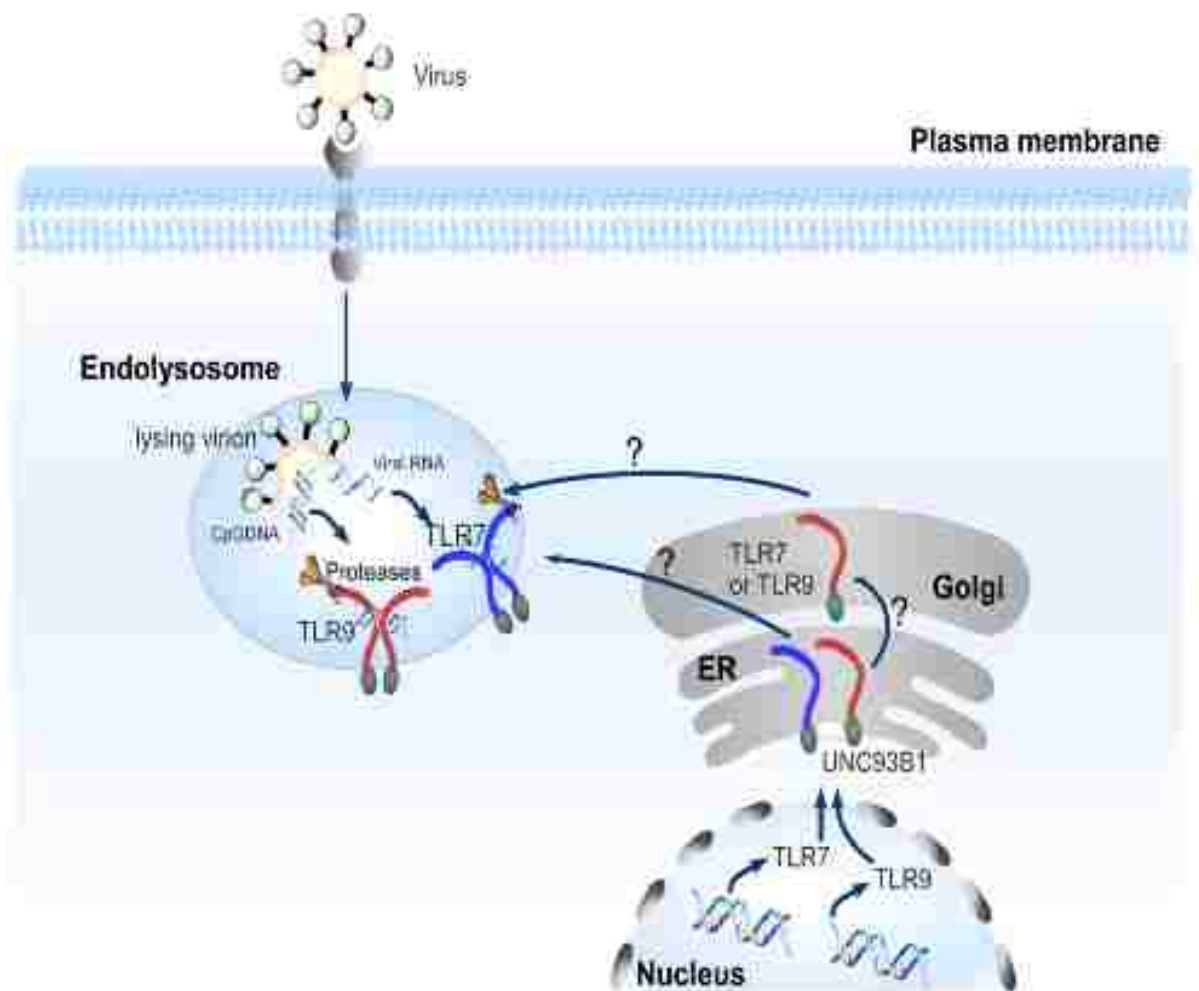
#### **1.2.4. TLR9 Family Members**

TLR7, TLR8 and TLR9 form a separate group of TLRs with high protein homology (Heil et al., 2003; Hornung et al., 2002; Kadowaki et al., 2001; Lund et al., 2004). All three receptors are located in the endolysosomal compartments, require endosomal acidification for activation and signal through the same adaptor molecule, MyD88. Additionally, all three receptors recognize nucleic acids, which require either intracellular infection of a pathogen or phagocytosis of these components from cellular debris, free virus or bacteria. The genes for TLR7 and TLR8 are located close to each other on the X chromosome and show 42.2% sequence identity and 72.7% protein homology.

##### **1.2.4.1. Trafficking and Processing of Toll-Like Receptors 7 and 9**

The intracellular localization of the endosomal TLRs vary with localization in early endosomes, late endosomes, lysosomes, multivesicular bodies, endoplasmic reticulum, golgi complex or plasma membrane (Chockalingam et al., 2009; Hacker et al., 1998; Latz et al., 2004; Leifer et al., 2004). TLR7 and TLR9 are synthesized in the endoplasmic reticulum (ER), pass through the golgi and are sorted to the endolysosomal compartments (Fig. 1.1.). The TLR7/9 trafficking is controlled by a multi transmembrane protein called Unc93B1, which resides in the

endoplasmic reticulum. A missense mutation in the Unc93B1 gene (H412R) inhibits its binding to the transmembrane domains of TLR 3/7/9 (Brinkmann et al., 2007; Tabeta et al., 2006). Unc93B1 was reported to deliver TLR7/9 from the ER to endolysosomes where TLR7/9 recognizes their ligands (Brinkmann et al., 2007; Tabeta et al., 2006) (Fig. 1.1.). Following their arrival in the endolysosomes, TLR7 and TLR9 are proteolytically cleaved which generates functionally competent receptors (Ewald et al., 2008; Park et al., 2008). As a result, delivery of ligands to these compartments appears to be a prerequisite for receptor activation. However,



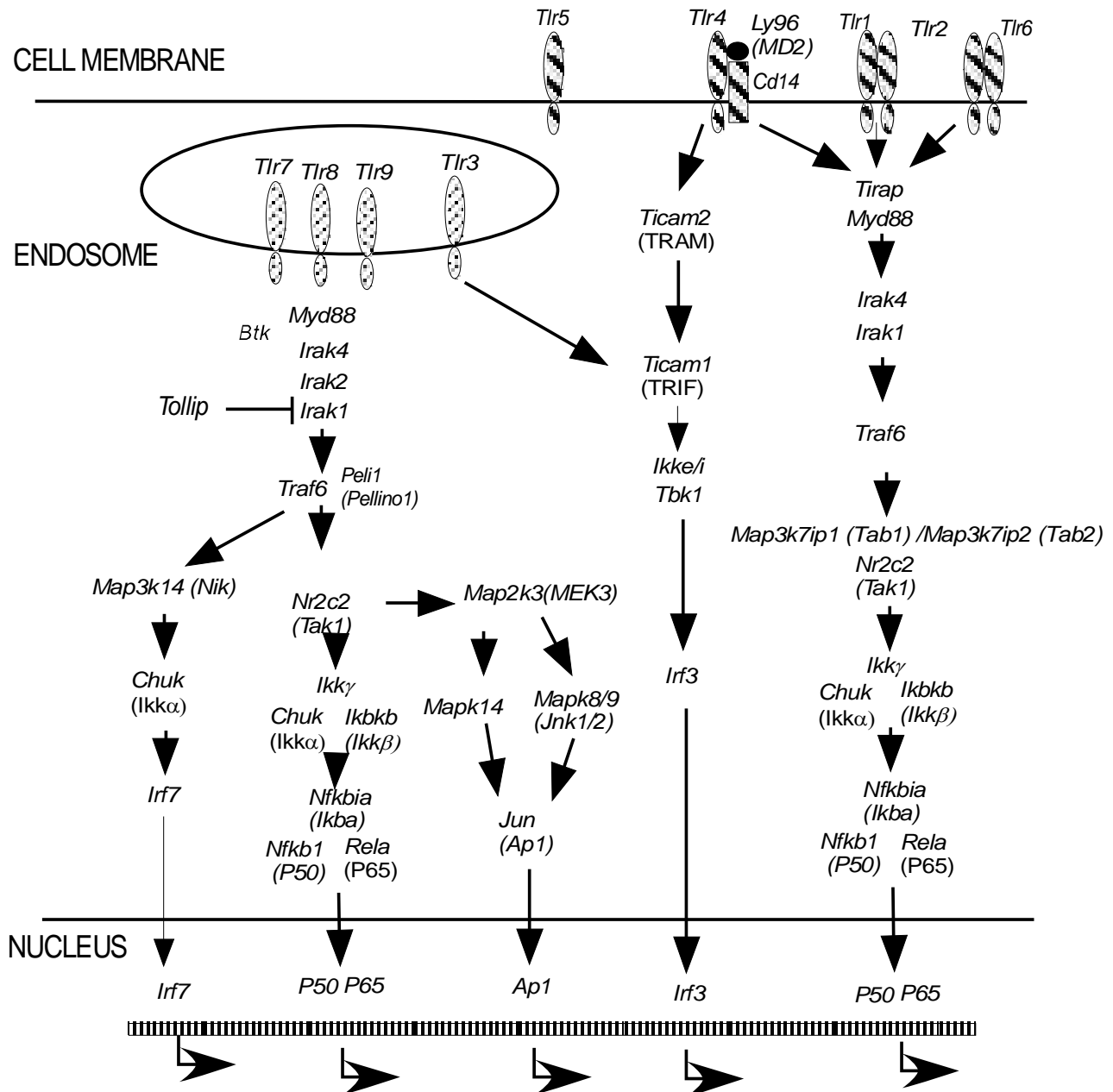
**Fig. 1.1. Trafficking and Processing of Toll-Like Receptors 7 and 9. Adapted from Barton and Kagan, Nature Reviews Immunology, 2009, 9:535-542.**

these receptors cannot be detected in the endolysosomal compartments prior to ligand stimulation. It is not known what signal leads to the translocation of these receptors into the endolysosomes and proteolytic cleavage of the receptor to generate a functional receptor. TLR8 is also expected to be delivered from the endoplasmic reticulum to endolysosomes through Unc93B1, and proteolytically cleaved to generate a functional receptor in a similar fashion to TLR7 and TLR9 because of their common features.

Interestingly, recent studies indicate that Unc93B1 inversely links TLR7 and TLR9 responses, although both receptors require Unc93B1 for their transportation to endolysosomes. A mutation in the N-terminal region of Unc93B1 enhanced TLR7 trafficking and responsiveness, but suppressed TLR9 trafficking and responsiveness in bone marrow derived dendritic cells, indicating that Unc93B1 represses TLR7 responses and enhances TLR9 responses (Fukui et al., 2009). However, it is not known how Unc93B1 is regulated in altering the TLR7 or TLR9 responses.

#### **1.2.4.2. TLR7, TLR8 and TLR9 Signaling**

All three receptors of the TLR9 family are dependent on recruitment of the adaptor protein MyD88 with the exception of TLR8 stimulation of neurons (Hemmi et al., 2002; Lund et al., 2004; Ma et al., 2006; O'Neill and Bowie, 2007). Binding of ligands to TLR9 was shown to facilitate formation of homodimers (Latz et al., 2007), and the interactions of the TLR9 family receptors with MyD88 may be dependent upon the conformational changes in the homodimers during ligand binding (Latz et al., 2007). MyD88 harbors a TIR domain and a death domain. The TIR domain of MyD88 interacts with the carboxy terminal of the TIR domain of the TLR. The amino terminal death domain of MyD88 interacts with the corresponding domain of IRAK4 (interleukin 1 receptor associated kinase 4), which then recruits IRAK1. Upon stimulation, IRAK4 and IRAK1 undergo sequential phosphorylation and dissociate from MyD88, resulting in



**Fig. 1.2. TLR7, 8, and 9 Signaling.** All TLR9 family members share a common pathway called the MyD88-dependent pathway, which activates either NF-κB to upregulate proinflammatory cytokine and chemokines, or IRF7 to induce interferons.

activation of the ubiquitin ligase, TRAF6 (tumor necrosis factor receptor associated factor 6) (Fig. 1.2.). TRAF6 activation leads to activation of p38 MAPK, interaction with the IKK complex, NF-κB activation and the upregulation of proinflammatory cytokine and chemokine

mRNA. MyD88 signaling of IRAK4 can also lead to type 1 interferons (IFN $\alpha$  and IFN $\beta$ ) through IRF7 phosphorylation and translocation into the nucleus (Kawai and Akira, 2006).

#### **1.2.4.3. TLR7, TLR8 and TLR9 Agonists**

- **TLR7/8 Agonists**

TLR7 was originally identified as eliciting antiviral effects when stimulated by the family of guanosine based imidazoquinoline compounds (Hemmi et al., 2002; Lee et al., 2003) which includes imiquimod (R837), resiquimod (R848) and loxoribine. The antiviral activity of imiquimod was first shown in guinea pigs infected with herpes simplex virus (Miller et al., 1999). Imiquimod was FDA approved (1977) for treatment of external genital warts caused by human papilloma virus infection.

Human TLR8 also recognizes R848 but surprisingly not R837. Neither of these compounds were recognized by mouse TLR8 suggesting that TLR8 may be nonfunctional in mice (Jurk et al., 2002). Recent studies indicate that murine TLR8 might be functional as poly-thymidine oligonucleotides (pT-ODN) in combination with imidazoquinoline compounds, such as R848 and CL075, induced NF- $\kappa$ B activation in human embryonic kidney cells (HEK293) transfected with murine TLR8- and primary TLR8-expressing mouse cells (Gorden et al., 2006a; Gorden et al., 2006b).

More synthetic agonists were recently identified that signal through either TLR7 or TLR8. CL087 (TLR7 agonist) is an adenine derivative that activates NF- $\kappa$ B and induces secretion of IFN $\alpha$  in human TLR7-expressing cells, but does not stimulate human TLR8 even at high concentrations (Kornbluth and Stone, 2006). CL097 (TLR7/8 agonist) is a highly water soluble derivative of imidazoquinoline compound R848 and stimulates both human TLR7 and human TLR8, similar to R848 (Salio et al., 2007). CL075 (TLR8/7 agonist) (also called 3M002) is a thiazoloquinoline derivative that stimulates TLR8 in human PBMC and induces TLR7 to a

lesser extent (Gorden et al., 2005). However, none of these compounds are tested in mouse cells and it is not known what receptors these molecules signal through.

Recent studies identified guanidine-uridine rich single stranded RNA (ssRNA) from viruses as the natural agonists of TLR7 and TLR8. ssRNA from influenza virus, Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) activate TLR7 *in vitro* (Diebold et al., 2004; Heil et al., 2004; Kato et al., 2005; Lund et al., 2004). IFN $\alpha$  production by VSV and influenza viruses was suppressed in TLR7 deficient mice (Diebold et al., 2004; Lund et al., 2004). Human TLR8 was shown to be involved in recognition of Coxsackie B virus and human parechovirus 1 (Triantafilou et al., 2005a; Triantafilou et al., 2005b). These studies suggest that TLR7 and TLR8 may play an important role in neuropathogenesis as several viral families with ssRNA; including flaviviruses, paramyxoviruses, rhabdoviruses and retroviruses, can infect the CNS and induce neurological disease (Beignon et al., 2005; Chambers and Rice, 1987; de la Torre, 2002; Mandl, 2005).

TLR7 was also shown to sense synthetic RNA oligonucleotides from HIV (ssRNA40), containing phosphothioate-protected RNA oligonucleotides with a GU-rich sequence from the U5 region of HIV-1 RNA. Upon stimulation with ssRNA40, murine TLR7 and human TLR8 induced the activation of NF- $\kappa$ B, whereas human TLR7 and murine TLR8 failed, implying a species specificity difference in ssRNA recognition. RNA41 and RNA42, two derivatives of RNA40 in which all U or G nucleotides were replaced with adenosine, are not recognized by either human TLR7 or murine TLR8 indicating TLR7 or TLR8 recognizes GU-rich ssRNA's (Heil et al., 2004).

#### • **TLR9 Agonists**

TLR9 was initially shown to recognize unmethylated DNA from viruses or bacteria that contains CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines (CpG-DNA)

(Bauer et al., 2001; Hemmi et al., 2000). Later, synthetic oligodeoxynucleotides (ODN) containing these CpG motifs were shown to mimic the immunostimulatory effects of bacterial DNA in macrophages and dendritic cells (DCs) (Krieg, 2002). *In vivo* production of proinflammatory cytokines and type-I IFNs in response to murine cytomegalovirus (MCMV), HSV1 and HSV2 were dependent on recognition by TLR9 (Barton, 2007). The TLR9 mediated IFN $\alpha$  secretion was shown to be limited to plasmacytoid dendritic cells that secrete high amounts of IFN $\alpha$  in response to viral infections (Hornung et al., 2002; Kadowaki et al., 2001; Krug et al., 2001).

CpG-ODNs are classified into three different classes, Class A, B and C, based on primary sequence motifs, secondary and tertiary structures and different responses in B-cells and plasmacytoid DCs (pDCs). The first described CpG-ODN belongs to class B, which contains CpG dinucleotide motifs with a phosphorothioate backbone. CpG-B ODNs strongly activate B-cells to develop into plasma cells, and induce TNF secretion from pDCs, but are weak inducers of IFN $\alpha$ . CpG-A ODNs contain a phosphodiester linked central palindromic motif and phosphorothioate poly-G tails that enable the formation of complexes and higher order structures. CpG-A ODNs induce high levels of IFN $\alpha$  by pDCs, but are poor activators of B-cells. CpG-C ODNs combine the properties of CpG-A and CpG-B ODNs, with a CpG containing palindromic motif and a phosphorothioate backbone respectively. CpG-C ODNs induce intermediate levels of IFN $\alpha$  in pDCs and directly activate B-cells (Jurk and Vollmer, 2007).

#### **1.2.4.4. TLR Agonists and Receptor Interactions**

The co-operative interactions among TLR9 family receptors may modulate the inflammatory response to their PAMPs (Berghofer et al., 2007; Marshall et al., 2007; Wang et al., 2006). Synergistic co-activation among multiple TLRs has been observed *in vitro*, including synergistic activation of TLR3 and 7 (Gautier et al., 2005; Napolitani et al., 2005; Roelofs et al.,

2005; Warger et al., 2006), TLR4 and 7 (Gautier et al., 2005; Napolitani et al., 2005; Roelofs et al., 2005), TLR3 and 9 (Whitmore et al., 2004) and TLR2/4 and 9 (Agren et al., 2006).

Simultaneous triggering of TLR7 and 9 in pathogen infection has been suggested. For example, the herpesviruses have been recently shown to activate both TLR7 and TLR9 signaling (Zucchini et al., 2008a). DNA and RNA derived from bacterial pathogens and dead cells are reported to stimulate TLR3, 7, and 9, as well as cell surface TLR2 and TLR4/MD-2 (Kariko et al., 2005).

Interaction between the TLR9 family has also been suggested *in vitro*. Co-expression of either TLR8 or TLR9 with TLR7 in HEK cells inhibited the response of these cells to TLR7 agonist stimulation, but not TLR8 or TLR9 agonist stimulation. Co-transfection of TLR8, but not TLR7, along with TLR9 inhibited TLR9 agonist-induced responses, while neither TLR7 nor TLR9 altered TLR8 agonist-induced responses in HEK cells (Wang et al., 2006). This suggests a hierarchy among TLR9 family receptors in suppressing responses, with TLR8 suppressing both TLR7 and TLR9 responses, TLR9 suppressing TLR7 responses and TLR7 having no suppressive effect on either TLR8 or TLR9 responses (TLR8>TLR9>TLR7).

TLR agonist-induced signaling may also impact other members of the TLR9 family. For example, TLR7/TLR9 co-stimulation demonstrated that synthetic and natural TLR7 agonists can suppress TLR9 (CpG-A and CpG-C ODN) induced IFN $\alpha$  production by pDCs and B cells (Berghofer et al., 2007; Marshall et al., 2007). This study also reported the down-regulation of TLR9 expression by TLR7 signaling in pDCs (Marshall et al., 2007). The predicted mechanism for these interactions is the generation of heterodimers between TLR7, TLR8, and TLR9, that could inhibit MyD88 binding and the subsequent signal transduction cascade (O'Neill and Bowie, 2007). Thus, the relative ratio of TLR7, TLR8 and TLR9 in the endosomes may influence receptor signaling and cytokine production.



### **1.3. TOLL-LIKE RECEPTORS AND THE CNS**

#### **1.3.1. Expression of TLRs in the CNS**

Microglia and astrocytes are endogenous cells in the CNS that respond to the infections caused by pathogens, and are responsible for production of proinflammatory cytokines and chemokines. So, it is expected that brain cells constitutively express TLRs. However, the expression of multiple TLRs in the cultured brain cells was mostly studied at mRNA level because of the unavailability of antibodies until recently.

Microglia express mRNA for all known TLRs including TLR9 family receptors. (Dalpke et al., 2002; Iliev et al., 2004; Olson and Miller, 2004; Zhang et al., 2005; Zuiderwijk-Sick et al., 2007). Expression of TLRs on astrocytes is not as clear because of inconsistent reports. Murine astrocytes were shown to express mRNA for TLRs from 1-10, with the exception of TLR8 (McKimmie and Fazakerley, 2005). However, others reported that astrocytes express TLR3 mRNA at robust levels, TLR 1, 4, 5, and 9 at low-level, and TLR 2, 6, 7, 8, and 10 mRNA at rare-to-undetectable by quantitative real-time PCR (Jack et al., 2005). Human astrocytes were shown to express only TLR2 and TLR4 mRNA (Carpentier et al., 2005). Adult neurons express only TLR3 mRNA (Prehaud et al., 2005).

*In vivo* immunofluorescence studies revealed the absence of TLR1, TLR3, TLR4 and TLR9 expression in normal mouse brain at protein level (Mishra et al., 2006). However, TLR2 expression was detected on microglia, astrocytes and neurons using respective cell specific markers (Mishra et al., 2006). TLR7 was primarily localized to brain capillary endothelial cells and ependymal cells of the choroid plexus (Lewis et al., 2008). TLR8 positive cells in the adult brain were mostly confined to cells in the white matter of periventricular, subcortical and cerebellar regions of the brain. Immunohistochemistry studies did not identify expression of TLR7, TLR8, or TLR9 on either astrocytes or microglia or neurons. However, embryonic

neurons expressed TLR8, both *in vitro* and *in vivo* (Ma et al., 2006). Thus, further investigation for the protein expression of TLRs using more sensitive techniques including flow cytometry or western blot techniques is needed.

### **1.3.2. Intrinsic Functions of TLRs in the CNS**

TLRs serve important functions in the brain development and homeostasis. TLR2 and TLR4 were recently shown to regulate adult hippocampal neurogenesis in rodents (Rolls et al., 2007). Adult neuroprogenitor cells (NPCs) express both TLR2 and TLR4, with these two receptors serving opposing functions in proliferation and differentiation of NPCs (Rolls et al., 2007). TLR2 activation promotes neuronal differentiation, while TLR2 inhibition suppresses neuronal differentiation and astroglial differentiation. In contrast, TLR4 activation inhibits both neuronal differentiation and self renewal of NSCs. *In vivo* activation of TLR4 in neonates was shown to alter memory functions in adult rats (Bilbo et al., 2006). TLR3 was also reported as a negative regulator of embryonic NPCs (Lathia et al., 2008).

TLRs also appear to play a role in brain development. TLR8 expression was recently detected on neurons at both embryonic and neonatal stages, and stimulation of TLR8 in neuronal cultures inhibit dendrite growth and induce caspase 3 activation, suggesting a role for TLR8 in regulating neuron growth during brain development (Ma et al., 2006). Certain TLRs may also have an active role in non-pathogen mediated diseases in the CNS. For example, the adaptor protein Myeloid Differentiation factor 88 (MyD88) which binds to several TLRs is an important mediator of brain injury (Koedel et al., 2007). These studies indicate the role of multiple TLRs in the intrinsic functions of the brain including brain development and homeostasis.

### **1.3.3. Role of TLRs in Neurologic Disease**

TLRs have been shown to have a role in disease pathogenesis in the brain. TLR2 and TLR4 play an important role in antibacterial responses elicited by microglia and astrocytes both

*in vitro* and *in vivo* including *Staphylococcus aureus*, or group B streptococci infections as well as meningitis induced by *E. coli* LPS (Lehnardt et al., 2006; Qin et al., 2005; Takeuchi et al., 2000; Zhou et al., 2006). TLR2 also plays a role in viral infections, including herpes simplex virus 1 (HSV1), and is necessary for the proinflammatory cytokines and chemokines production by microglia following HSV1 infection (Aravalli et al., 2005). TLR3 plays an important role in the neuropathogenesis of West Nile virus (WNV) infection and herpes simplex virus infections (Wang et al., 2004). Furthermore, TLR3 signaling in astrocyte and microglia cells is activated by multiple demyelinating diseases including Coxsackie virus, Theiler's murine encephalitis virus (TMEV) and HIV infections (Aravalli et al., 2007). TLR3 also has a significant role in breaking the BBB during virus infections as demonstrated in WNV infection (Wang et al., 2004).

Less is known about the role of TLR9 family members, TLR7, TLR8, and TLR9, in response to pathogens. TLR7 may play an important role in neuropathogenesis as several viral families with ssRNA, including flaviviruses, paramyxoviruses, rhabdoviruses and retroviruses, can infect the CNS and induce neurological disease (Beignon et al., 2005; Chambers and Rice, 1987; de la Torre, 2002; Mandl, 2005). TLR7 was shown to mitigate lethal WNV encephalitis (Town et al., 2009). It is postulated that microglia cells sense brain invading WNV through TLR7 dependent recognition of ssRNA and secrete IL-23 promoting viral clearance (Town et al., 2009). Recent studies on retroviral infections utilizing murine polytropic retroviral infection demonstrated a role of TLR7 in inducing significant proinflammatory cytokines and chemokines and astrocyte activation early in the infection (Lewis et al., 2008). These studies show that TLR7 may play a role in the pathogenesis of other neurovirulent ssRNA viruses, both in eliciting innate immune responses and in recruitment of inflammatory cells.

TLR9 recognizes the DNA genomes from neurovirulent viruses like HSV-1 and mouse CMV that are rich in CpG motifs. TLR9 was shown to act in synergy with TLR2 in recognizing

HSV-1 to induce early cytokine and cellular response and thus restrict viral load in the brain and prevent lethal HSV encephalitis (Sorensen et al., 2008).

#### **1.3.4. Therapeutics**

Agonists of both TLR7 and TLR9 are also being investigated for the potential use in treating CNS-related diseases and in inducing innate immune responses in the CNS (Butchi et al., 2008; El Andaloussi A. et al., 2006; Pedras-Vasconcelos et al., 2006a; Pedras-Vasconcelos et al., 2008; Prins et al., 2006). Treatment of CNS-tumor bearing mice with 5% imiquimod (along with melanoma-associated Ag-pulsed DC vaccination) resulted in synergistic reduction in CNS tumor growth (Prins et al., 2006) and intraperitoneal inoculation of CpG-ODN protects newborn mice from a lethal challenge with neurotropic tacaribe arena virus (Pedras-Vasconcelos et al., 2006a). However, the mechanisms behind these responses are not known.

Systemic injection of TLR7 agonist can induce microglia activation (Zhang et al., 2005) and repeated intraperitoneal administration of CpG-ODN have a sustainable effect of immune effectors in the brain including TNF production (Wagner et al., 2007). This suggests that TLR7 and TLR9 agonists may be able to cross the BBB in cases of insult or injury. However TLR agonists may have pathogenic properties in the CNS. Intracerebral inoculation of CpG DNA in neonatal mice induces death in a few hours after inoculation (Pedras-Vasconcelos et al., 2006b). Intracisternally administered bacterial DNA containing CpG motifs induces meningitis in mice (Deng et al., 2001). Understanding the cell types in the brain that can be activated by TLR7 and TLR9 and the downstream affects of this activation is important for understanding both viral pathogenesis as well as potential use of TLR agonists in the treatment of neurological diseases.

Several studies have demonstrated the ability of microglia to respond to TLR7/TLR9 ligands (Gurley et al., 2008). Microglia stimulated with ssRNA and CpG-ODNs can produce IL-12 (p40), CXCL2, CCL2 and/or TNF (Gurley et al., 2008). TLR9 stimulated microglia can also

produce nitric oxide (NO), and have enhanced phagocytic activity and induce neuronal damage (Dalpke et al., 2002; Iliev et al., 2004). Less is known on astrocytes, but they were shown to produce IL-12, CXCL2 and CCL2 in response to either ssRNA or CpG-ODN stimulation. These studies demonstrated that both astrocytes and microglia can respond to TLR7 or TLR9 agonist stimulation. However, an in depth comparison of the innate immune responses elicited by these cell types in response to TLR7 or TLR9 stimulation has not been completed. A better understanding of the role of these TLRs and the innate immune system itself in the CNS is important in developing new avenues in the treatment of neuroinflammatory conditions.

#### **1.4. SUMMARY**

TLR7 and TLR9 are evolutionarily closely related, highly homologous, confined to the endosomal membranes, recognize nucleic acids that they encounter in these compartments, and signal via the adaptor molecule MyD88. Despite the high levels of similarities between these receptors, direct stimulation of TLR9 in the brain causes death of the mice, while TLR7 agonist inoculation does not (Pedras-Vasconcelos et al., 2006b). Cells in the brain including astrocytes, microglia, meninges, and neurons may vary in their expression of these TLRs and respond differently to TLR7 or TLR9 stimulation by selective signal transduction pathways. However, there is a lack of basic understanding of which cell types in the brain respond to stimulation of TLR7 and TLR9 ligands, and which cellular pathways are activated following TLR7 and TLR9 stimulation. Thus, defining the responses of these cell types to TLR7 and TLR9 stimulation will provide a more clear understanding of the neuroinflammatory responses to pathogenic insult in the brain.

The interactions between TLR7 and TLR9, and their agonists may also modulate the inflammatory signaling in response to their PAMPs. An antagonistic relationship between TLR7 and TLR9 receptor responses was observed in human embryonic kidney (HEK) cells (Wang et

al., 2006). In addition TLR7/TLR9 co-stimulation studies suggested that TLR7 agonists can suppress TLR9-induced IFN $\alpha$  production by plasmacytoid dendritic cells (pDCs) and B cells (Berghofer et al., 2007; Marshall et al., 2007). TLR7 and TLR9 interactions are also important in relevance to infection as some viruses like cytomegalovirus, herpes simplex virus-1 and HIV can induce both TLR7 and TLR9 activation during infection (Beignon et al., 2005; Mandl et al., 2008; Zucchini et al., 2008b). Thus, interactions between these receptors and agonists may play an important role in regulating innate immune responses to infection and in the use of these agonists in the therapeutic treatment of diseases.

The objective of the present study is to understand the functional effects and interactions of TLR7 and TLR9, and their agonists in the central nervous system. We hypothesize that, despite the high similarity between TLR7 and TLR9, the activation of these receptors result in substantially different neuroinflammatory responses in the CNS. Furthermore, we hypothesize that different cell types in the CNS have distinct responses to TLR7 and TLR9 agonist stimulation and that there is cross-regulation between these receptors in controlling the innate immune responses by brain cells. A combination of *in vivo* studies, by inoculating TLR7 and TLR9 agonists intracerebroventricularly, and *in vitro* studies, by culturing and stimulating different cells in the brain, was utilized to analyze the neuroinflammatory pathways following stimulation of TLR7 and TLR9. Furthermore, we analyzed the functional interactions between TLR7 and TLR9 agonists and their receptors in regulating neuroinflammation in the brain by co-stimulation studies and by utilizing mice deficient in TLR7.

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**CHAPTER 2**  
**MATERIALS AND METHODS**

## 2.1. SOLUTIONS

### Common Buffers

- Phosphate Buffered Saline (4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>). 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> were dissolved in 800 ml distilled water and adjusted pH to 7.4. Volume adjusted to 1L with additional distilled water and sterilized by autoclaving.

- Tris Buffered Saline (TBS) (50 mM Tris-HCl pH 7.5, 150 mM NaCl). 30 ml of 5 M NaCl and 50ml of 1M Tris-HCL, pH7.5 were added in 920ml of RNase-free water and adjusted pH to 7.5.

### Solutions for TLR agonist Inoculations *in vivo*

- 4% Trypan Blue (20X): 4 g trypan blue (Sigma-Aldrich T6146) powder was dissolved in 100 ml of distilled water, sterile filtered through 0.22 µm filter and stored at RT.

### Solutions for Immunohistochemistry / Immunocytochemistry/ Flow Cytometry

- 10% Neutral Buffered Formalin: (Sigma-Aldrich # 533998).

- 2% Paraformaldehyde: 1 ml 16% paraformaldehyde (Electron Microscopy Sciences # 15710) added to 7 ml PBS.

- 1% Saponin/10X PBS Stock: 0.5 g saponin (Sigma-Aldrich # 47036) was dissolved in 10X PBS with gentle stirring (will foam) and volume is adjusted to 50 ml with deionized water.

- 0.1% Saponin/PBS: 1% saponin/10X PBS stock solution was diluted 1 in 10 in distilled water.

- 2% BSA:(Bovine serum albumin) (Sigma-Aldrich # A9647). 2g BSA dissolved in 100 ml PBS.

- 2% Donkey Serum: (Sigma-Aldrich # D9663). 2 ml donkey serum added to 100 ml PBS.

- Normal Donkey Serum Blocking Solution: 2% donkey serum, 1% bovine serum albumin, 0.1% cold fish skin gelatin (Sigma-Aldrich # G7765), 0.1% triton X-100 (Sigma-Aldrich # T8532), and 0.05% tween 20 (Sigma-Aldrich # P9416) in 1X PBS, pH 7.2. Stored at 4°C.

- Citrate Target Retrieval Solution: 9 ml 0.1M citric acid (1.92 g citric acid, 100 ml distilled water), 41 ml 0.1M sodium citrate dihydrate (14.7 g sodium citrate, 500 ml distilled water) and 450 ml ultrapure water, pH 6.0.
- DAPI Stock Solution (5 mg/ml): 10 mg DAPI was dissolved in 2 ml dimethyl-formamide and stored at -20°C.
- DAPI Working Solution (100 ng/ml): 2 µl DAPI stock solution added to 100 ml PBS.

### **Solutions for *in situ* Hybridization Analysis**

- Xylene: (Sigma-Aldrich # 534056).
- 4% Paraformaldehyde: 10 ml 16% paraformaldehyde added to 30 ml RNase-free water.
- 200 mM HCl: 826 µl conc. HCl (Sigma-Aldrich H1758) added to 50 ml RNase-free water.
- Proteinase K Buffer: (2 mM CaCl<sub>2</sub> in TBS): 0.1 ml of 1 M CaCl<sub>2</sub> (Sigma-Aldrich # 21115) added to 50 ml of TBS. Proteinase K: (Roche # 3115887001)
- 2X SSC: (Standard Saline Citrate). 55 ml 20X SSC (Sigma-Aldrich Cat #: 15557-044), added to 500 ml RNase-free water (Invitrogen # 10977-023).
- Hybridization Buffer: 2X SSC, 10% dextran sulfate (Sigma-Aldrich # D8906), 0.01% sheared salmon sperm DNA (Invitrogen # 15632-011), 0.02% SDS (Invitrogen # 15553-027), 50% formamide (Ambion # AM9342).
- RNase Buffer: (500 mM NaCl, 100 mM Tris, pH 8.0). 5 ml 5 M NaCl (Sigma-Aldrich # S5150), 0.5 ml 1 M Tris pH 8.0 (Invitrogen # 15568-025), 45 ml RNase-free water.
- RNase A: (Roche Cat # 10109142001).
- 1X SSC/ 50% Formamide: 25 ml 2X SSC added to 25 ml Formamide (Ambion # AM9342).
- 1X SSC: 25ml 20XSSC (Sigma-Aldrich Cat #: 15557-044) added to 475ml RNase-free water.
- Maleic Acid (1 X): 55 ml 10X maleic acid stock (Roche Blocking Reagent Kit, Cat #

11585762001) added to 495 ml RNase-free water.

- Blocking Buffer: 45 ml 1X maleic acid added to 5 ml blocking reagent (Roche, Cat #

11585762001).

- Anti-Dig Antibody- Alkaline Phosphatase: (Roche, Cat # 1 093 274).
- Detection Buffer: 0.1 M Tris-HCl, pH 8.0.
- Fast Red Detection Solution: Fast red tablet (from Roche, Cat # 1 496 549) was crushed, added to 2 ml of detection buffer, and filtered through 0.22 or 0.45  $\mu$ m filter to remove particulate.
- DAB (3,3'-diaminobenzidine): (Pierce, Fisher scientific # 34002).
- Mayer's Hematoxylin: (Electron Microscopy Sciences # 26043-05).
- Tacha's Bluing Solution: (BioCare Medical # HTBLU-MX).
- Shur/Mount Water Based Mounting Medium(Electron Microscopy Sciences # 17992-01).

### **Solutions for Astrocytes and Microglia Cultures**

- 70% Percoll: (prepare 1 ml/ mouse): 7 ml Percoll (Sigma-Aldrich # P4937), 1 ml sterile 10X PBS were added to 2 ml sterile water. 30  $\mu$ l of 0.4% cresol red (Sigma-Aldrich # 114472) solution was added per 20 ml of 70% percoll. Filter sterilized and stored at 4°C for a maximum of 1 week.
- 30% Percoll: (prepare 1 ml/ mouse): 3 ml Percoll and 1 ml sterile 10X PBS were added to 6 ml sterile water. Filter sterilized and stored at 4°C for a maximum of 1 week.
- 0% Percoll: (prepare 1 ml/ mouse): 1 ml sterile 10X PBS was added to 9 ml sterile water. 30  $\mu$ l of 0.4% trypan blue (Sigma-Aldrich # T8154) solution was added per 20 ml of 0% percoll. Filter sterilized and stored at 4°C for a maximum of 1 week.
- 2% Glucose in PBS: 10 g D-glucose (Sigma-Aldrich # G5146) was dissolved in 500 ml PBS. Filter sterilized and stored at 4°C.



- Astrocyte Culture Media: Dulbecco's modified Eagle's medium (Sigma-Aldrich # D6429) containing 4,500mg glucose/L, 110mg sodium pyruvate/L, 0.584g L-glutamine/L, was supplemented with 10% inactivated fetal bovine serum (Hyclone, Fisher Scientific # SH3007003IH) and 1% penicillin-streptomycin (Invitrogen # 15140-122).
- Microglia Culture Media: Astrocyte media with 20 % LADMAC culture supernatant (mouse bone marrow cells producing macrophage colony stimulating factor/M-CSF).
- Meningeal Culture Media: Same as astrocyte media.

### **Solutions for Primary Cortical Neuron Cultures**

- Dissection Media for Neuron Cultures (CMF-HBSS: Calcium magnesium free-Hanks Balanced Salt Solution buffered with 10 mM HEPES, pH 7.3): 20 ml of 10X CMF-HBSS (Invitrogen # 14185-052) and 2 ml of 1M HEPES (Invitrogen # 15630-080) (100X) were added to 178 ml water. One set was kept ice cold and other set at room temperature.
- Digestion Media for Neuron Cultures: 0.5 ml of warm 2.5% trypsin (Invitrogen # 15090-046) was added to 4.5 ml of CMF-HBSS with HEPES just before use.
- Neuronal Plating Media: 5 ml 12% D-glucose (20x) and 5 ml 5% inactivated fetal bovine serum (IFBS) were added to 90 ml Opti-MEM with L-glutamine (Gibco # 31985).
- Neuronal Maintenance Media: 4 ml B-27 supplement (2%) (Invitrogen # 17504-044) and 0.5 ml glutamax-I 200 mM (0.5 mM final concentration) (Invitrogen # 35050-061) were added to 195.5 ml neurobasal medium (Invitrogen # 21103-049).
- NMDA (N-methyl-D-aspartic acid): (Sigma-Aldrich # M3262).

### **Solutions for MTT Cell Viability Assay**

- MTT: 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (Invitrogen # M6494). Stock concentration: 5 mg/ml in PBS. 50 mg MTT powder was dissolved in 10 ml PBS, and allowed 5-

10 min on magnetic stirrer to get yellow colored solution. Filter sterilized through 0.22  $\mu\text{m}$  filter and stored at 4°C in dark up to several months.

### **Solutions and Reagents for RNA Extraction and Real-Time RT-PCR**

Trizol reagent (Invitrogen # 15596-018), 2-propanol (Sigma-Aldrich # 19516), Chloroform (Sigma-Aldrich # 496189), Mini RNA isolation kit (Zymo Research # R1031), RNA cleanup columns (Zymo research # R1018), iScript Reverse Transcription kit (Bio-Rad # 1708891), SYBR Green Supermix with ROX (Bio-Rad # 1715852), 384-well clear plates (Applied Biosystems # 4343370).

- Master Mix for DNase Treatment: **Reaction volume**: 100  $\mu\text{l}$ . **RNA sample**: (1-2 $\mu\text{g}$ ) 5  $\mu\text{l}$  for brain tissue, (1 $\mu\text{g}$ ) 10  $\mu\text{l}$  for the cells. RNA was added to 10  $\mu\text{l}$  DNase buffer (Ambion # AM2224), 15  $\mu\text{l}$  DNase I and quantities sufficient to 100  $\mu\text{l}$  with RNase-free water.

- Master Mix for Reverse Transcription (RT): **Reaction Volume**: 20  $\mu\text{l}$ . 10  $\mu\text{l}$  RNase-free water, 4  $\mu\text{l}$  5X iScript reaction mix (Bio-Rad # 1708891), and 1  $\mu\text{l}$  iScript Reverse Transcriptase were added per sample. 15  $\mu\text{l}$  reaction mix was dispensed to appropriate wells and added 100 ng (5  $\mu\text{l}$ ) DNase treated RNA to each reaction.

- Master Mix for Real-Time PCR: **Reaction Volume**: 35  $\mu\text{l}$ . 9  $\mu\text{l}$  RNase-free water, 17.5  $\mu\text{l}$  2X iTaq SYBR Green Supermix with Rox (Bio-Rad # 1725852), 0.5  $\mu\text{M}$  forward primer, and 0.5  $\mu\text{M}$  reverse primer were added per sample. 30  $\mu\text{l}$  reaction mix was dispensed to appropriate wells and added 10 ng (5.0  $\mu\text{l}$ ) cDNA to each reaction.

### **PCR Array**

First strand cDNA kit (SA Biosciences # C-03), RT<sup>2</sup>-SYBR Green/ROX PCR master mix (SA Biosciences # PA-012-24), 384-well clear plates (Applied Biosystems # 4343370)

### **Multiplex Bead Assays**

- Cell Lysis Buffer: 40  $\mu$ l factor 1 (-20°C, useable upto 5 freeze/thaws), 20  $\mu$ l factor 2 (-20°C, useable upto 5 freeze/thaws), 40  $\mu$ l 500 mM PMSF (Sigma-Aldrich # P7626) diluted in dimethyl sulfoxide, and protease inhibitor cocktail tablet (Bio-Rad # 171-304012) or Complete Mini (Roche # 11836153001) were added to 10 ml lysis buffer (Bio-Rad #171-304012).

## **2.2. AGONISTS OF TLR4, TLR7, TLR8 AND TLR9**

The TLR7 and/or TLR8 agonists, imiquimod (R837), loxoribine, 3M002 (CL075), CL087 and CL097; TLR9 agonist type B CpG-ODN 1826 [5'- tcc atg acg ttc ctg acg tt -3' 20 mer] and FITC labeled CpG-ODN 1826, as well as the TLR4 agonist LPS were purchased from Invivogen (San Diego, California) (Gorden et al., 2005; Heil et al., 2003; Hemmi et al., 2002; Lee et al., 2003; Lee et al., 2006; Wille-Reece et al., 2006). All the agonists were suspended in endotoxin-free water, aliquoted, and stored at -20°C until use (Table 2.1).

For the intracerebroventricular (i.c.v.) inoculations, agonists were diluted in endotoxin-free 1X PBS/0.2% trypan blue, just before use (Table 2.1). The vehicle control, 0.2% trypan blue in PBS was used for mock i.c.v. inoculations. For the *in vitro* cell stimulations, agonists were diluted in media specific for astrocytes, microglia, or neurons just before use (Table 2.1). Media without any agonists were used as controls.

## **2.3. IN VIVO EXPERIMENTS**

### **2.3.1. Mice**

Inbred Rocky Mountain White (IRW) mice at 48 h post-birth were used for the present study. TLR7-deficient C57BL/6 mice (Hemmi et al., 2002) were kindly provided by Shizuo Akira and were backcrossed with IRW mice for ten generations (Lewis et al., 2008).

C57BL/6 mice were purchased from Charles River. C57BL/6/TLR9KO mice (Hemmi et al., 2000) were kindly provided by Shizuo Akira. All the animal procedures were conducted in accordance with the Louisiana State University Institutional Animal Care and Use Committee

**Table. 2.1. Different TLR Agonists Used in the Study.**

<u>ligand</u>	<u>Agonist</u>	<u>chemical group</u>	<u>Stock</u>	<u>Preparation</u>	<u>IN VIVO use</u>	<u>IN VITRO use</u>
<b>1. LPS</b>	TLR 4	Lipopoly-saccharide	1 µg/µl	dissolved 5 mg in 5 ml water	<b><u>2µg/pup</u></b> 2 µl LPS (1µg/ul) 6.5 µl water 1µl 10X PBS 0.5µl 20X Trypan blue	<b><u>10 µg/ml</u></b> 10 µl LPS stock 1 ml media
<b>2. Loxoribine</b>	TLR7	Guanosine analog	20 mM	Added 0.74 ml ethanol and 6.66 ml water to 50mg, heat at 55°C,5 min	<b><u>100 nmoles</u></b> As per imiquimod	<b><u>100 µM</u></b> 5 µl loxoribine 1 ml media
<b>3. Imiquimod (R837)</b>	TLR7	Imidazo-quinoline compound	5 mg/ ml (20mM)	dissolved 5 mg in 1ml water	<b><u>100 nmoles</u></b> 5 µl imiquimod (20 mM) 3.5 µl water 1µl 10X PBS 0.5µl 20X Trypan blue	<b><u>5 µM</u></b> 0.25 µl imiquimod (20 mM) 1 ml media
<b>4. CL 075</b>	TLR8/7	Thiazolo-quinoline	20mM	dissolved 0.5 mg in 103 µl water	<b><u>100 nmoles</u></b> As per imiquimod	<b><u>20 µM</u></b> 1 µl CL075 (20 mM) in 1 ml media
<b>5. CL 087</b>	TLR7	Adenine derivative	20mM	dissolved 0.5 mg in 79.5 µl DMSO	<b><u>100 nmoles</u></b> As per imiquimod	not tested
<b>6. CL 097</b>	TLR8/7	Imidazo-quinoline compound	20mM	dissolved 0.5 mg in 103 µl water	<b><u>100 nmoles</u></b> As per imiquimod	not tested
<b>7. CpG-ODN</b>	TLR9	ODN 1826	1 µg / µl (155 µM)	dissolved 200 µg in 200 µl water	<b><u>0.5 µg/pup (80 pmoles)</u></b> 0.5 µl ODN 1826 8 µl water 1µl 10X PBS 0.5µl 20X Trypan blue	<b><u>0.5 µg/ml (80nM)</u></b> 0.5µl ODN 1826 1 ml media
	Type B					

guidelines or in accordance with the regulations of the Rocky Mountain Laboratories Animal Care and Use Committee and the guidelines of the National Institutes of Health.

### **2.3.2. Intracerebroventricular (i.c.v.) Inoculations of Newborn Mice**

Mice, at approximately 48 h post-birth, were anesthetized by hypothermia, and 10  $\mu$ l (5  $\mu$ l per hemisphere) of the appropriate TLR agonist or vehicle control was inoculated into the lateral ventricles using a 33 gauge needle and a Hamilton syringe following a previously established protocol (Peterson et al., 2004; Poulsen et al., 1999). Correct ventricular inoculation was confirmed by observing trypan blue staining in the ventricles (Peterson et al., 2004; Poulsen et al., 1999). Mice were inoculated i.c.v. with either 2  $\mu$ g of LPS, 50  $\mu$ g (200 nmoles) of imiquimod, 0.5  $\mu$ g (80 pmoles) of CpG-ODN 1826 or 10  $\mu$ l of vehicle control (0.2% trypan blue in PBS). For the dose curve analysis, imiquimod was used at 20, 100, 200 or 500 nmoles per mouse. The neuroinflammatory responses to imiquimod at the 100 nmole dosage were compared with the responses induced by other TLR7/8 agonists: loxoribine, 3M002 (CL075), CL087 and CL097 at 100 nmole dosage. The survival curve analysis of CpG-ODN 1826 was tested at 77.5 pmoles (0.5  $\mu$ g), 155 pmoles (1 $\mu$ g), 755 pmoles (5  $\mu$ g), 1550 pmoles (10  $\mu$ g), 2325 pmoles (15  $\mu$ g), and 3100 pmoles (20  $\mu$ g) per mouse.

### **2.3.3. Preparation of Brain Tissue for Histological and Molecular Analysis**

Animals inoculated i.c.v. with TLR4, TLR7, or TLR9 agonists, or vehicle control were anesthetized by deep inhalation anesthesia followed by axillary incision and cervical dislocation at the specified time points or at the end of the experimental protocol. Brains were removed and immediately cut into two halves by mid-sagittal dissection, snap frozen in liquid nitrogen, and stored at -80°C for molecular analysis. One half was

used for RNA extraction and the other half was used for multiplex analysis of cytokine and chemokine production. Brains removed from the mice inoculated for *in situ* hybridization and/or immunohistochemistry were divided into four coronal sections using a brain matrix and fixed in 10% neutral buffered formalin for 48 hours prior to processing for histological analysis. Tissues were embedded in paraffin, cut in 4- $\mu$ m sections, adhered to coated microscope slides, and stained with hematoxylin and eosin (H&E) using an automated histological stainer. Stained sections were blindly graded for inflammatory changes and neural/glial degeneration and necrosis by a board certified veterinary pathologist.

#### **2.3.4. *In situ* Hybridization Analysis**

Due to the secreted nature of cytokines and chemokines, it can be difficult to detect the expression of these proteins or determine their cellular source. *In situ* hybridization detects mRNA expression inside individual cells, thereby allowing for the detection of the cellular source of gene expression (Perlman and Argyle, 1992). In brief, the slides were dewaxed by incubating in xylene overnight on a rocker, followed by rehydration of sections in 100%, 95% and 70% ethanol respectively. The sections were fixed in 4% paraformaldehyde for 20 min at room temperature and then incubated with 200 mM HCl for 10 min at RT to denature the proteins. The sections were then incubated with 10  $\mu$ g/ml proteinase K in Tris-buffered saline with 2 mM calcium chloride for 20 min. The proteinase K activity was stopped by incubating in cold TBS for 10 min followed by dehydration of the sections in 70%, 95% and 100% ethanol respectively. The sections were then incubated in chloroform for 20 min at RT, followed by rehydration as described above. The sections were then incubated in 2X SSC buffer for 10 min,

followed by incubation with hybridization buffer at 56°C for 1-4 h. At the end of the incubation, the appropriately diluted digoxigenin (DIG)-labeled RNA anti-sense or sense probes were added to the sections, covered with hybridization coverslips and incubated overnight at 56°C. The slides were incubated in 2X SSC to remove the cover slips, followed by incubation in 50% formamide in 1X SSC for 60 min. Sections were incubated in blocking buffer for 30 min at room temperature and then incubated in a 1:100 dilution of Alkaline-Phosphatase anti-DIG antibody for 60 min at RT. The slides were then washed in blocking buffer, 1X TBS, and incubated in detection buffer for 10 min at RT. The slides were then incubated in Fast Red solution using gasket cover slips (Grace Bio-Labs, Hatfield, PA) until color developed in the slides from treated animals. No staining was observed in mock controls or with non-specific probes.

### **2.3.5. Immunohistochemistry Analysis of *in situ* Sections**

When the sufficient color was developed by *in-situ* hybridization, gasket cover slips were removed and the slides were washed in 1X PBS. The sections were blocked in normal donkey serum blocking solution at 37°C for 60 min, and incubated overnight at 4°C with polyclonal rabbit anti-GFAP antibody (Dako) or rabbit anti-Iba1 antibody (Wako) in normal donkey serum blocking solution. Following two washes of 10 min in PBS, goat anti-rabbit IgG antibody labeled with HRP conjugate (Zymed) were applied and incubated at RT for at least 30 min. Slides were washed twice in PBS and incubated in metal enhanced Diamino benzidine (DAB) (Pierce, Rockford, IL). After sufficient color development, slides were washed twice in PBS, counterstained with hematoxylin, and covered with a glass cover slip using aqueous mounting medium (Lerner laboratories, Pittsburgh, PA).

### **2.3.6. Antigen Retrieval and Immunohistochemistry**

Tissue sections were dewaxed in Xylene twice for 15 min each at RT followed by rehydration in 100%, 95%, and 70% Ethanol respectively for 5 min each. Tissue sections were washed in PBS twice for 5 min each and antigen retrieval was carried out with citrate target retrieval solution in a Decloaker at 120°C for 20 min, following manufacturer's instructions. When Decloaker cooled to 90°C, bring tissue sections to room temperature gradually by replacing half of the citrate buffer with ultra pure water for at least 4 times. Tissue sections were washed in 0.5% Fish Skin Gelatin (FSG)/ PBS twice for 10 minutes each on a rocker and then slides were incubated with donkey serum blocking solution for 30 minutes at RT (175 µl per slide). Tissue sections were incubated with rabbit anti-florescein antibody (Invitrogen) or Rat anti-mouse CD54 (ICAM-1) antibody (eBioscience) in normal donkey serum blocking solution for 30 min at RT. Following two washes of 10 min in PBS-FSG, AlexaFluor 488-conjugated goat anti-rabbit or goat anti-rat secondary antibodies (Invitrogen) were applied and incubated for at least 30 min. Slides were washed twice in PBS-FSG and stained with DAPI (100 ng/ml) solution for 20 min. Slides were washed twice in PBS-FSG and covered with a glass cover slip using ProLong Gold anti-fading mounting medium.

### **2.3.7. Blood-Brain Barrier Permeability**

Blood-brain barrier permeability changes following TLR7 or TLR9 stimulation in the brain were determined by measuring Evans blue diffusion into the brain. Mice were inoculated with imiquimod, CpG-ODN or vehicle control, intracerebroventricularly as described, but without trypan blue. 10 µl of 1% (wt/vol) Evans blue solution was injected intra peritoneally, 4 h or 1h before testing BBB permeability. Mice were anesthetized and



perfused via intracardiac puncture with 1 ml of neutral buffered formalin. Brains were subsequently removed and analyzed for the blue color under a stereo-microscope.

## **2.4. *IN VITRO* EXPERIMENTS**

### **2.4.1. Isolation and Culturing of Cortical Astrocytes and Microglia**

Astrocyte and microglia cultures were prepared from the brain cortex of 1-2 day old IRW, IRW/TLR7<sup>-/-</sup>, C57BL/6, C57BL/6/TLR9<sup>-/-</sup> mice in accordance with the regulations of the Louisiana State University Institutional Animal Care and Use Committee guidelines or in accordance with the regulations of the Rocky Mountain Laboratories Animal Care and Use Committee and the guidelines of the National Institutes of Health. 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 a single cell suspension. Cells were pelleted by centrifugation at 500g for 5 min at 4°C. 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 with no brakes at 4°C. Cells between 0% and 30% percoll layers were rich in astrocytes and were seeded at  $2 \times 10^5$  cells in BD-Primaria T-25 flasks (VWR international # 29184-801) containing astrocyte specific media. The microglia cell populations collected between 30% and 70% percoll layers were seeded at  $5 \times 10^5$  in Primaria T-25 flasks containing microglia specific media. When cells reached confluency, in 7-10 days, 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) and

reseeded in Corning 12-well/24-well Cell Bind plates (ISC Bio-express # T-3406-12) until 80-90% confluency. Microglia were removed from confluent T-25 flasks using a cell scraper and reseeded in 12-well/24-well Cell Bind plates.

#### **2.4.2. Stimulation of Astrocytes and Microglia Cultures with Imiquimod or CpG-ODN**

To determine the optimal concentration for activation of astrocytes and microglia, imiquimod was used at 5 nM, 50 nM, 500 nM, 5  $\mu$ M and 50  $\mu$ M, and CpG-ODN 1826 was used at 0.5 nM, 5 nM, 50 nM, 80 nM, 500 nM. For all further studies, multiplex analysis of cytokines and chemokines (12 hours), and Super Array analysis (6 hours), imiquimod and CpG-ODN 1826 were used at 5  $\mu$ M (1.25  $\mu$ g/ml) and 80 nM (0.5  $\mu$ g/ml) respectively. All the experiments were conducted in triplicate wells for each time point or concentration, and with astrocyte specific or microglia specific media as mock control.

#### **2.4.3. Flow Cytometry Analysis**

Semi confluent cultures of primary astrocytes and microglia were analyzed for purity by intracellular staining for GFAP (glial fibrillary acidic protein), an astrocyte marker, and F4/80, a microglia/macrophage marker. These cells were also analyzed for expression of TLR7 and TLR9 proteins by intracellular flow cytometry. Briefly, cells were trypsinized or gently scraped off the plate, washed in PBS and fixed for 20 min in 2% paraformaldehyde (Electron Microscopy Sciences). Cells were permeabilized with 0.1% saponin in PBS (pH 7.0) and were then incubated with polyclonal rabbit anti-bovine GFAP (Dako), monoclonal anti-mouse F4/80 (eBioscience), polyclonal rabbit anti-TLR7 (Zymed) or monoclonal mouse anti-TLR9 (Imgenex) for 30 min. Cells were washed twice with 0.1% saponin in PBS and incubated with Alexa Fluor 488 conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG (BD Biosciences) for 30

min. Cells were washed twice with 0.1% saponin in PBS, resuspended in PBS and analyzed on a FACSAria flow cytometer (BD Bioscience) using FACSDiva software (BD Bioscience). Data analysis was performed using FCS Express V3 software (De Novo). Specificity of the TLR7 antibody was verified by immunocytochemical staining of TLR7-transfected HEK cells and non-transfected HEK cells. A rabbit anti-green fluorescent protein (GFP) polyclonal antibody was utilized as an additional negative control and demonstrated no increase in staining compared to the no-primary antibody control (data not shown).

#### **2.4.4. Mouse Primary Cortical Neuron Cultures**

For culturing neurons, 14-well Cell Bind plates were coated with 20 µg/ml Poly L- Ornithine hydro bromide (Sigma-Aldrich # P3655) in sterile distilled water overnight at 37°C and 5% CO<sub>2</sub>, followed by extensive washing with sterile distilled water. Plates were then coated with 2.5 µg/ml of Laminin (Sigma-Aldrich # L2020) in sterile HBSS for 3-4 hours at 37°C and 5% CO<sub>2</sub>. Following incubation, all the wells were extensively washed with sterile HBSS and used immediately.

Cortical neurons were isolated from mouse embryos at 16-18 days gestation (E16-E18). Brains from the E16-E18 mice were dissected out, meninges were peeled off and cortices were collected in cold neuron dissection media. Neurons were dissociated in neuron digestion media for 15 min at 37°C and made into a single cell suspension by gentle trituration using flame polished glass Pasteur pipettes. Cells were seeded at 4x10<sup>5</sup> cells per well of 24 well plates or 8 chamber slides in neuronal plating media. Following initial attachment of the cells to the plates (4h), neuron plating media was replaced with

neuronal maintenance media along with TLR7 or TLR9 ligands or stimulated cell culture supernatants and incubated at 37°C and 5% CO<sub>2</sub>.

At 4 h post culture, apoptosis was induced in some wells by adding 300 µM NMDA + 5µM glycine for 15 min in HBSS (Ca, Mg free), rinsed with HBSS at the end of incubation and replaced with neuronal maintenance media. Supernatants from TLR7 or TLR9 stimulated astrocytes and microglia were added to all the wells at a 1:1 ratio of neurobasal media and stimulated supernatants, and incubated for 72 h at 37°C and 5% CO<sub>2</sub>. To determine the effect of direct TLR agonist stimulations on neurons, cells were stimulated with either 5 µM imiquimod and/or 80 nM CpG-ODN 1826. Cells were also exposed to NMDA as described above for comparison. Neuronal survival or death was measured by MTT assay or staining neurons for beta tubulin.

#### **2.4.5. MTT Assay**

MTT [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan] was added into all the wells directly into the medium at a final concentration of 0.5 mg/ml and incubated at 37°C for 3-4 h. All the wells were rinsed twice with PBS and the insoluble purple colored formazan produced from MTT by mitochondrial reductases was solubilized in DMSO to get a homogenous color. The absorbance was measured at 540 nm on a Spectramax 190 plate reader with Softmax Pro 5 software, with DMSO as a reagent blank. The percent viability of the cells was calculated from the mean absorbance of mock controls (OD of the test sample/average OD of mock samples X 100).

#### **2.4.6. Immunofluorescence Assay**

Neuronal cultures in poly-D-lysine coated 8 chamber slides (BD Biosciences # 354632) were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100

and 0.1% sodium citrate in 1X PBS for 30 min. Cells were then treated with 0.1 M glycine for 30 min and incubated with donkey serum blocking solution in PBS for 30 min. Cells were incubated with primary antibodies, monoclonal anti-tubulin beta III isoform or polyclonal rabbit anti-bovine GFAP (Dako) for 30 min at room temperature. Cells were rinsed with PBS twice and incubated with goat anti-mouse Alexa Fluor 488 (Invitrogen) or goat anti rabbit Alexa Fluor 555 (Invitrogen) for 30 min at room temperature in the dark. Finally, cells were washed twice in PBS and slides were mounted in Fluorogel II with DAPI (Electron Microscopy Sciences) and kept in the dark at 4°C. All images were taken using a Nikon Eclipse 55i fluorescent microscope. GFAP detection was minimal in neuronal cultures with less than one GFAP positive cell detected per every 2 fields using a 10X objective.

#### **2.4.7. Meningeal Cultures**

The meninges dissected out during preparation of astrocyte and microglia cultures were collected into DMEM containing 10% inactivated fetal bovine serum and were chopped into small pieces using cell scrapers and were plated in Primaria T-25 flasks. At confluency, meningeal cells were trypsinized and replated into 12-well Cell Bind plates and allowed to grow to 80-90% confluency before stimulating with TLR agonists.

#### **2.4.8. Cell Entry Assay for CpG-ODN 1826**

Glial cells were grown in 96-well plates to near confluency and were stimulated with mock or imiquimod 5 $\mu$ M or 50 $\mu$ M  $\pm$  80 nM FITC labeled CpG-ODN 1826. Cells were incubated for 30 min at 37°C and 5% CO<sub>2</sub>, washed 3 times in PBS and then analyzed for FITC uptake. All images were taken using an Olympus IX71 inverted fluorescent microscope. Further, cells were lysed in cell lysis buffer (0.5% Triton X-100,

0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris HCl, pH 7.4 and 8 mM EDTA) to release FITC into solution, and the fluorescence intensity was quantitated using a microplate reader (Polar Star Omega, BMG Labtech).

#### **2.4.9. TLR7 Protein Expression in Astrocytes (C8D1A) and Macrophages (RAW 264.7) by Flow Cytometry Analysis**

RAW264.7 (ATCC # TIB-71) and C8D1A (ATCC # CRL-2541) were obtained from ATCC and maintained according to ATCC guidelines. Semi-confluent cultures of C8D1A and RAW264.7 cells were analyzed for TLR7 protein expression by intracellular staining. In short, cells were gently scraped off the plate, washed in PBS and fixed for 20 min in 2% paraformaldehyde. Cells were permeabilized with 0.1% saponin in PBS (pH 7.0) and incubated with primary antibodies, either polyclonal rabbit anti-bovine GFAP (Dako) or polyclonal rabbit anti-mouse TLR7 (Zymed) for 30 min at room temperature. Cells were washed twice with 0.1% saponin in PBS and incubated with AlexaFluor 488 conjugated goat anti-rabbit IgG (Molecular Probes) for 30 min at room temperature in the dark. Cells were washed twice with 0.1% saponin in PBS, resuspended in PBS and analyzed on a FACSAria flow cytometer (BD Bioscience) using FACSDiva software (BD Bioscience). Data analysis was performed using Flowjo software (Treestar).

#### **2.4.10. Stimulation of Astrocytes and Macrophages *in vitro* with TLR7 Agonists, Imiquimod and Loxoribine**

Semi-confluent cultures of C8D1A astrocyte cells or RAW 264.7 cells were seeded into six-well plates and stimulated with imiquimod or loxoribine at 100  $\mu$ M in 1 ml. Astrocyte cells were stimulated with imiquimod, loxoribine or media alone for 6, 12, 24 or 48 h, in triplicate wells for each time point. The supernatant was collected from each well at specified time points, and triplicate aliquots were stored at -80°C until use

for multiplex analysis of cytokine and chemokine production. At each time point, astrocyte cells were lysed within the well, and total RNA was extracted from the cells using a mini RNA isolation kit (Zymo Research) following the manufacturer's instructions.

## **2.5. MOLECULAR METHODS**

### **2.5.1. RNA Extraction and Reverse Transcription (RT)**

Astrocyte, microglia, meningeal, or neuron cultures were lysed and processed for RNA extraction using a mini RNA isolation kit (Zymo Research) following manufacturer's instructions. Total RNA from the brain tissue was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA from cells or brain was treated with DNase (Ambion, Austin, TX) for 30 min at 37°C and purified over RNA cleanup columns (Zymo Research) before use. RNA samples were converted to cDNA using iScript Reverse Transcription kit (Bio-Rad) following manufacturer's instructions. (B-RT Program: 5min at 25°C, 30 min at 42°C, 5 min at 85°C, cool to 20°C). Prior to analysis by real-time PCR, following the reverse transcriptase reaction, samples were diluted five-fold in RNase-free water for use in real-time PCR reactions.

### **2.5.2. Analysis of mRNA Expression by Real-Time PCR**

The primers to detect *App*, *Bdnf*, *Cd3e*, *Cd14*, *Ccl2*, *Cxcl10*, *F4/80*, *Gapdh*, *Gfap*, *Icam-1*, *Ifnb1*, *Prnp*, *S100b*, *Slc1a2*, *Slc1a3* and *Tnf* cDNA were designed using Primer3 software (Rozen and Skaletsky, 2000) with a T<sub>m</sub> of 60°C for all primers (Table 2.2). All primer pairs were confirmed to be specific for the gene of interest, and no homology to other genes was detected when the primers were blasted against the National Center for Biotechnology Information (NCBI) database. A cDNA pool produced from mRNA from

**Table 2.2. Primers Used for Real-Time RT-PCR Analysis.** (<sup>a</sup> NCBI, National Center for Biotechnology Information.)

<b>Common name</b>	<b>NCBI<sup>a</sup> Gene Symbol &amp; ID#</b>	<b>Forward primer</b>	<b>Reverse primer</b>
Amyloid beta (A4) precursor	<i>App</i> : 11820	ACCGTTGCCTAGTTGGTGAG	CATGCCATAGTCGTGCAAGT
Brain derived neurotrophic factor	<i>Bdnf</i> : 12064	ATTAGCGAGTGGGTCACAGC	ACTGCTTCAGTTGGCCTTTG
CD3 antigen, epsilon polypeptide	<i>Cd3e</i> : 12501	GAGCACCTGCTACTCCTTG	TGAGCAGCCTGATTCTTTCA
Cd14 antigen	<i>Cd14</i> :12475	AACCTGGAAGCCAGAGAACA	CCAGAAGCAACAGCAACAAG
Chemokine ligand 2, MCP-1	<i>Ccl2</i> : 20296	TCCCAATGAGTAGGCTGGAG	CCTCTCTCTTGAGCTTGGTGA
Chemokine ligand 10, IP-10	<i>Cxcl10</i> : 15945	CAGTGAGAATGAGGGCCATAGG	CTCAACACGTGGGCAGGAT
F4/80	<i>Emr1</i> : 13733	TTACGATGGAATTCTCCTTGTATATCA	CACAGCAGGAAGGTGGCTATG
Glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i> : 14433	TGCACCACCAACTGCTTAGC	TGGATGCAGGGATGATGTTC
Glial fibrillary acidic protein	<i>Gfap</i> : 14580	CGTTTCTCCTTGTCTCGAATGAC	TCGCCCCGTGTCTCCTTGA
Intercellular adhesion molecule-1,	<i>Icam1</i> : 15894	AGGGCTGGCATTGTTCTCTA	CTTCAGAGGCAGGAAACAGG
Interferon beta	<i>Ifnb1</i> : 15977	AGCACTGGGTGGAATGAGAC	TCCCACGTCAATCTTTCTC
Prion protein	<i>Prnp</i> : 19122	GGACCGCTACTACCGTGAAA	TCATCTTCACATCGGTCTCG
S100 protein, beta polypeptide,	<i>S100b</i> : 20203	GGTGACAAGCACAAGCTGAA	ACGAAGGCCATGAACTCCT
Solute carrier family 1 member 2, GLUT1, Eaat2	<i>Slc1a2</i> : 20511	TCTGAGGAGGCCAATACCAC	TTCATCCCGTCCTTGAAC
Solute carrier family 1 member 3, GLAST, Eaat1	<i>Slc1a3</i> : 20512	GCCTATCCAGTCCAACGAAA	CGAAGCACATGGAGAAGACA
Tumor necrosis factor	<i>Tnf</i> : 21926	CCACCACGCTCTTCTGTCTAC	GAGGGTCTGGGCCATAGAA



a macrophage cell line, an astroglia cell line, brain and spleen tissue was used to analyze the specificity of primers. SYBR Green Supermix with ROX was used for measurement of real-time PCR amplification (2min at 50°C, 3min at 95°C, 15sec at 95°C, 40 cycles of (1min at 60°C and 15sec at 95°C), 15sec at 60°C, 15 sec at 95°C). All samples were run in triplicate on a 384-well plate using a 7900 Applied Biosystems PRISM machine with an automatic set baseline and a manual set CT of 0.19, which intersects the mid-log phase of curves for all of the PCR pairs. The dissociation curves were used to confirm amplification of a single product for each primer pair per sample. A known positive control sample was run for the corresponding gene on all assays. RNA that did not undergo reverse transcription (DNA contamination control) and water were used as negative controls. Data for each sample was initially calculated as the percent difference in  $C_T$  value with the house keeping gene *Gapdh* ( $\Delta C_T = C_T \text{ Gapdh} - C_T \text{ gene of interest}$ ). The mean percent *Gapdh* values of mock samples for each time point were calculated and used to generate fold changes relative to mock expression for each group at each time point.

### **2.5.3. Multiplex Analysis of Cytokine and Chemokine Proteins**

Supernatants from stimulated and unstimulated astrocyte, microglia, and meningeal cultures were collected at 12 hps and were stored in triplicate aliquots at -80°C until use. Just before use, supernatants were thawed and centrifuged at 4500g for 15 min at 4°C to remove any cellular debris. Culture supernatants were analyzed for cytokine and chemokine protein production using Linco's 22 plex kit (Millipore) or BioSource 20-plex assay (Invitrogen) on a Luminex 100 instrument (Bio-Rad) following manufacturer's instructions. All the samples were run in duplicate. Samples were calculated as pg/ml of

supernatant using a standard curve generated from in-plate standards. For the majority of the positive samples, values were within the linear range of the standard curve.

To generate tissue homogenates for analysis by multiplex bead array, brain samples were weighed and then homogenized in 200  $\mu$ l Bio-plex cell lysis solution containing Complete Mini Protease inhibitors (Roche) and 2 mM phenylmethylsulfonyl fluoride. Samples were homogenized using Kontes disposable pellet pestles (Fisher Scientific, Hampton, NH) and volumes were adjusted to 300 mg/ml of tissue with lysis buffer. Cellular debris was removed by centrifugation at 4,500g for 15 min at 4°C. Samples were analyzed for cytokine and chemokine protein expression using a BioSource 20-plex assay (Invitrogen) on a Luminex 100 instrument (Bio-Rad) following manufacturer's instructions. Samples were calculated as pg/ml using a standard curve from in-plate standards and subsequently converted to fg/mg of brain tissue. For the majority of the positive samples (e.g. cytokine and chemokines at 12 hours post imiquimod stimulation), the pg/ml concentration value was in the linear range of the standard curve.

#### **2.5.4. Mouse Toll-Like Receptor Signaling Pathway PCR Array**

Astrocytes and microglia were treated with either 5  $\mu$ M imiquimod or 80 nM of CpG-ODN 1826 or both. At 6 hours post stimulation, RNA was isolated and treated with DNase I as described earlier (2.5.1). First strand cDNA was synthesized using 100 ng of cleaned up RNA and analyzed for a mouse-TLR pathway-specific gene expression profile as per the manufacturer's instructions (SABiosciences) on a 7900 Applied Biosystems PRISM instrument. A total of 84 genes related to mouse TLR-mediated signal transduction were analyzed in a 384-well format. The CT (cycle threshold) values

from both control and treatment groups were obtained from real-time 384-well PCR Array results and analyzed using RT<sup>2</sup> Profiler PCR Array data analysis template (SA Biosciences). The samples were analyzed only if the test passed all the quality controls including RT efficiency and lack of DNA contamination. Data were calculated as fold difference for the treatment groups compared to mock groups.

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## **CHAPTER 3**

### **ANALYSIS OF THE NEUROINFLAMMATORY RESPONSE TO TLR7 STIMULATION IN THE BRAIN: COMPARISON OF MULTIPLE TLR7 AND/OR TLR8 AGONISTS\***

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### 3.1. INTRODUCTION

Neuroinflammation, including astrogliosis and production of proinflammatory cytokines and chemokines, is a common finding following viral, bacterial and parasitic infections of the CNS in both children and adults (Asensio and Campbell, 1997; Dickson et al., 1993b; Griffin, 2003; Hunt et al., 2006; Kelder et al., 1998; McCoig et al., 2004; Nau and Bruck, 2002; Szklarczyk et al., 2007; Wilson and Hunter, 2004). Neuroinflammatory responses are also observed in autism, Alzheimer's disease and other neurological diseases of unknown etiology (Ahlsen et al., 1993; Cohly and Panja, 2005; Konsman et al., 2007; Sun et al., 2003; Vargas et al., 2005). The neuroinflammatory response may be a common mechanism of pathogenesis leading to neuronal damage and long-term neurological disorders (Dickson et al., 1993a; Hornig and Lipkin, 2001; Hornig et al., 2002; Minagar et al., 2002).

The initiation of inflammation is often associated with the recognition of pathogen-associated molecular patterns (PAMPs), the repeated structural motifs that are unique to microorganisms (Akira et al., 2001; Janeway, Jr., 1992; Medzhitov and Janeway, Jr., 1997). These PAMPs are recognized by transmembrane-bound TLRs as well as cytoplasmic or mitochondrial associated pattern recognition receptors (PRRs) (Akira et al., 2001; Janeway, Jr., 1992; Medzhitov and Janeway, Jr., 1997). There are at least 11 identified TLRs in humans and 12 TLRs in mice (Kaisho and Akira, 2006). Multiple TLRs are upregulated in the CNS in response to pathogen infection (Aravalli et al., 2007; McKimmie and Fazakerley, 2005; McKimmie et al., 2005; Mishra et al., 2006). Of these receptors, several have been shown to contribute to neuroinflammatory responses and pathogenesis including TLR2, TLR3, TLR4 and TLR9 (Aravalli et al., 2005; Dalpke et al., 2002; Iliev et al., 2004; Kurt-Jones et al., 2004; Pedras-Vasconcelos et al., 2006; Wang et al., 2004; Zhang et al., 2007). Intracerebroventricular

(i.c.v.) administration of agonists for either TLR4 or TLR9 induced strong neuroinflammatory responses and damage in the CNS (Dalpke et al., 2002; Xia et al., 2006).

Two other TLRs that may play an important role in initiating innate immune responses in the CNS are TLR7 and TLR8. These receptors were originally identified as eliciting antiviral effects when stimulated by the family of guanosine based imidazoquinoline compounds (Hemmi et al., 2002; Lee et al., 2003) which includes imiquimod, loxoribine and R-848. More recent studies identified the natural PAMP of TLR7 and TLR8 to be guanidine-uridine rich single stranded RNA from viruses, suggesting that these receptors may be important modulators of the immune response to certain neurotropic viruses such as flaviviruses, paramyxoviruses, rhabdoviruses and retroviruses. However, there remains a lack of basic understanding of the neuroinflammatory properties of TLR7 and TLR8.

Functional differences between mouse TLR7 and TLR8 have not been described; however, TLR7 deficient mice do not respond to imiquimod, R-848 or viral ssRNA. Furthermore, VSV and influenza A- induced IFN $\alpha$  responses are suppressed in TLR7  $-/-$  mice (Diebold et al., 2004; Lund et al., 2004). This suggested that murine TLR8 may be biologically inactive in mice (Heil et al., 2004; Hemmi et al., 2002). However, recent studies have demonstrated that murine TLR8, but not TLR7, is expressed on neurons in utero and in the first two weeks post-birth in the neonatal brain (Ma et al., 2006). Stimulation of neurons with TLR7/8 agonists induced caspase 3 activation and inhibited dendrite growth suggesting that TLR8 may be functional in neonates and serve an important role in brain development (Ma et al., 2006).

In the present study, we analyzed the downstream responses to TLR7 and/or TLR8 stimulation in the developing brain. For this, we administered different TLR7/8 agonists by i.c.v inoculation in newborn mice and analyzed the neuroinflammatory responses within the CNS.

These results revealed that TLR7/8 agonists differed in their ability to induce neuroinflammation. The agonists that did induce neuroinflammatory responses induced pronounced activation of astrocytes and the production of chemokines by several cell types in the CNS.

## **3.2. RESULTS**

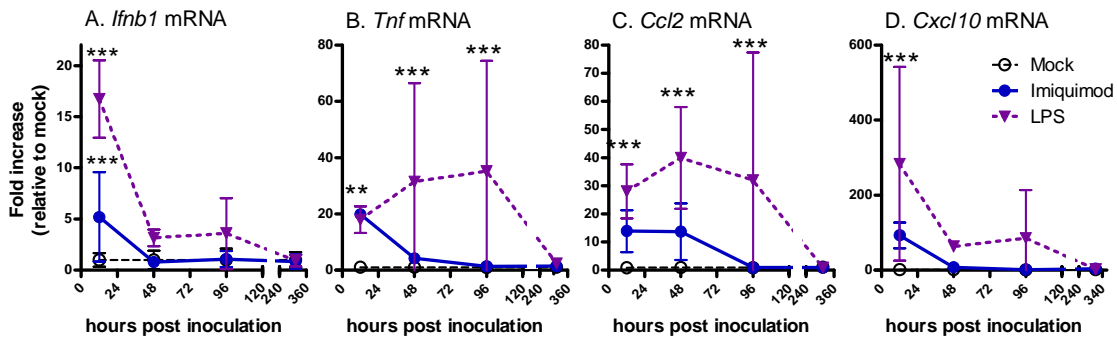
### **3.2.1. ICV Inoculation of TLR7 Agonist Induces Pronounced Neuroinflammatory Response in the CNS**

The first week post-birth of the neonatal mouse brain corresponds to the late second and early third trimester development of a human brain in terms of limbic and cortex development (Clancy et al., 2001). The first few days post-birth in the mouse are ideal for i.c.v. inoculations due to ease of inoculation and the ability to readily confirm inoculation in lateral ventricles. We examined the ability of TLR7 stimulation at this time point to induce neuroinflammatory responses using the TLR7 agonist, imiquimod. We compared these responses to stimulation with the TLR4 agonist, LPS which is known to induce severe neuroinflammation and damage to the neonatal brain (Cunningham et al., 2005). In addition, the comparison between TLR7 and TLR4 allows us to analyze the predicted differences in response between an intracellular PRR that recognizes viral products and extracellular PRR that primarily recognizes bacterial surface components.

Administration of the TLR7 agonist, imiquimod, induced a pronounced neuroinflammatory response in the brain at 12 hours (h) post inoculation with the upregulation of mRNA for *Ifnb1* (*Interferon beta*), *Tnf* (*Tumor necrosis factor*), *Ccl2* (*Monocyte chemoattractant protein-1/MCP-1*) and , *Cxcl10* (*Interferon inducible protein-10/IP-10*) (Fig. 3.1), cytokines and chemokines commonly associated with virus-induced neuroinflammation (Kerr et al., 2002; McManus et al., 2000; Peterson et al., 2001). In most cases, imiquimod-induced mRNA



expression returned to basal levels by 48 h post stimulation, suggesting a short term response. In contrast, LPS stimulation generally induced a longer term response with *Ccl2* and *Tnf* mRNA expression still significantly upregulated at 96 h post inoculation (Fig. 3.1). The increase in cytokine and chemokine expression did not correlate with any obvious clinical symptoms in the mice following i.c.v. inoculation with either LPS or imiquimod.

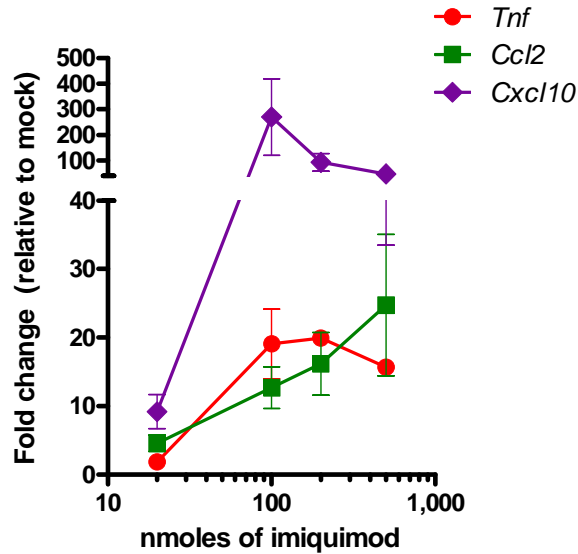


**Fig. 3.1. Induction of Neuroinflammatory Responses Following Imiquimod or LPS Inoculation in the Brain.** Mice at 48 h of age were inoculated by intracerebroventricular injection with 2  $\mu$ g of LPS, 50  $\mu$ g of imiquimod, or 10  $\mu$ l of vehicle control (0.2% trypan blue in PBS). Brain tissues were removed at 12, 48, 96 or 312 h post inoculation and snap frozen for RNA or protein analysis as described. RNA samples were processed for real-time quantitative RT-PCR analysis and values calculated relative to expression of *Gapdh* controls. Data are presented as the fold induction of each gene of interest relative to mock-inoculated controls. Mock expression levels were calculated as the mean of 6 animals per time point. Data represent the mean  $\pm$  standard deviation of 3-7 mice per group per time point and represent the combined data from 2 independent experiments. Statistical analysis was completed by two-way ANOVA using Bonferroni post-test. \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

### 3.2.2. Optimal Dose for TLR7-Mediated Inflammation

The ability of TLR7 to stimulate innate immune responses in the neonatal brain may be dose dependent. Imiquimod was administered at 20, 100, 200 and 500 nmoles per mouse. No dose above 500 nmoles was administered due to insolubility of the agonist. 100 to 200 nmoles of imiquimod were optimal for eliciting neuroinflammatory responses in the CNS at 12 h post inoculation as demonstrated by upregulation of mRNA for multiple cytokines such as *Tnf*, *Ccl2* and *Cxcl10* (Fig. 3.2). Imiquimod at the 20 nmoles dose did not stimulate any

neuroinflammatory response, whereas 500 nmoles of imiquimod induced higher responses for some cytokines and chemokines, but not others (Fig. 3.2).

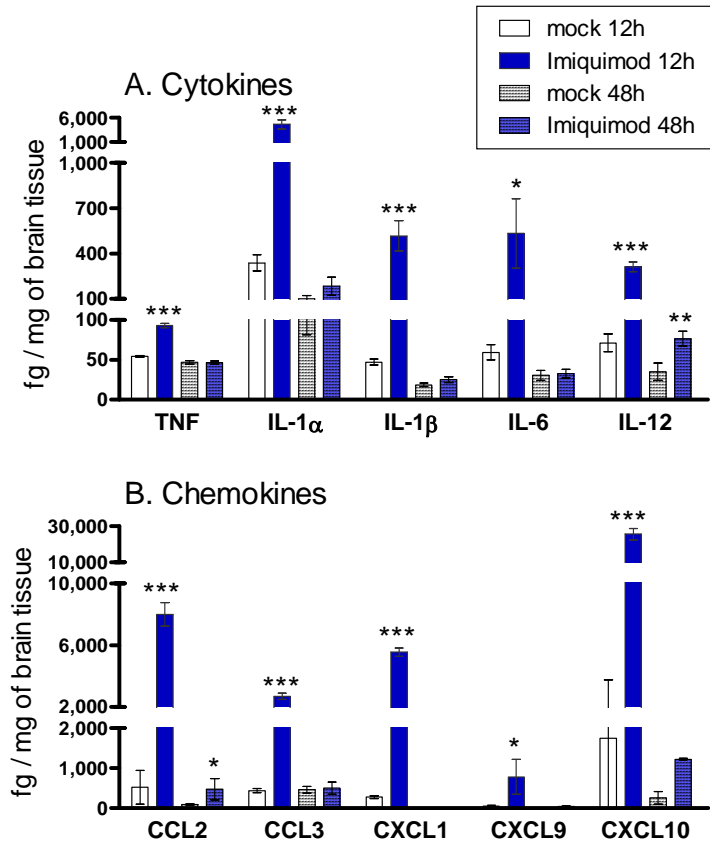


**Fig. 3.2. Cytokine Response to Imiquimod Is Dose Dependent.** Mice were treated as described in Fig. 3.1, but using varying concentrations of imiquimod in 10  $\mu$ l of PBS/0.2% trypan blue. At 12 hpi brain tissue was removed and processed for RNA. Samples were analyzed as described in Fig. 3.1. Data represent the mean  $\pm$  standard deviation of 3 to 7 mice per group and represent the combined data from 2 independent experiments.

### 3.2.3. Analysis of Cytokines and Chemokines Induced by Imiquimod

Multiplex bead assays were performed to determine which cytokines and chemokines were upregulated by stimulation of TLR7. Proinflammatory cytokines TNF, IL1 $\alpha$  (Interleukin-1 $\alpha$ ), IL1 $\beta$ , IL6, IL12 and chemokines CCL2, CCL3 (Macrophage inflammatory protein-1 $\alpha$ /MIP-1 $\alpha$ ), CXCL1 (Neutrophil activating protein-3/NAP-3/KC), CXCL9 (Monokine induced by gamma interferon/MIG), and CXCL10 were upregulated in the CNS following stimulation with imiquimod at 12 hpi (Fig. 3.3, A and B). By 48hpi, only IL12, CCL2, CCL3 and CXCL9 remained elevated compared to mock controls (Fig. 3.3, A and B). Imiquimod administration did not induce the upregulation of GM-CSF (granulocyte macrophage – colony stimulation

factor), IFN $\gamma$ , IL5, IL10, IL13 or IL17 at either time point (data not shown). LPS administration induced similar cytokines and chemokines to imiquimod, but with the additional upregulation of IL2 and IL13 (data not shown). The fold increase of cytokine and chemokine production was substantially higher in the LPS inoculated mice for almost all cytokines and chemokines, the exception being CXCL1 (data not shown).

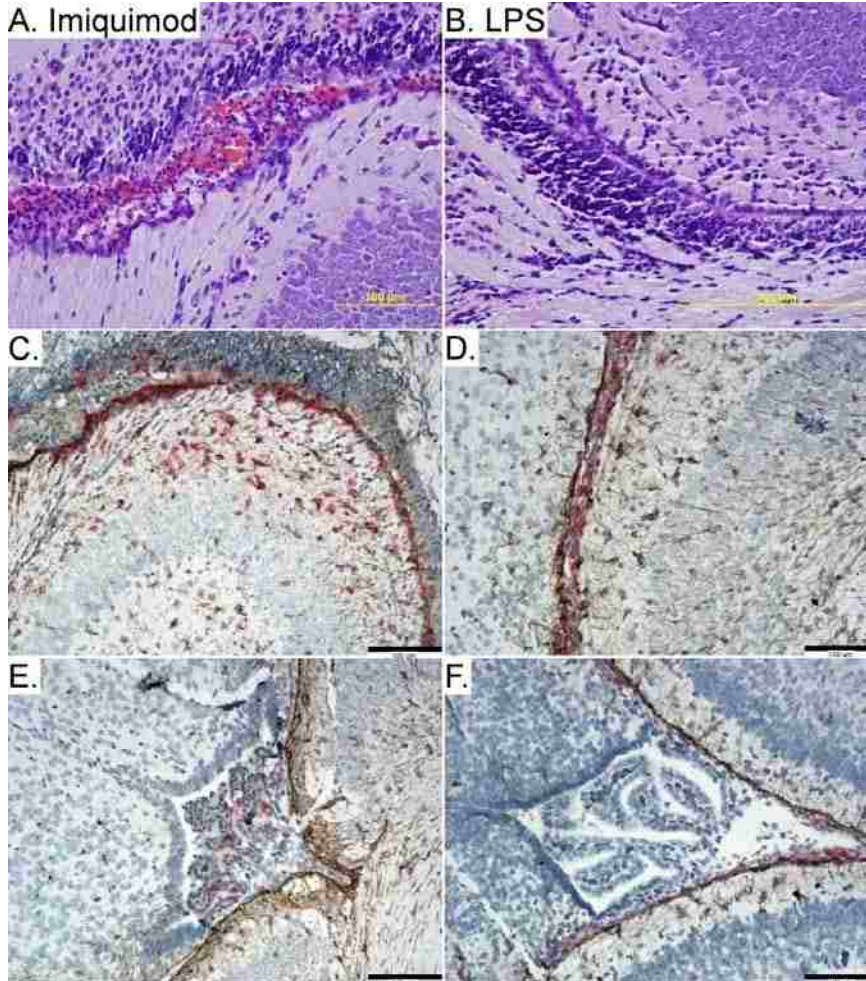


**Fig. 3.3. Increased Protein Expression of (A) Proinflammatory Cytokines and (B) Chemokines in the CNS Following Stimulation with Imiquimod.** Mice were inoculated as described in Fig. 1 and tissues were removed at 12 or 48 hpi. One half of sagittally divided brain was homogenized in lysis buffer containing protease inhibitors and analyzed for protein expression using a Biosource 20-plex bead array on a Bioplex Luminex system. Samples were calculated as pg/ml using a standard curve from in-plate standards and subsequently converted to fg/mg brain tissue. Data represent the mean SD of three mice per group. Statistical analysis was completed by one way ANOVA with Newman-Keuls post-test. \*P<0.05, \*\*P<0.01. \*\*\*P<0.001.

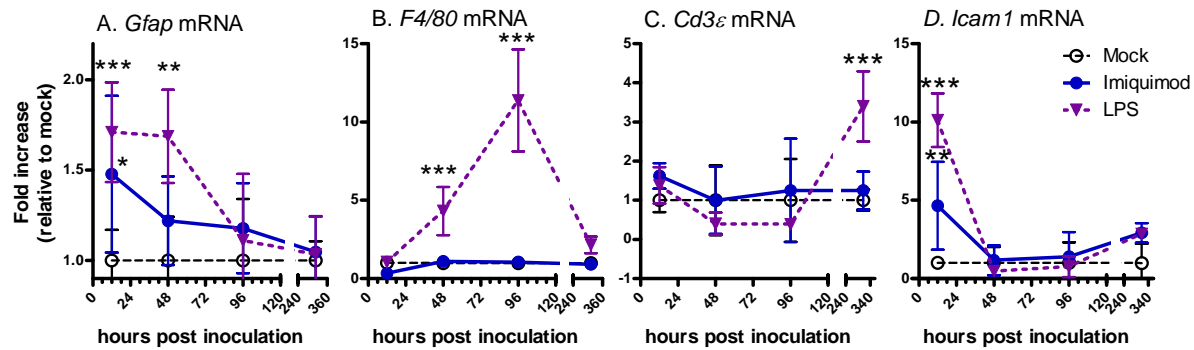
### 3.2.4. Cellular Responses in the CNS Following TLR7 Agonist Administration

As 12 hpi was the peak time for cytokine mRNA and protein production, we examined tissue samples from this time point for histologic changes. All mice including mock controls had minimal to mild meningitis, characterized by infiltration of the meninges by variable numbers of neutrophils and lymphocytes. The cause of the minimal to mild meningitis in all mice is uncertain, but may be in response to the i.c.v. needle inoculations or the volume of liquid (10  $\mu$ l) injected. The mice inoculated with imiquimod developed, in addition to suppurative meningitis, moderate to severe suppurative ventriculitis, characterized by mild hemorrhage and infiltration of the lateral ventricles by neutrophils and lymphocytes (Fig. 3.4A). The suppurative ventriculitis was the only consistent pathologic change associated with imiquimod inoculation and was not observed in mock or LPS-treated animals (Fig. 3.4 B, data not shown).

To examine cellular changes at the molecular level, we analyzed the mRNA expression of Glial Fibrillary Acidic Protein (*Gfap*), which is upregulated by astrocyte activation. Additionally, we analyzed mRNA expression of F4/80, a macrophage and microglia marker; Cd3 epsilon polypeptide (*Cd3 $\epsilon$* ), a marker for T cell infiltration; and Intercellular adhesion molecule-1 (*Icam-1*), which is upregulated on peripheral endothelial cells following stimulation with TLR7 agonists (Gunzer et al., 2005). Administration of imiquimod induced *Gfap* and *Icam1* mRNA expression at 12 hpi, but did not significantly alter the expression of either *F4/80* or *Cd3 $\epsilon$*  mRNA at any of the time points analyzed (Fig. 3.5). In contrast, LPS administration induced prolonged upregulation of F4/80 mRNA and increased expression of *Cd3 $\epsilon$*  mRNA at 13 days post inoculation (dpi) (Fig. 3.5). Thus, stimulation of either TLR7 or TLR4 resulted in astrocyte activation and upregulation of adhesion molecules, but only TLR4 stimulation induced mRNA upregulation of the microglia/macrophage marker *F4/80*.



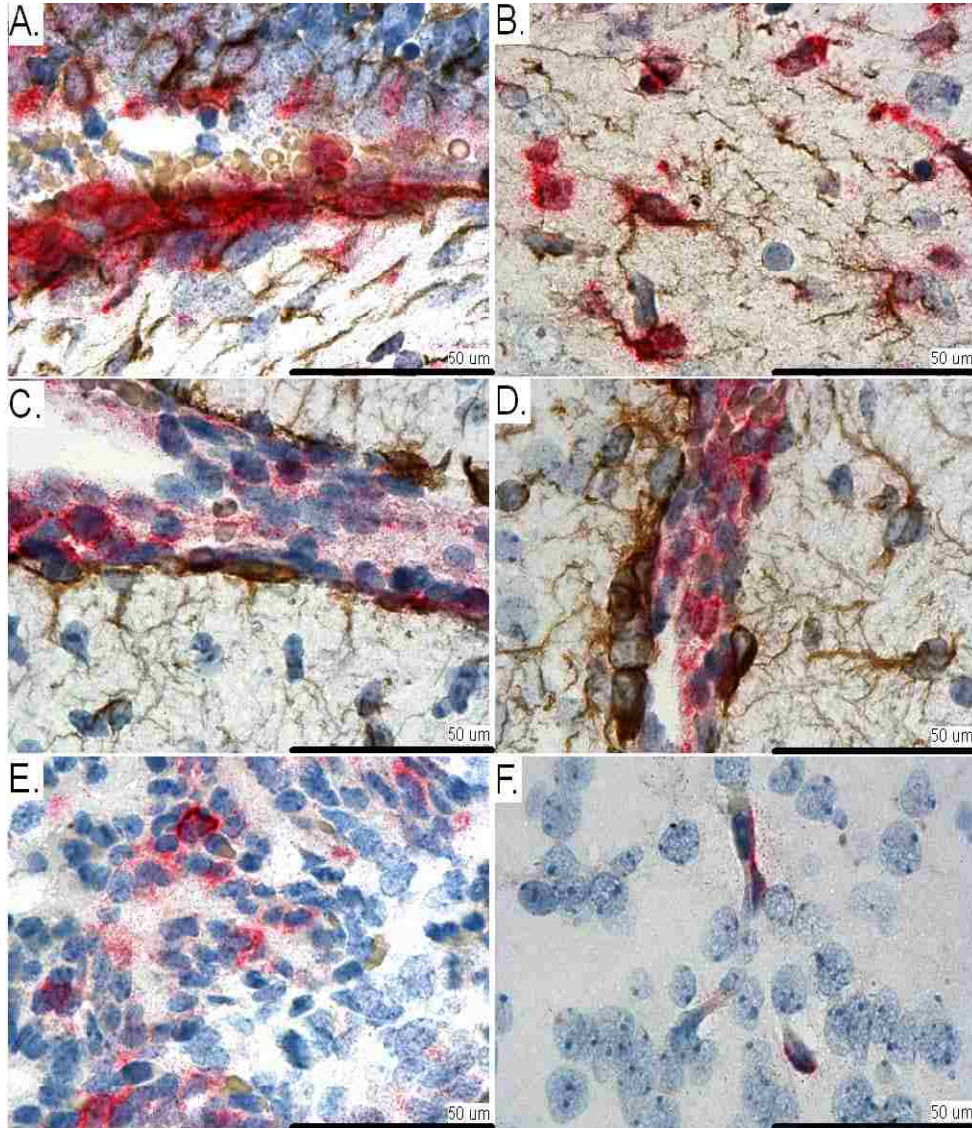
**Fig.3.4. Comparison Between Imiquimod and LPS-Treated Mice.** (A-B) H&E stained mid-coronal sections of brain tissue from (A) imiquimod and (B) LPS-treated mice at 12 hpi. Both images are of lateral ventricles and are representative of the three mice in each group. The imiquimod treated mice have increased numbers of neutrophils, lymphocytes, and erythrocytes within the lateral ventricles (suppurative ventriculitis) (A) as compared to the LPS-treated mice, which have no appreciable inflammatory infiltrates (B). (C-F) Analysis of brain tissue sections from (C,E) imiquimod and (D,F) LPS-treated mice at 12 hpi for *Ccl2*-mRNA expressing cells (bright red stain). *Ccl2* mRNA was detected using digoxigenin (DIG)-labeled RNA anti-sense probes and stained with Fast Red substrate. Cells were then stained with anti-goat GFAP antibody, followed by secondary antibodies labeled with Alexa Fluor 488 and metal enhanced DAB (brown stain). (C-D) Location of *Ccl2*-positive cells varies dramatically between (C) imiquimod and (D) LPS-treated mice. Images are of same region and from same mice as in A and B. (E,F) *Ccl2*-positive cells were also detected in the choroid plexus of (E) imiquimod, but not (F) LPS-treated mice. All images were taken with a digital camera attached to an Olympus scope. Scale bar is shown for all images (100  $\mu\text{m}$  for A,C-F, 200  $\mu\text{m}$  for B). Data are representative of two separate experiments. Non-specific RNA probes and no-primary antibody controls were used as controls for C-F.



**Fig. 3.5. Kinetics of Cellular Responses (A-D) in the CNS Following Imiquimod or LPS Inoculation in the Neonatal Brain.** Brain tissues at 12, 48, 96 or 312 h post inoculation were processed for real-time RT-PCR analysis using primers specific for (A) *Gfap* (B) *F4/80* (C) *Cd3ε* and (D) *Icam-1* mRNA. Values were calculated relative to the expression of *Gapdh* controls. Data are analyzed as described in Fig. 3.1. Data represent the mean  $\pm$  standard deviation of 3-7 mice per group per time point and represent the combined data from 2 independent experiments. Statistical analysis was completed by two-way ANOVA using Bonferonni post-test., \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

To better understand which cells in the CNS were responding to agonist administration, we analyzed tissue samples from mice at 12 hpi by *in situ* hybridization. We analyzed cells for *Ccl2* mRNA expression as this chemokine is often associated with viral infection in the CNS (McManus et al., 2000; Peterson et al., 2004). *Ccl2*-positive cells in imiquimod treated mice were located at the edges of the ventricles and spread out into the tissue (Fig. 3.4C). Dual staining with GFAP demonstrated that the *Ccl2* positive cells lining the ventricles (Fig. 3.6A) as well as in the tissue (Fig. 3.6B) were primarily astrocytes. *Ccl2*-positive cells were also detected in the choroid plexus (Fig. 3.4E), with *Ccl2*-expressing ependymal cells and endothelia (Fig. 3.6E, data not shown) observed. Brain capillary endothelia located in the thalamus, were also positive for *Ccl2* (Fig. 3.6F). Thus, multiple cell types respond to imiquimod stimulation including astrocytes, ependymal and endothelial cells. In contrast, *Ccl2*-positive cells in LPS-treated mice appeared to be primarily infiltrating cells within the ventricles (Fig. 3.4, D and F, 3.6C). Astrocytes were not positive for *Ccl2* mRNA in LPS-treated mice (Fig. 3.6D). Thus,



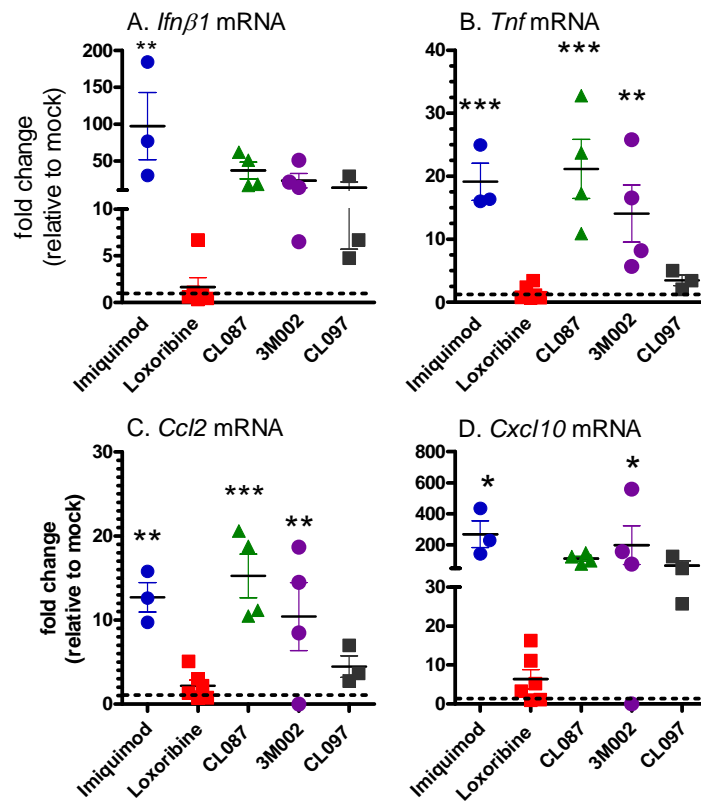


**Fig. 3.6. *In situ* Hybridization-Immunohistochemistry Analysis of Mid-Coronal Sections of (A, B, E, F) Imiquimod, and (C,D) LPS Inoculated Neonatal Brain.** Tissue sections were stained as described for Fig. 3.4C-F. (A,B) GFAP-positive astrocytes (brown stain) express *Ccl2*-mRNA (red stain) in imiquimod treated mice. Images are from same region shown in Fig. 3.4A and C. (C,D) Infiltrating cells, not GFAP-positive astrocytes, express *Ccl2* mRNA in LPS-treated mice. Images are from same region shown in Fig. 3.4B and D. (E) *Ccl2*-positive cells from choroid plexus include ependymal and epithelial cells. (F) Capillary endothelial cells in thalamus are also positive for *Ccl2* mRNA expression in imiquimod treated mice. All images were taken with a digital camera attached to an Olympus scope. Scale bars are shown for all images (50 µm). Images are representative of cells in the surrounding area. Data are representative of two replicate experiments. Non-specific RNA probes and no-primary antibody controls were used as controls for all experiments.

despite the common upregulation of *Ccl2* mRNA and protein by imiquimod and LPS, the cellular source of *Ccl2* at 12 hours post infection was different between these two agonists.

### 3.2.5. Comparison Between TLR7/8 Agonists

Recent studies have indicated that murine TLR8 is functional and that murine TLR8 can be stimulated on neurons (Gorden et al., 2006a; Gorden et al., 2006b). Differences in the chemical structure were shown to influence binding of agonists to human TLR7 and human TLR8 (Table 3.1) (Hemmi et al., 2002; Lee et al., 2003). To investigate possible differences between these agonists in inducing neuroinflammation, we compared three TLR7 agonists

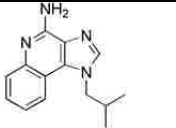
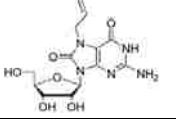
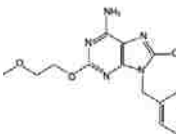
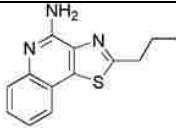
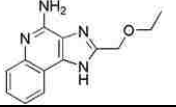


**Fig. 3.7. *In vivo* Response to Stimulation by Various TLR7/8 Agonists in the Developing Brain.** 100 nmoles of the appropriate agonist was inoculated i.c.v. into 2 day old mice as described in Fig. 3.1. At 12 hpi, brain tissue was removed and snap frozen in liquid nitrogen. Samples were analyzed as described in Fig. 3.1. Fold increase in (A) *Ifnb1* (B) *Tnf* (C) *Ccl2* and (D) *Cxcl10* mRNA as compared to mock-infected controls. Each symbol represents an individual animal for the group. Data represent the mean +/- standard deviation for 3 to 6 mice per group and are representative of two replicate experiments. Statistical analysis was completed by one way ANOVA with Newman-Keuls post-test. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.



(imiquimod, loxoribine and CL087), a TLR8 agonist (3M002) and a TLR7/8 agonist (CL097) (Table 3.1). Interestingly, the ability of these agonists to induce cytokine/chemokine responses was not divided between TLR7 or TLR8 stimulation capabilities. Two TLR7 agonists (imiquimod and CL087) and the TLR8 agonist (3M002) induced similar levels of proinflammatory cytokines and chemokines (Fig. 3.7). The TLR7/8 agonist, CL097, induced *Ifnb1* and *Cxcl10* mRNA expression, but did not induce the expression of *Tnf* or *Ccl2* mRNA. Loxoribine, a TLR7 agonist similar to imiquimod induced only minimal expression of proinflammatory cytokines and chemokines compared to the other agonists. Thus, TLR7/TLR8 agonists differed in their ability to induce proinflammatory responses in the CNS in mice.

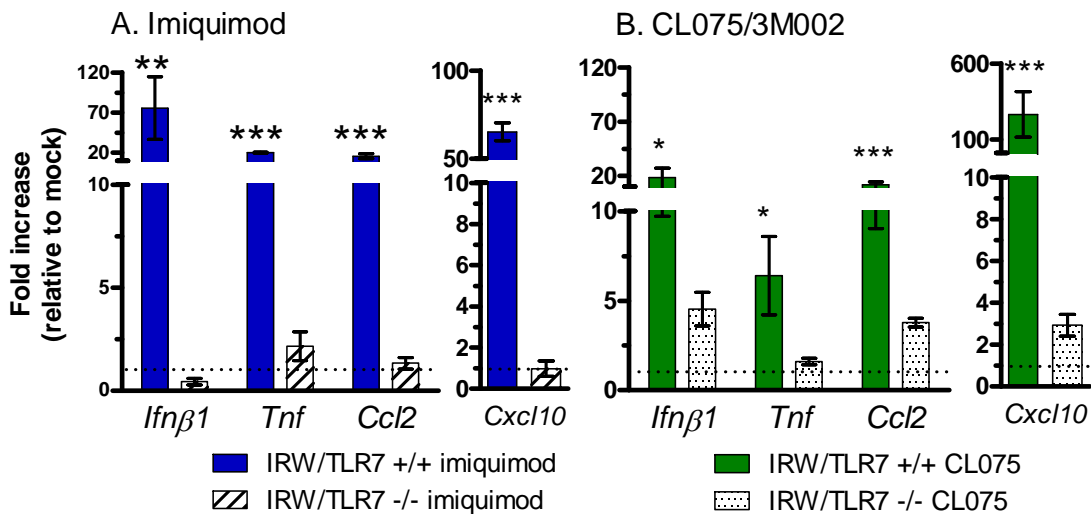
**Table 3.1. Properties of TLR7 and TLR8 Agonists Used.**

Ligand	Description	Stimulation of		Structure
		hTLR7	hTLR8	
Imiquimod (R837)	imidazoquinoline	+	-	
Loxoribine (7-Allyl-8-oxoguanosine)	guanosine analog	+	-	
CL087	adenine analog	+	-	
3M002 (CL075)	thiazoloquinoline	-	+	
CL097	imidazoquinoline	+	+	

### 3.2.6. TLR7 Contributes to Both Imiquimod and 3M002-Induced Response

3M002 stimulated human TLR8 or mouse TLR8 transfected-HEK cells, but not human TLR7 transfected-HEK cells (Gorden et al., 2005). To investigate whether 3M002-induced

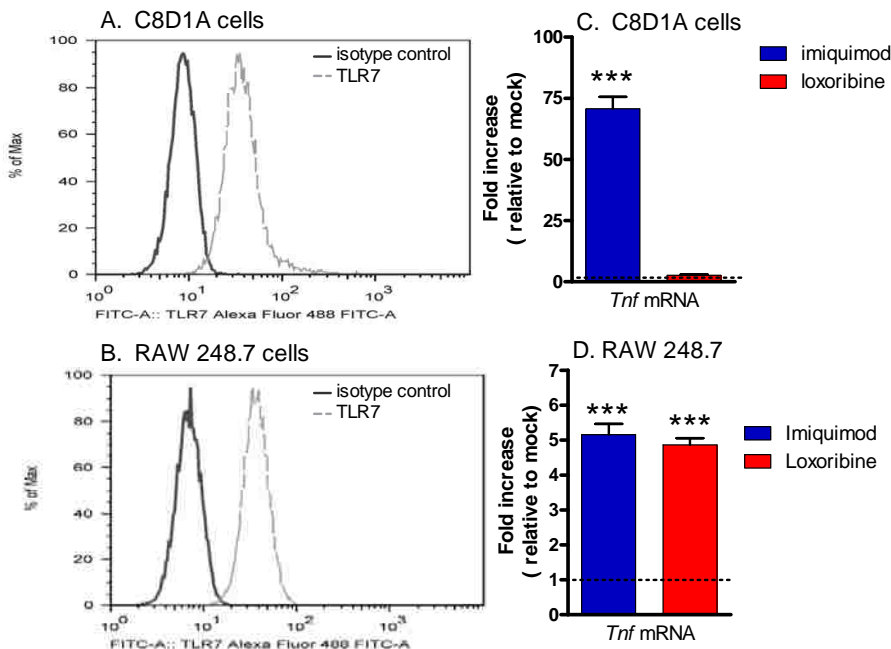
neuroinflammation was mediated by TLR7 or TLR8, we utilized TLR7 deficient mice. As a control, we also analyzed imiquimod-induced neuroinflammatory responses in TLR7 sufficient and deficient mice. Both imiquimod and 3M002 induced significant upregulation of *Ifnb1*, *Tnf*, *Ccl2* (*MCP1*) and *Cxcl10* (*IP-10*) mRNA levels in the brain of TLR7 sufficient (+ or +/-) mice at 12 hpi (Fig. 3.8). In contrast, imiquimod did not upregulate any of the cytokines or chemokines in TLR7 deficient (-/- or -) mice. This confirms that imiquimod upregulated the proinflammatory cytokines and chemokines in the brain through the TLR7 pathway, not by TLR8. 3M002-induced cytokine and chemokine expression was greatly reduced in TLR7 deficient compared to TLR7 sufficient mice (Fig. 3.8B). However, 3M002 did induce a low level of *Ccl2* and *Ifnb1* mRNA expression indicating that 3M002 may also be stimulating through TLR8, albeit at a much reduced level than TLR7 (Fig. 3.8B).



**Fig. 3.8. Influence of TLR7 Deficiency on Cytokine and Chemokine mRNA Expression Following (A,B) Imiquimod or (C,D) 3M002 Inoculation.** TLR7 wild type/homozygous positive (+/+, +) or heterozygous positive (+/-) or TLR7 deficient mice were inoculated as described Fig. 3.1. Brain tissues were removed at 12 h post inoculation, processed and analyzed as described in Fig. 3.1. Data represent the mean +/- standard deviation for 3 to 4 mice per group and are representative of two replicate experiments. Statistical analysis was completed by one way ANOVA with Newman-Keuls post-test. \*P<0.05, \*\*P<0.01. \*\*\*P<0.001.

### 3.2.7. Astrocyte Response Differs Between Imiquimod and Loxoribine.

The difference between the ability of imiquimod and loxoribine to induce cytokine and chemokine responses *in vivo* (Fig. 3.7) was surprising since both agonists stimulate TLR7 in dendritic cells and macrophages (Heil et al., 2003; Hemmi et al., 2002; Lee et al., 2003). Therefore, we compared the ability of these agonists to stimulate cells *in vitro*. As astrocytes were one of the primary cell populations to respond to imiquimod stimulation, we examined the ability of imiquimod and loxoribine to stimulate the astrocyte cell line, C8D1A. As a positive control, we used the macrophage cell line, RAW 264.7.



**Fig. 3.9. Expression of TLR7 on (A) Astrocytes (C8D1A), and (B) Macrophage Cells (RAW) by Intracellular Flow Cytometry Analysis.** Data was collected on a FACSaria and analyzed with FlowJo software. *Tnf* mRNA expression in astrocyte cultures (C) and macrophage cultures (RAW) (D) following stimulation with the TLR7 agonists, imiquimod, and loxoribine. Semi-confluent cultures of astrocytes or macrophages were stimulated with 100  $\mu$ Moles/ml of imiquimod or loxoribine or mock stimulated. Cells were lysed at 48hrs post stimulation and the RNA was isolated. Real-time quantitative RT-PCR analysis was performed using primers specific for *Tnf*, and *Gapdh* mRNA, and analyzed as described in Fig. 3.1. Data represent the mean  $\pm$  standard deviation for 3 samples per group and represent one of three replicate experiments. Statistical analysis was completed by one way ANOVA with Newman-Keuls post-test. \*\*\*P<0.001.

Both cell types express TLR7 as detected by intracellular flow cytometry staining (Fig. 3.9, A and B). Imiquimod, but not loxoribine, stimulation of C8D1A cells induced *Tnf* mRNA (Fig. 3.9C). As expected, both loxoribine and imiquimod induced upregulation of *Tnf* mRNA expression in a macrophage cell line (Fig. 3.9D). Thus, the difference between the responses to imiquimod and loxoribine *in vivo* may be due to their abilities to activate certain cell types of the CNS, such as astrocytes.

### **3.3. DISCUSSION**

Although TLR7 is a known mediator of immune responses to ssRNA viruses, very little is known about the role of this receptor in neuroinflammation. In the present study, we demonstrated that a single inoculum of a TLR7 agonist was sufficient to induce a pronounced neuroinflammatory response including the production of multiple proinflammatory cytokines and chemokines, as well as the activation of astrocytes. The duration of the response was limited as both cytokine and chemokine induction and astrocyte activation waned by 48 h post stimulation. Thus TLR7 stimulation induced a short, but pronounced, neuroinflammatory response in the CNS. This contrasts with other TLR agonists such as LPS or CpG DNA which can induce neuronal apoptosis and/or animal death with a single i.c.v. administration (Macagno et al., 2006; Pedras-Vasconcelos et al., 2006).

The neuroinflammatory responses induced by imiquimod stimulation in the brain were of short duration compared to LPS stimulation (Fig. 3.1). One possible reason for this is the anatomical location of these toll-like receptors on the cells. TLR4 is located on the cell surface whereas TLR7 is present within the cell on endosomal membranes. The polysaccharide side chains of LPS located on the bacterial surfaces can stimulate TLR4 located on the cell membranes directly. In order to stimulate TLR7 during viral infections, ssRNA must directly interact with the endosomal membranes either by direct uncoating in the endosomal membranes

or by autophagy from the cytoplasm (Lee et al., 2007). This would limit the activation of cells to either infected cells or phagocytic cells surrounding the site of infection.

Another possible reason for the differences in neuroinflammatory responses induced by TLR7 and TLR4 agonists is that the structural complexity of the TLR7 agonists is quite different from that of the TLR4 agonists. The TLR4 agonist LPS is a large complex molecule, consisting of lipids and polysaccharides. The TLR7 agonists used in the present study are small synthetic compounds which may be degraded rapidly. This may also be pertinent to the responses to bacterial versus viral infections, with bacterial LPS remaining stable and viral ssRNA being degraded by RNases. Thus, a robust viral infection with generation of substantial RNA particles may be needed to elicit an equivalent viral mediated neuroinflammatory response. This is supported by observations of the inflammatory response to retrovirus infection in the CNS where peak chemokine mRNA expression correlated with peak viral RNA expression (Peterson et al., 2004; Peterson et al., 2006; Peterson et al., 2001). Interestingly, the chemokine mRNA response started to decline at the approximate time when virus levels plateaued in the brain (Peterson et al., 2004; Peterson et al., 2006; Peterson et al., 2001).

One of the primary differences in the response between imiquimod and LPS stimulation was the source of *Ccl2* mRNA (Fig 3.4, 3.6). In imiquimod-treated animals, the predominant cell type producing *Ccl2* mRNA was astrocytes, although not astrocytes expressed *Ccl2* mRNA. CCL2 expression by astrocytes has been observed with virus infection, multiple sclerosis and Alzheimer's disease (Johnstone et al., 1999; McManus et al., 1998; Peterson et al., 2004). In contrast, astrocytes from LPS-stimulated animals were not positive for *Ccl2* mRNA (Fig. 3.4 and 3.6), although it is possible that these cells do express *Ccl2* mRNA at later time points following infection. However, the majority of the *Ccl2*-producing cells in the LPS-inoculated mice were

infiltrating cells, suggesting that most of the inflammatory response was also from infiltrating cells rather than the resident glial cells.

Dose curve analysis of imiquimod administration indicated that 100-200 nmoles induced neuroinflammation, while 5-fold higher or lower concentrations did not. Possibly high concentrations of agonist overwhelm cells resulting in the limitation of the production of proinflammatory cytokines and chemokines. Alternatively, at higher concentrations individual agonists may aggregate resulting in a diminished uptake by cells in the CNS.

In the current study, not all of the TLR7/8 agonists used induced neuroinflammatory responses. The ability to induce neuroinflammation in the brain was not discernable by preference for TLR7 or TLR8. For example, the TLR7 agonist, imiquimod, induced astrocyte responses both *in vivo* and *in vitro*, whereas the TLR7 agonist, loxoribine, did not. In contrast, both imiquimod and loxoribine induced responses in macrophages, as have been reported for plasmacytoid dendritic cells and B cells (Hemmi et al., 2002; Lee et al., 2003). These variations in neuroinflammatory responses among TLR7/8 agonists used in the present study could be due to differential uptake of individual agonists by certain cell types. Possibly, loxoribine is not endocytosed by astrocytes as readily as imiquimod. Thus, a comparison between complementary agonists may be necessary to differentiate the functional responses to TLR7 or TLR8 in different cell types.

TLR7 and TLR8 are highly homologous and their distinct roles in innate immune responses are still being analyzed (Heil et al., 2004; Lee et al., 2003). In our current study, the human TLR8 agonist, 3M002, induced similar neuroinflammatory responses to the TLR7 agonists. However, this response appeared to be mediated primarily by TLR7, not TLR8. This agrees with *in vitro* studies in which 3M002 stimulation of human TLR8 but not mouse TLR8

induced NF- $\kappa$ B activation (Gorden et al., 2005). Thus, in the mouse, 3M002 appears to stimulate primarily through TLR7, rather than TLR8.

TLR agonists are currently being studied as immune activators or immune response modifiers (IRMs) to enhance vaccines, improve the immune response to different cancers and treat viral infections (Murad et al., 2007; Wang et al., 2005; Wille-Reece et al., 2005; Zaks et al., 2006). In the present study, certain TLR7/8 agonists induced substantial pro-inflammatory responses in the CNS in terms of cellular activation and production of proinflammatory cytokines and chemokines, without inducing overt damage. This suggests that the TLR7/8 agonists may be used as immune response modifiers for diseases and conditions affecting the central nervous system. These studies also demonstrate that TLR7/8 agonists can differ widely in their ability to induce neuroinflammation, as some of the agonists induced only minimal responses in the brain. Thus, agonists such as loxoribine may be effective in enhancing peripheral immune responses yet limit activation of glial cells and production of proinflammatory cytokines in the CNS. The differences between these IRMs in inducing inflammatory responses may be beneficial when targeting a specific organ or cell type.

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## **CHAPTER 4**

### **COMPARISON OF THE NEUROINFLAMMATORY RESPONSES FOLLOWING TLR7 AND TLR9 ACTIVATION IN THE CNS**

## 4.1. INTRODUCTION

Neuroinflammation is a common response to infection or injury of the central nervous system (CNS) and includes the induction of proinflammatory cytokines, the activation of glial cells and the recruitment of inflammatory cells to the CNS (Asensio and Campbell, 1997; Dickson et al., 1993; Griffin, 2003; Kelder et al., 1998; McCoig et al., 2004). This response can be detrimental to neurons in the CNS and lead to neurological disorders. Neuroinflammatory responses may be dependent on the initiation of innate immune responses triggered by the stimulation of intrinsic brain cells by pathogen-associated molecular patterns (PAMPs), the repeated structural motifs generated by microbes that are not normally found in the host or by debris from apoptotic or necrotic cells following injury. Determining the initiation of these responses and the downstream consequences of activation of the innate immune responses will lead to a better understanding of the underlying mechanisms of neuronal damage following infection in the brain.

Recent studies demonstrated that toll-like receptor (TLR) 7, which recognizes viral single-stranded RNA, and TLR9, which recognizes unmethylated DNA with CpG motifs generated by certain viruses and bacteria, can play an important role in both the activation of innate immune responses and in the viral pathogenesis in the central nervous system (Lewis et al., 2008; Sorensen et al., 2008; Town et al., 2009). Agonists of both TLR7 and TLR9 are also being investigated for the potential use in treating CNS-related diseases (Butchi et al., 2008; El Andaloussi A. et al., 2006; Pedras-Vasconcelos et al., 2006; Pedras-Vasconcelos et al., 2008; Prins et al., 2006). However, there appears to be some differences in the CNS response to activation of these receptors. For example, peripheral administration of TLR9 agonists, but not TLR7 agonists, were protective against arenavirus induced neurological disease (Pedras-Vasconcelos et al., 2006). Different responses were observed with the intracerebroventricular

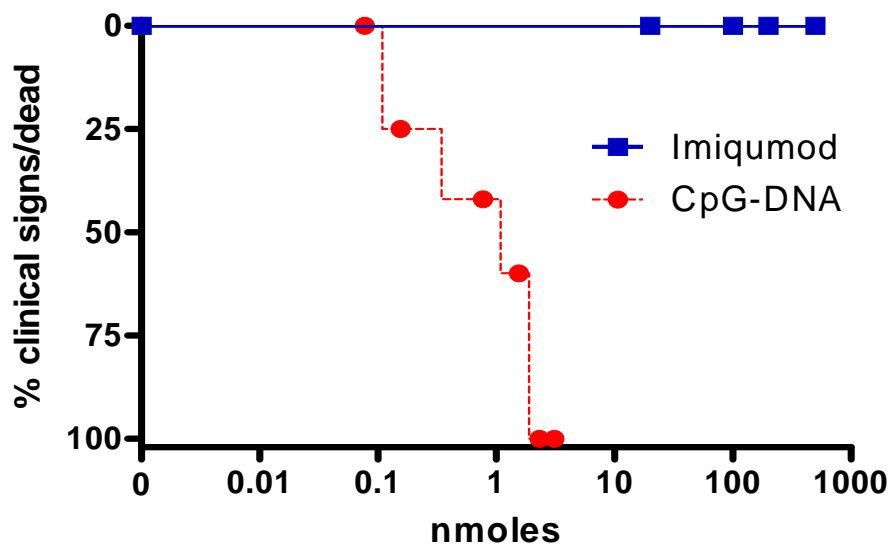
(i.c.v.) inoculation of agonists, as TLR9 agonist inoculation was lethal in newborn mice, while TLR7 agonist was not (Chapter 3, Fig 3.2) (Butchi et al., 2008; Pedras-Vasconcelos et al., 2006). Understanding the similarities and differences of TLR7 and TLR9-induced cell activation in the brain is important for understanding viral pathogenesis as well as for the potential use of TLR agonists in the treatment of neurological diseases.

In the present study, we compared the responses of TLR7 and TLR9 agonist stimulation in the CNS using intracerebroventricular (i.c.v.) inoculation of imiquimod and CpG-ODN respectively. We examined why TLR9 agonist have a substantially stronger inflammatory response in the CNS compared to TLR7 agonists and compared the responses to agonist stimulation both at cellular and molecular level.

## **4.2. RESULTS**

### **4.2.1. Comparison of Dose Response between TLR7 and TLR9 Agonists in the CNS**

Studies with the use of TLR9 agonists for the treatment of arenavirus infection demonstrated that TLR9 agonist administered i.c.v. was fatal in neonatal mice (Pedras-Vasconcelos et al., 2006; Pedras-Vasconcelos et al., 2008). This response was not observed in neonatal mice inoculated with TLR7 agonists. To understand the differences in the CNS response to these two agonists, which bind highly similar receptors, we inoculated neonatal mice within 48 hours of birth with different concentration of either the TLR7 agonist, imiquimod, or the TLR9 agonist, CpG-ODN 1826. Interestingly, TLR9 agonist induced death within 3-4 hours in all the mice at 3100 pmoles (20 µg). Mice inoculated with CpG-ODN between 155 pmoles-2325 pmoles (1µg-15µg) induced clinical signs of severe respiratory distress and unresponsiveness within 12 hours (Fig. 4.1). In contrast, 500 nmoles of imiquimod, injected i.c.v. did not induce any detectable sign of discomfort in neonatal mice. Thus, CpG-ODN induces death in neonatal mice, while imiquimod does not.



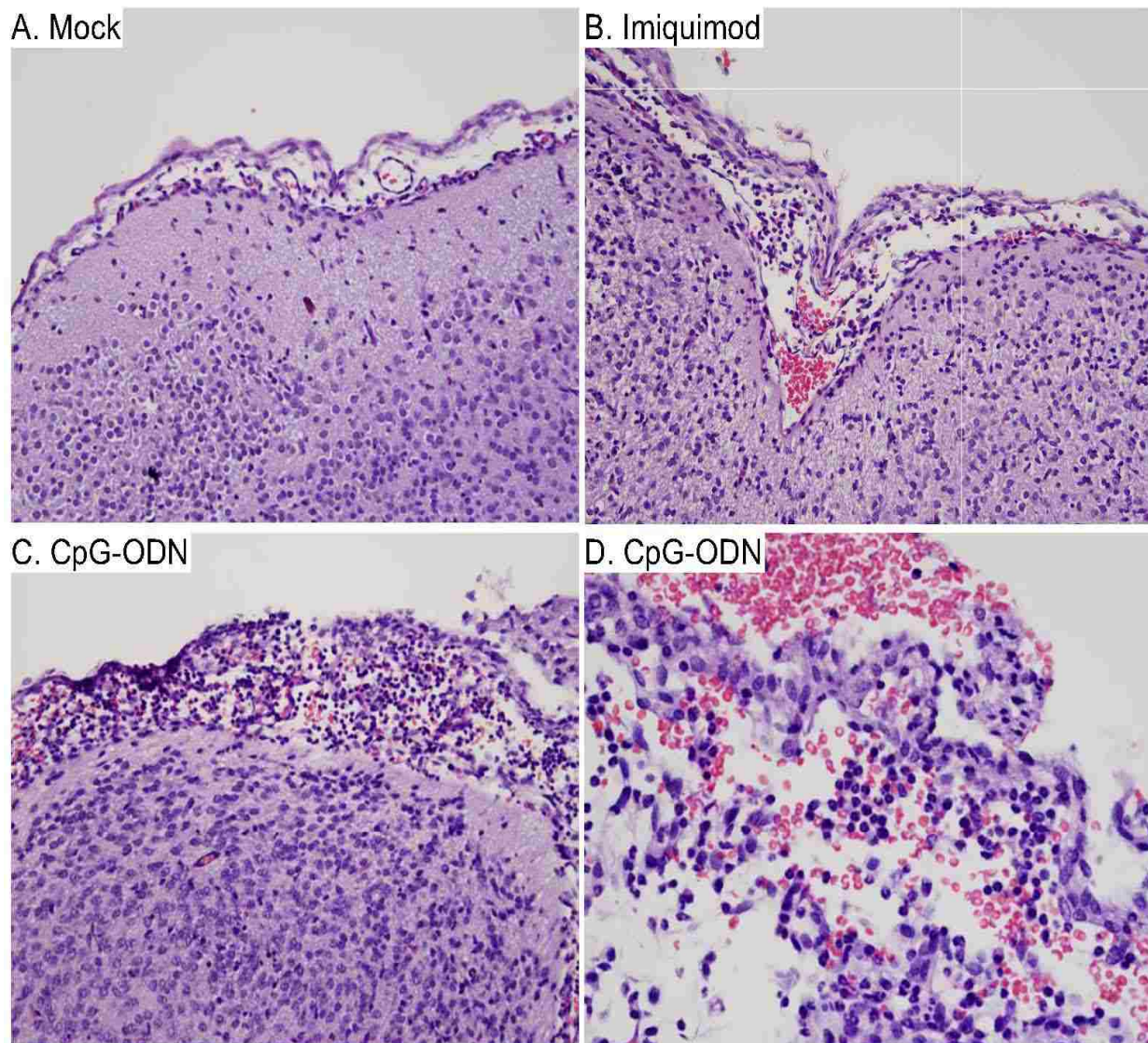
**Fig. 4.1. Intracerebroventricular Inoculation of TLR9 Agonist, But Not TLR7 Agonist Induces Death in Neonatal Mice.** Mice were inoculated with the indicated concentration of the TLR7 agonist, imiquimod, or the TLR9 agonist CpG-ODN 1826 and followed for clinical signs of substantial distress or death within the first 12 hours following inoculation. Each symbol represents the % mice with clinical signs or death for each dose with an n of 5-7 mice per dose.

#### 4.2.2. Inflammatory Response to TLR7 and TLR9 Agonist Administration in the CNS

To compare the inflammatory responses induced by imiquimod and CpG-ODN stimulation in the CNS, we utilized the highest concentration of CpG-ODN that was not lethal to mice (80 picomoles) and the concentration of imiquimod that induced the strongest inflammatory response (100 nanomoles) (Chapter 3, Fig. 3.2) (Butchi et al., 2008). Histological analysis of tissue sections from CpG-ODN, imiquimod and mock-inoculated brain tissues was completed in a blinded study for meningitis and perivascular cuffing. Meningitis was observed in CpG-ODN and imiquimod-inoculated animals as well as some mild infiltrate in some of the mock (vehicle control)-treated animals (Fig. 4.2). neutrophils (N), plasma cells (P) and lymphocytes (L) were all observed in the meninges of imiquimod and CpG-ODN inoculated animals (Table 4.1), however, a higher level of cellular inflammation was associated with the CpG-ODN inoculated mice than imiquimod or mock-inoculated samples (Fig. 4.2). Despite the substantial meningeal



inflammatory response, only minor perivascular cuffing was observed in any of the inoculated mice (Table. 4.1).



**Fig. 4.2. Meningeal Inflammation in the Brain Tissue Following I.C.V. Inoculation of Imiquimod or CpG-ODN.** Brain tissue was isolated from inoculated mice at 12 hps, cut in four coronal sections and processed for histology. Hematoxylin and Eosin sections were analyzed in blinded fashion for inflammation. Tissues sections from mice inoculated with (C) CpG-ODN showed pronounced meningeal inflammation compared to mice inoculated with (A) mock-inoculum (PBS) or with (B) imiquimod. (D) Higher power image of inflammatory cells in the meninges of CpG-ODN inoculated mice. (A-C) 100X, (D) 400X.

**Table. 4.1. Inflammation Following I.C.V. Inoculation of Imiquimod or CpG-ODN.**

Inoculum <sup>a</sup>	Meningitis <sup>b</sup>	Meningeal infiltrate <sup>c</sup>	Perivascular cuffing <sup>d</sup>
no inoculum	1	N	0
no inoculum	1	N	0
mock (PBS)	2	N	0
mock (PBS)	2	N	0
mock (PBS)	1	L/P	0
imiquimod	3	N, L/P	0
imiquimod	2	N, L/P	0
imiquimod	4	N, L/P	0
CpG-ODN	4	N, L/P	0
CpG-ODN	4	N, L/P	0
CpG-ODN	4	N, L/P	0
CpG-ODN	4	N, L/P	0
CpG-ODN	4	N, L/P	0

a: mice inoculated with 10 µl of mock inoculum (PBS with 0.2% trypan blue), 100 nmoles (25 ug) of imiquimod or 80 pmoles (0.5 µg) of CpG-ODN in a 10 µl volume of PBS with 0.2% trypan blue. Tissue sections were removed as 12 hps.

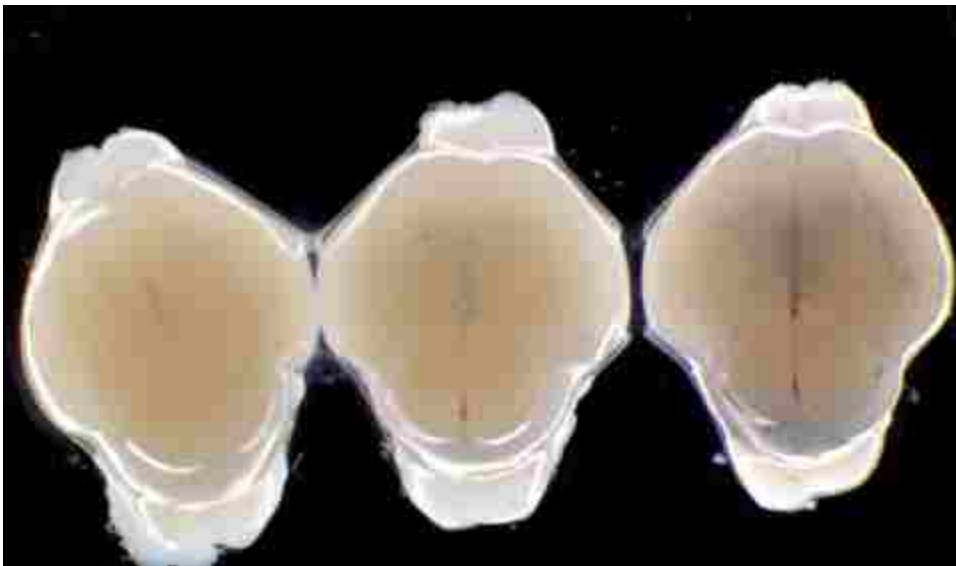
b: Meningitis was scored on a scale of 0-4 with the following scale: 0, no infiltrate; 1, minimal cellular infiltrate; 2, mild cellular infiltrated; 3, moderate infiltrate; 4, severe infiltrate. Slides were randomly numbered to generate a scrambled ordered before being blindly analyzed.

c: N: neutrophils; L: lymphocytes, P: Plasma cells

d: perivascular cuffing was scored on a 0-4 with 0 being no signs of inflammatory cells in the perivascular space; 1, a few areas of perivascular cuffing with a low cellular infiltrate; 2, multiple regions of low level perivascular cuffing or a few areas of moderate infiltrate; 3, multiple areas of moderate infiltrate; 4, multiple areas of prominent perivascular cuffing. Slides were randomly numbered to generate a scrambled ordered before being blindly analyzed.

To examine if the BBB was compromised by either imiquimod or CpG-ODN inoculation, we inoculated mice intraperitoneally with Evans blue dye at 1h or 4h prior to analysis, whether the dye crossed the blood-brain barrier at 12 and 24 hpi. No crossing of Evans blue dye was detectable by gross examination of brain tissue at 12 hpi, however, Evans blue dye was observed in brain tissue from CpG-ODN inoculated mice at 24 hpi (Fig. 4.3). Slight to undetectable levels

of Evans blue dye was observed in imiquimod inoculated mice suggesting that imiquimod inoculation did not alter the BBB to the same degree as CpG-ODN inoculation.

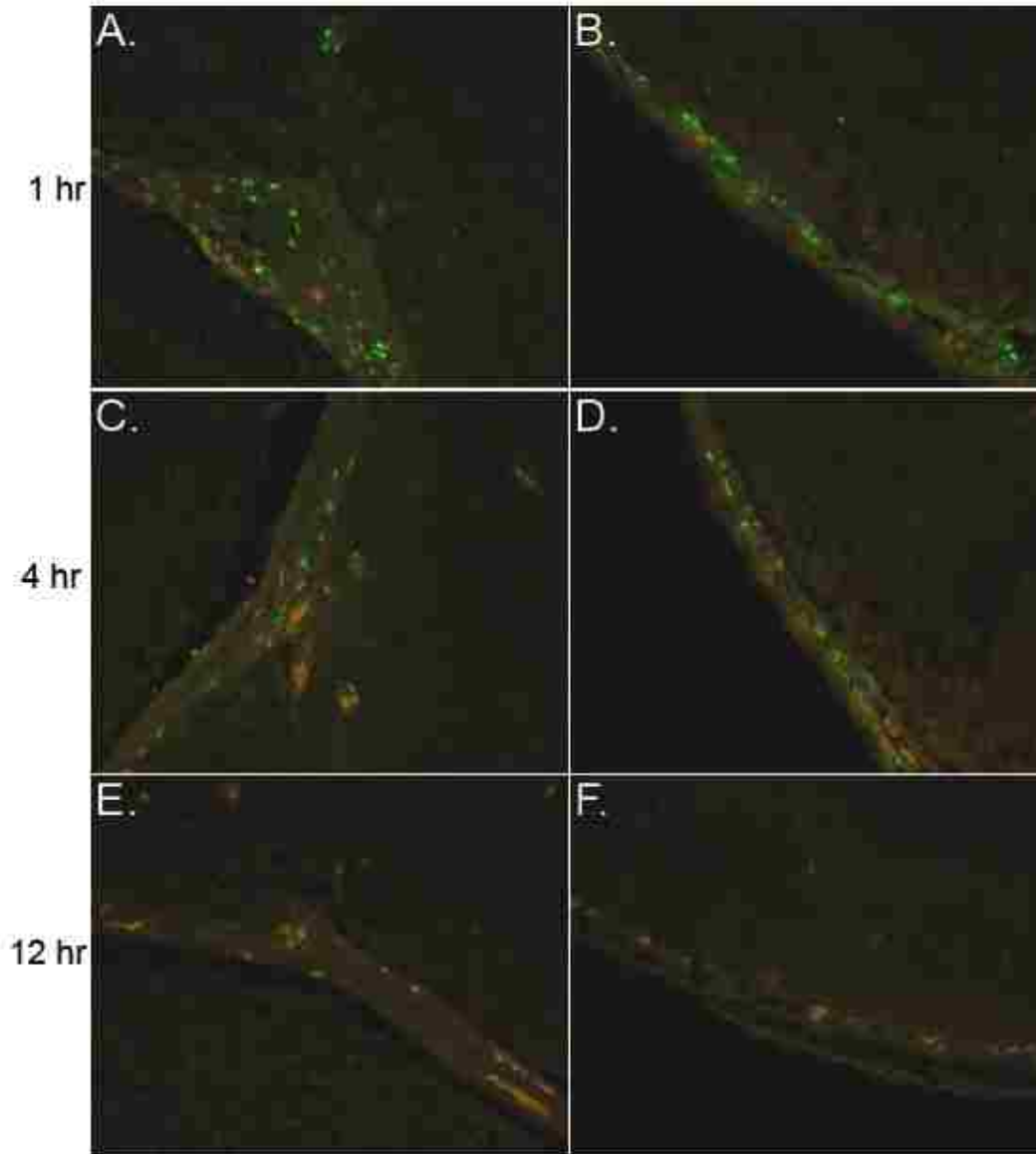


**Fig. 4.3. Evans Blue Analysis of BBB Breakdown in Inoculated Mice.** Mice were inoculated with agonists or controls. At 20 hpi, mice were given Evans blue dye intraperitoneally. At 24 hpi, mice were perfused with PBS and brain tissue removed. Brain tissue was analyzed under a stereo-microscope.

#### **4.2.3. Localization of CpG-ODN in the CNS Following I.C.V. Inoculation**

Imiquimod is a small molecule, which induced activation in multiple regions of the brain, most likely due to the ability of imiquimod to spread within the CNS (Chapter 3, Fig 3.4, 3.6). In contrast, CpG-ODN is a larger molecule (20 mer) and therefore may not migrate out of the ventricles as well as imiquimod or other TLR7 agonists. To examine CpG-ODN localization after i.c.v. inoculation we examined the localization of FITC-labeled CpG-ODN at one, four and twelve hours post-inoculation using an anti-FITC antibody. A non-labeled CpG-ODN inoculated mouse was utilized as a control for non-specific staining. CpG-ODN was primarily localized in the ventricles and meninges at 1 hps (Fig. 4.4A, B), with spread to brain capillary endothelia and cells around blood vessels by 4 hps (Fig. 4.4C,D). Interestingly, no CpG-ODN was detected in

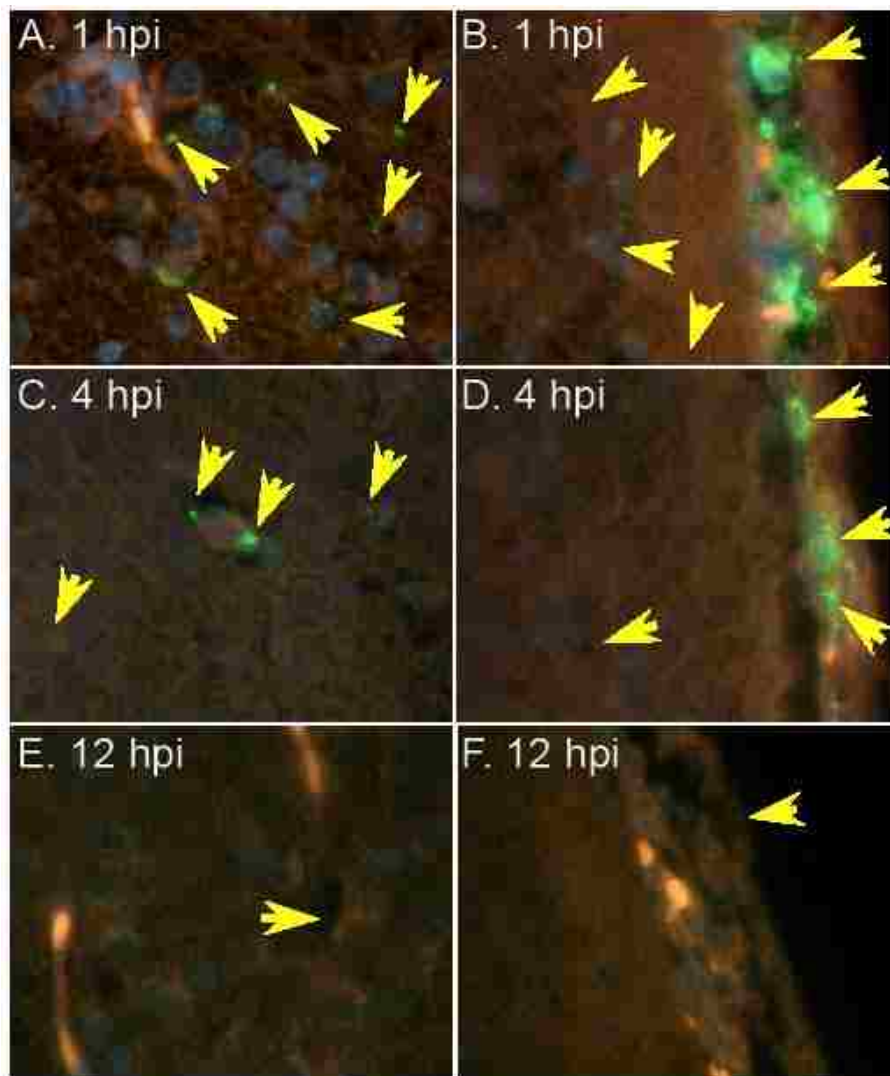
any regions of the brain at 12hps, indicating that the majority of CpG-ODN was taken up by cells and processed by this time point.



**Fig. 4.4. CpG-ODN (green) in the Brain Tissue at One, Four, and 12 Hours Post Inoculation.** Mice were inoculated with 80 pmoles of (A-F) FITC-labeled CpG-ODN or unlabeled CpG-ODN (not shown) and brain tissue removed at (A,B) one, (C,D) four or (E,F) twelve hours post-inoculation. Brain tissue was isolated from inoculated mice, cut in four coronal sections and processed for histology. Tissue sections were stained for FITC using rabbit anti-fluorescein primary antibody and goat anti-rabbit AlexaFluor 488 secondary antibody. Images are 100X magnification.



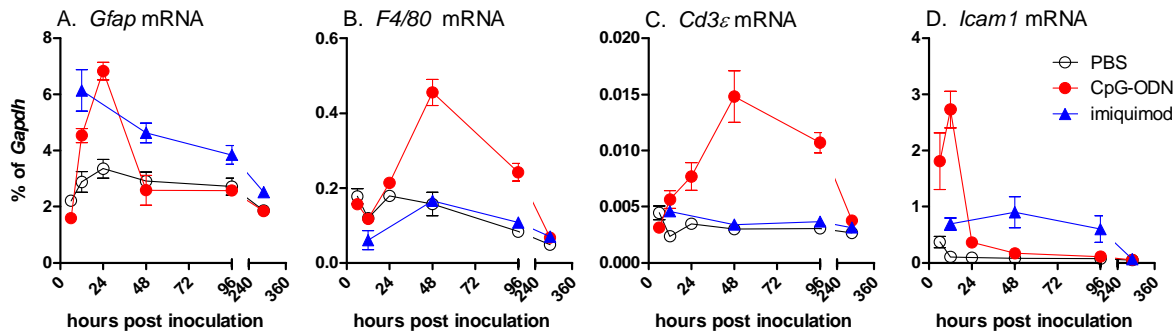
Although the majority of CpG-ODN was detected in the ventricles and meninges, low amounts of CpG-ODN was detected in numerous cells throughout the brain at one hpi (Fig. 4.5A). At four hours, only a few cells outside the ventricles and meninges were positive for CpG-ODN and these were generally around blood vessels (Fig. 4.5C,D). At twelve hpi, only a few random dots of CpG-ODN were detected (Fig. 4.5E,F). Control non-labeled CpG-ODN sections were negative for fluorescence (data not shown).



**Fig. 4.5. CpG-ODN (green) in the Brain Tissue at (A,B) One, (C,D) Four, and (E,F) 12 Hours Post Inoculation.** Images are from the same sections as described in Fig. 4.4. Yellow arrows point to labeled CpG-ODN, which is primarily cell associated but was also found non-cell associated. Magnification 1,000X.

#### 4.2.4. Cell Marker Expression Following TLR Agonist Inoculation.

We previously found that i.c.v. inoculation of imiquimod induced only low level glial cell activation and did not induce T cell recruitment to the CNS, as detected by gene expression of glial activation markers and T cell markers (Chapter 3, Fig. 3.5). Increased astrocyte activation, as detected by *Gfap* mRNA expression, was only detected in CpG-ODN inoculated mice at 24 hpi and returned to basal levels by 48 hpi (Fig. 4.6A). However, *F4/80* and *Cd3ε* mRNA expression peaked at 48 hpi and remained elevated at 4 days post inoculation (Fig. 4.6B, C). Thus, CpG-ODN inoculation induced a pronounced inflammatory response in the CNS that is maintained for several days, despite the clearance of CpG-ODN by 12 hpi.

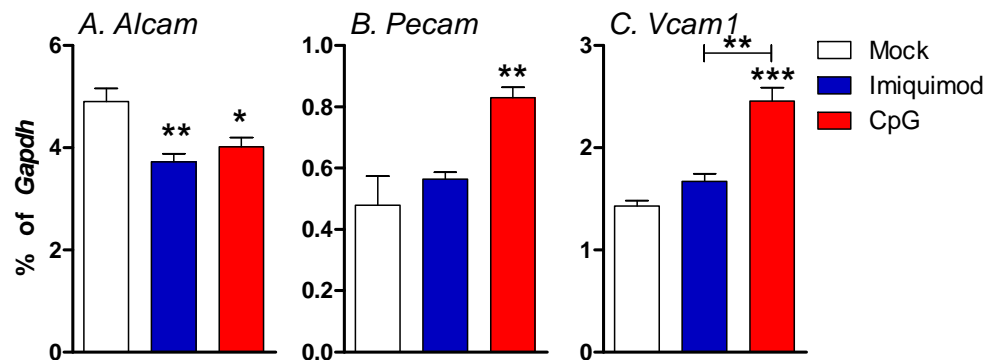


**Fig. 4.6. Kinetics of Cellular Responses (A-D) in the CNS Following Imiquimod or CpG-ODN Inoculation in the Neonatal Brain.** Mice at 48 h of age were inoculated as described in Table 4.1. Brain tissues were removed at 6, 12, 24, 48, 96 or 312 hpi and snap frozen for RN analysis. RNA samples were processed for real-time quantitative RT-PCR analysis using primers specific for (A) *Gfap* (B) *F4/80* (C) *Cd3ε* and (D) *Icam1* mRNA. Values were calculated relative to expression of *Gapdh* controls. Data represent the mean +/- standard error of 3-7 mice per group per time point and represent the combined data from 2 independent experiments.

#### 4.2.5. Alteration of Adhesion Molecule Expression.

Adhesion molecules ICAM1 (CD54, Cellular adhesion molecule 1), PECAM (CS31, Platelet endothelial cell adhesion molecule), VCAM (CD106, Vascular cell adhesion molecule) and ALCAM (CD166, Activated leukocyte cell adhesion molecule) play a role in the migration

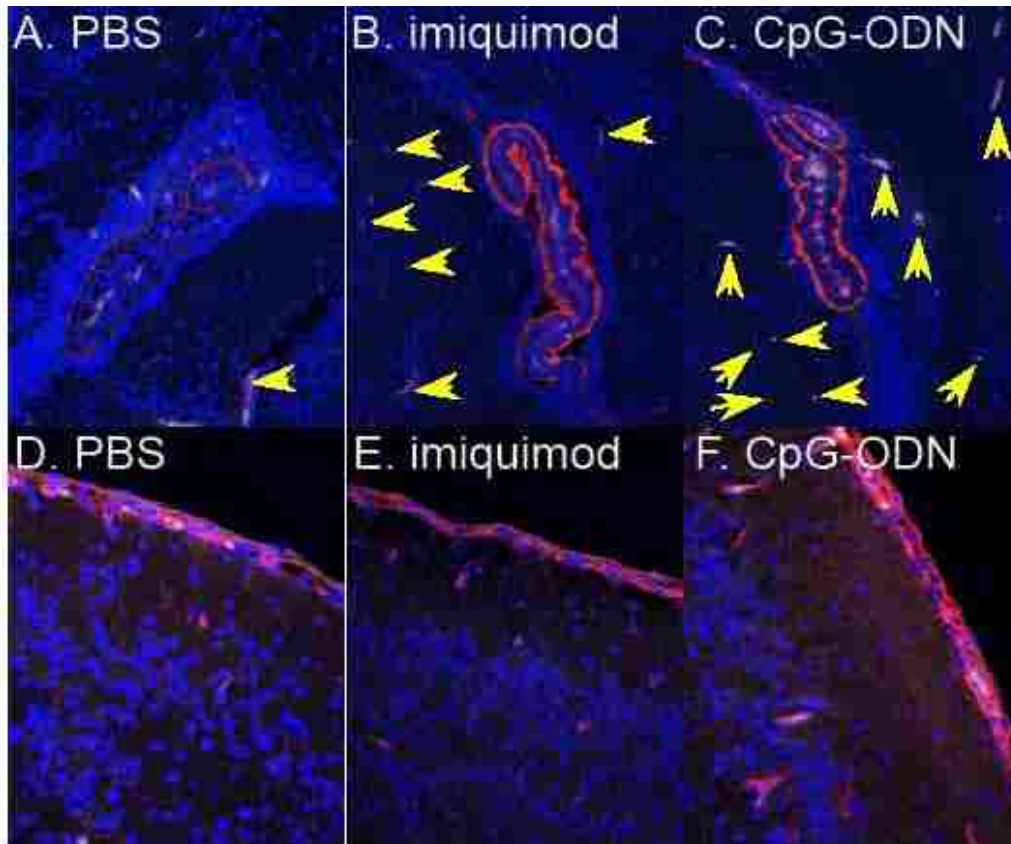
of lymphocytes and other inflammatory cells across the blood-brain barrier. Examination of the mRNA expression of *Icam1* demonstrated that both agonists induced upregulation of *Icam1* mRNA, but at substantially higher levels in CpG-ODN inoculated mice compared to imiquimod inoculated mice (Fig. 4.6D). Expression of *Icam1* mRNA was elevated early following agonist stimulation and then rapidly fell to basal levels by 48 hps. Analysis of other adhesion molecules at the 12 h time point demonstrated that *Pecam* (CD31) and *Vcam* (CD106) mRNA expression was elevated only by CpG-ODN inoculation and not imiquimod inoculation (Fig. 4.7). In contrast, mRNA expression of *Alcam* was not upregulated (Fig. 4.7).



**Fig. 4.7. Upregulation of Adhesion Molecules Following CpG-ODN or Imiquimod Inoculation in the CNS.** RNA samples from the 12 hour time point in Fig. 4.4 were analyzed for expression of other adhesion molecules by real-time quantitative RT-PCR analysis using primers specific for (A) *Alcam* (B) *PeCam* and (C) *Vcam1* mRNA. Values were calculated relative to expression of *Gapdh* controls. Data represent the mean +/- standard error of 3-7 mice per group per time point and represent the combined data from 2 independent experiments. Statistical analysis was completed by one-way ANOVA using Bonferonni post-test., \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

Since *Icam1* mRNA expression was upregulated to a greater extent than the other adhesion molecules and since ICAM1 has been implicated as an important mediator of neuroinflammation, we examined the cellular source of ICAM1 expression by immunohistochemistry. ICAM1 was readily detected in all tissue sections and was expressed at

similar levels in the meninges (Fig. 4.8 D-F), however, there was a substantial difference in the intensity level between mock (PBS) and agonist inoculated mice in the ventricles and blood vessels (yellow arrows) surrounding the ventricles (Fig. 4.8 A-C). No substantial difference was observed in ICAM1 localization between imiquimod and CpG-ODN inoculated mice.



**Fig. 4.8. ICAM1 Expression Following Agonist Inoculation in the Brain.** Mice were inoculated with (A,D) PBS control, (B,E) 100 nmoles of imiquimod or (C,F) 80 pmoles of CpG-ODN. Brain tissues were removed at twelve hpi, cut in four coronal sections and processed for histology. Tissue sections were stained for ICAM1 using a rat anti-mouse ICAM1 antibody followed by an AlexaFluor 488-conjugated anti-rat secondary antibody. Image magnifications are (A-C) 200X (D-F) 400X. Yellow arrows indicate ICAM1 positive blood vessels.

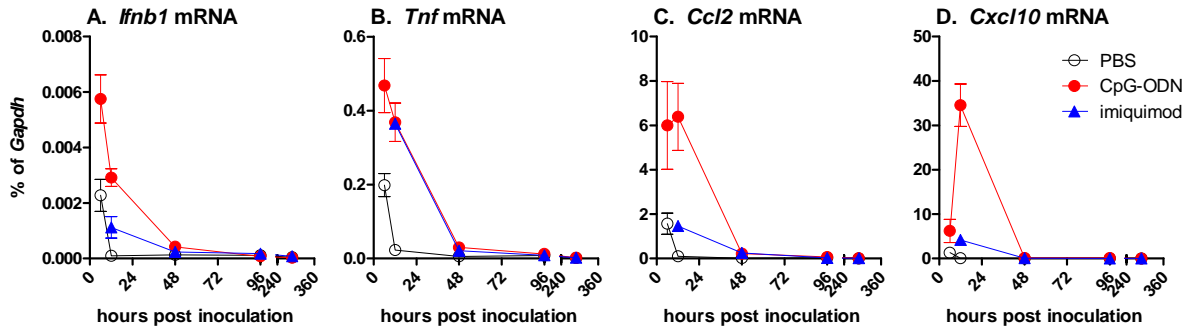
#### 4.2.6. Comparison of Cytokine Responses.

One explanation for the differences in adhesion molecule expression and cell recruitment would be the cytokine and chemokine response to the TLR agonist stimulation in the CNS.

Comparison of mRNA expression of cytokines and chemokines normally associated with

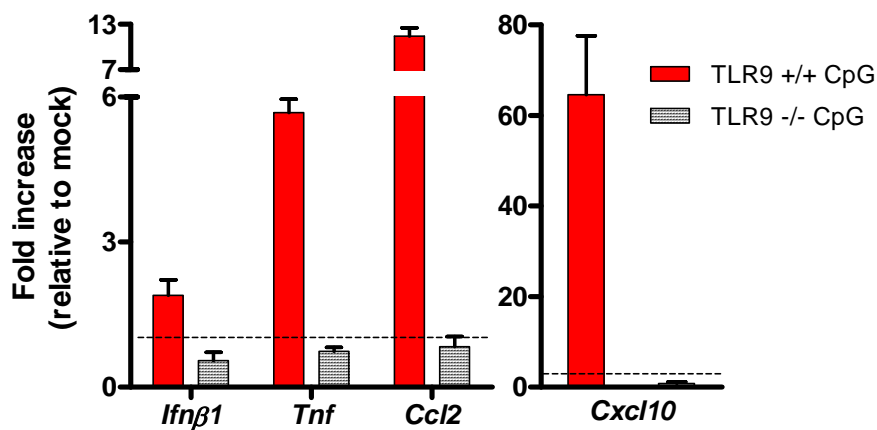


inflammation in the CNS demonstrated that CpG-ODN inoculation induced substantially higher levels of cytokines and chemokines than imiquimod inoculation, with the exception of *Tnf* mRNA (Fig. 4.9). Similar to the expression of adhesion molecules, the expression levels of most cytokine and chemokine mRNA returned to near basal levels by 48 hps.



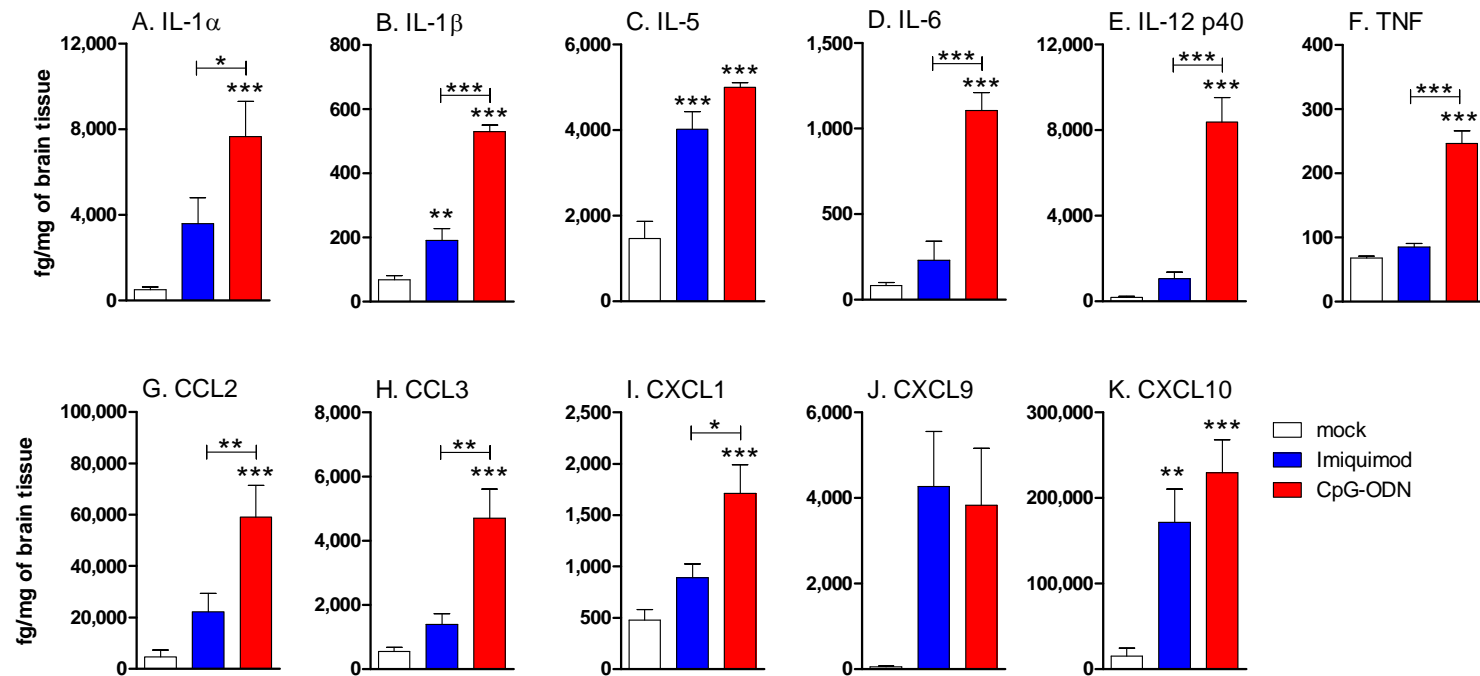
**Fig. 4.9. Cytokine Responses Following Imiquimod or CpG-ODN Inoculation In the Brain.** Mice at 48 h of age were inoculated as described in Table 4.1. Brain tissues at 6, 12, 48, 96 or 312 h post inoculation were processed for real-time RT-PCR analysis using primers specific for (A) *Ifnb1* (B) *Tnf* (C) *Ccl2* and (D) *Cxcl10* mRNA. Data were analyzed as described in Fig. 4.6. Values were calculated relative to the expression of *Gapdh* controls. Data represent the mean  $\pm$  standard error of 3-7 mice per group per time point and represent the combined data from 2 independent experiments. Statistical analysis was completed by two-way ANOVA using Bonferonni post-test., \*P <0.05, \*\*P <0.01. \*\*\*P <0.001.

To confirm that the induction of these responses to CpG-ODN was mediated by TLR9, we analyzed the response of CpG-ODN stimulation in TLR9 deficient mice. A similar analysis with imiquimod had been previously completed demonstrating that imiquimod induced responses in the CNS were dependent on TLR7 (Butchi et al., 2008). For this experiment, we utilized C57BL/6 wild-type mice to aid as a direct comparison to C57BL/6 TLR9<sup>-/-</sup> mice. i.c.v. inoculation of CpG-ODN in TLR9 deficient mice did not induce cytokine or chemokine expression, while inoculation of C57BL/6 wild-type mice did induce strong upregulation of cytokines and chemokines (Fig. 4.10). Thus, CpG-ODN induced inflammatory responses are mediated via TLR9.



**Fig. 4.10. CpG-ODN-Induced Responses are Dependent on TLR9.** Wild-type C57BL/6 and TLR9 deficient C57BL/6 mice were inoculated as described in Table 4.1. Brain tissues were removed at 12 h post inoculation, processed and analyzed for mRNA expression. Data represent the mean +/- standard error for 4 to 5 mice per group and are representative of two replicate experiments.

To determine if there was a difference in the cytokine or chemokine profile induced by imiquimod or CpG-ODN inoculation, we analyzed protein levels of 20 different cytokines in brain tissue from inoculated mice. A time point of 12 hours was chosen since this is the peak of cytokine and chemokine mRNA expression (Fig. 4.9) and is also the peak of cytokine and chemokine protein expression for TLR7 inoculated mice (chapter 3, Fig. 3.3) (Butchi et al., 2008). Imiquimod and CpG-ODN inoculation induced expression of similar proinflammatory cytokines and chemokines including IL-1 $\alpha$ , IL-1 $\beta$ , IL-5, IL-12, CCL2, CCL3, CXCL1, CXCL9 and CXCL10 (Fig. 4.11). Despite the relative similar level of *Tnf* mRNA expression between imiquimod and CpG-ODN inoculated mice, protein levels of TNF were substantially higher in CpG-ODN inoculated mice compared to imiquimod inoculated mice (Fig. 4.11 F). Similarly, most of the cytokines and chemokines analyzed were substantially higher in the CpG-ODN inoculated mice compared to imiquimod inoculated mice. The enhanced cytokine response by CpG-ODN stimulation versus imiquimod stimulation may be responsible for both the increased



**Fig. 4.11. Comparison of Proinflammatory Cytokine Response Following Imiquimod or CpG-ODN Inoculation in the Brain.** Mice were inoculated as described in Table 4.1 and tissues were removed at 12 hpi. One half of the sagittally divided brain was homogenized in lysis buffer containing protease inhibitors and analyzed for protein expression using a multiplex bead array on a Bioplex Luminex system. Samples were calculated as pg/ml using a standard curve from in-plate standards and subsequently converted to fg/mg of brain tissue. Data represent the mean  $\pm$  standard error of 4-8 mice per group. Statistical analysis was completed by one way ANOVA with Newman-Keuls post-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.

cellular infiltration associated with CpG-ODN inoculation as well as the high level of fatality following CpG-ODN inoculation of newborn mice.

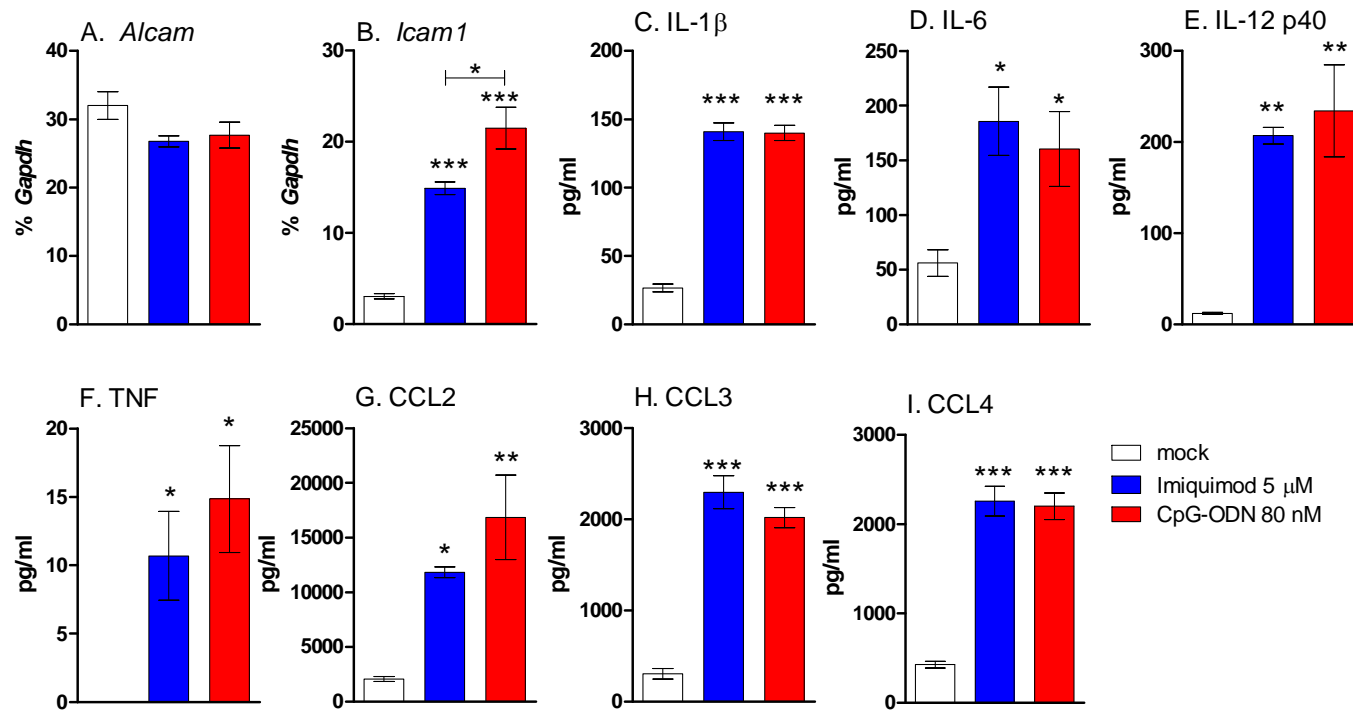
#### **4.2.7. Similar Responses to TLR7 and TLR9 Agonists by Meningeal Cells.**

One possible explanation for the differences in cytokine responses and meningeal inflammation following imiquimod and CpG-ODN stimulation would be a difference in the response of the cells in the meninges to TLR7 and/or TLR9 agonist stimulation. We therefore generated cultures of mixed meningeal cells using the meninges of two day old mice. Interestingly, no differences were observed in the level of the response of meningeal cells to CpG-ODN versus imiquimod stimulation (Fig. 4.12). Thus, *in vitro*, meningeal cells respond similarly to both TLR7 and TLR9 agonists. This is analogous to the similarity in cytokine responses by microglia and astrocytes stimulated with TLR7 or TLR9 agonists and suggest that these cells are not responsible for the heightened response to CpG-ODN stimulation in the CNS.

#### **4.2.8. Source of Cytokine Expression Following *in vivo* Inoculation of TLR Agonists.**

The difference in cytokine production may be due to the type of cell being activated by the agonist or the level of the response of a particular cell type to the agonist. To examine which cells were responsible for cytokine production, we utilized *in situ* hybridization analysis of tissue sections. Since TNF was one of the cytokines that was upregulated by both imiquimod and CpG-ODN at mRNA level, but differentially induced at protein level, we analyzed if *Tnf* mRNA is expressed by different cell types. Interestingly, the primary cell types expressing *Tnf* mRNA following TLR7 or TLR9 agonist stimulation were ependymal cells in the lateral ventricles, as well as in the 3<sup>rd</sup> ventricle in CpG-ODN inoculated mice (Fig. 4.13).

As a comparison for the *Tnf* expression, we also analyzed expression of *Ccl12* mRNA, a chemokine that is produced by infiltrating macrophages during retrovirus infection



**Fig. 4.12. Response of Meningeal Cells to Imiquimod or CpG-ODN Stimulation.** Meningeal cells were generated from the meningeal tissue of mice at 24-48 hours of age. Cells were cultured as described in the methods and stimulated with 80 nM of CpG-ODN or 5 μM of imiquimod. After 12 h, RNA was isolated from the cells and supernatant collected for cytokine analysis. (A,B) Real-time quantitative RT-PCR analysis for (A) *Alcam* and (B) *Icam1*. Values were calculated relative to expression of *Gapdh* controls. Data are presented as gene expression as a percentage of *Gapdh* expression. (C-K) Supernatants were analyzed for cytokine production by multiplex bead array. (A-K) Data represent the mean +/- standard error of 4 separate samples per group and are representative of two independent experiments. Statistical analysis was completed by one-way ANOVA using Bonferroni post-test., \*P<0.05, \*\*P<0.01. \*\*\*P<0.001. Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.

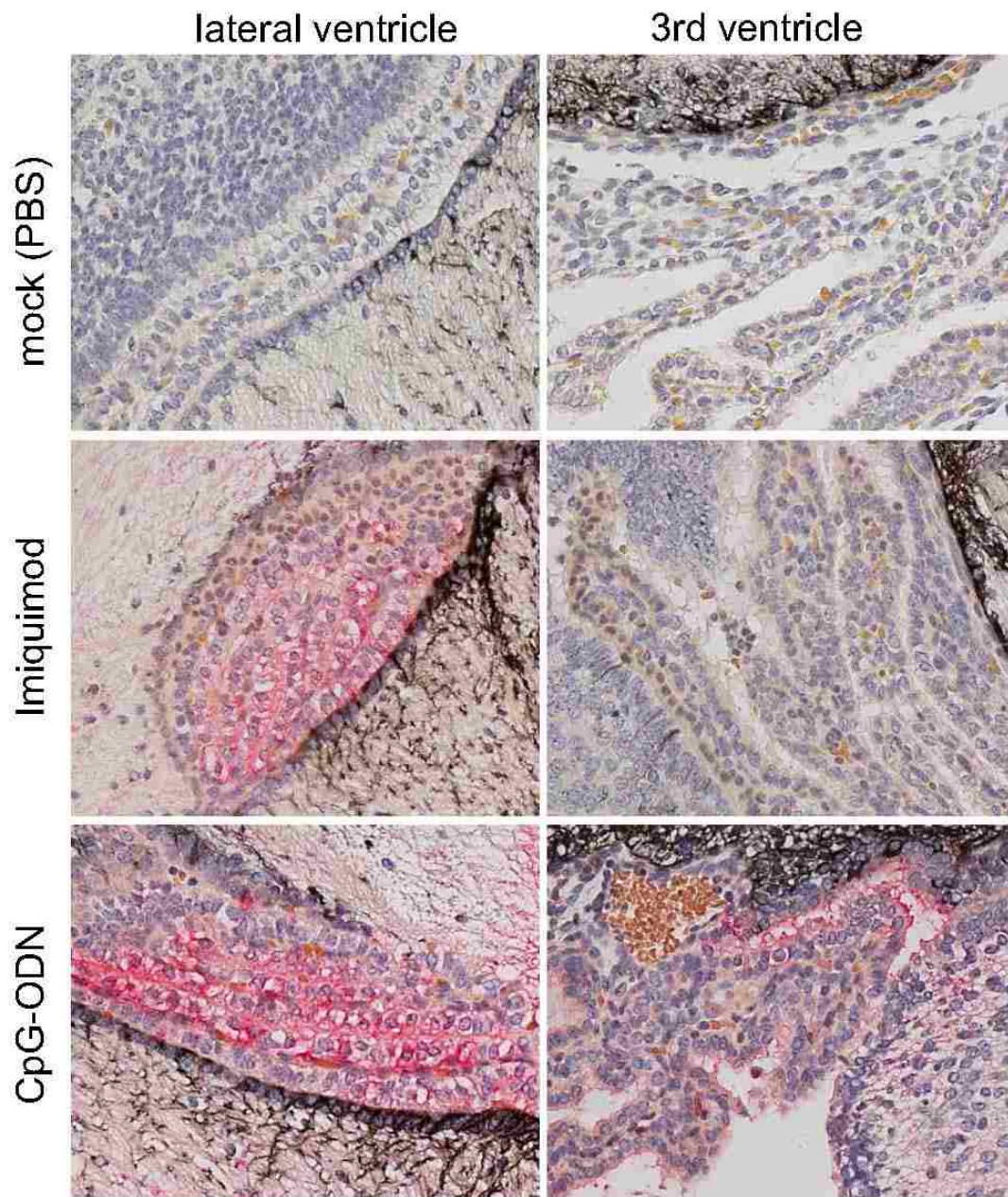
(Peterson et al., 2004). *Ccl12* mRNA-expressing cells were primarily detected in areas around the meninges and ventricles and were positive for IBA1, suggesting that these cells were infiltrating macrophages or activated microglia (Fig. 4.14). These cells were detected at high numbers in imiquimod and CpG-ODN inoculated mice, but were also detected in low numbers in the mock-inoculated group. Thus, the influx of *Ccl12* expressing IBA1 positive cells may be a generalized response to injury in the CNS and not be dependent recruitment for cytokines or chemokines induced by TLR stimulation.

#### **4.2.9 Comparison of Apoptosis Following TLR7 and TLR9 Agonist Stimulation.**

Since TNF expression is often associated with apoptosis, we examined brain tissue from mock, imiquimod and CpG-ODN inoculated mice for active-caspase 3 expression, which is a marker of cells undergoing apoptosis. Random apoptotic cells were found in all three inoculation groups, which correlate with the active death process in the developing brain. However, in the thalamus region directly below the 3<sup>rd</sup> ventricle, there was an increase in the number of apoptotic cells compared to either mock or imiquimod inoculated mice (Fig. 4.15). A slight increase in apoptotic cells in CpG-ODN inoculated mice was observed around the ventricles and in the choroid plexus (data not shown).

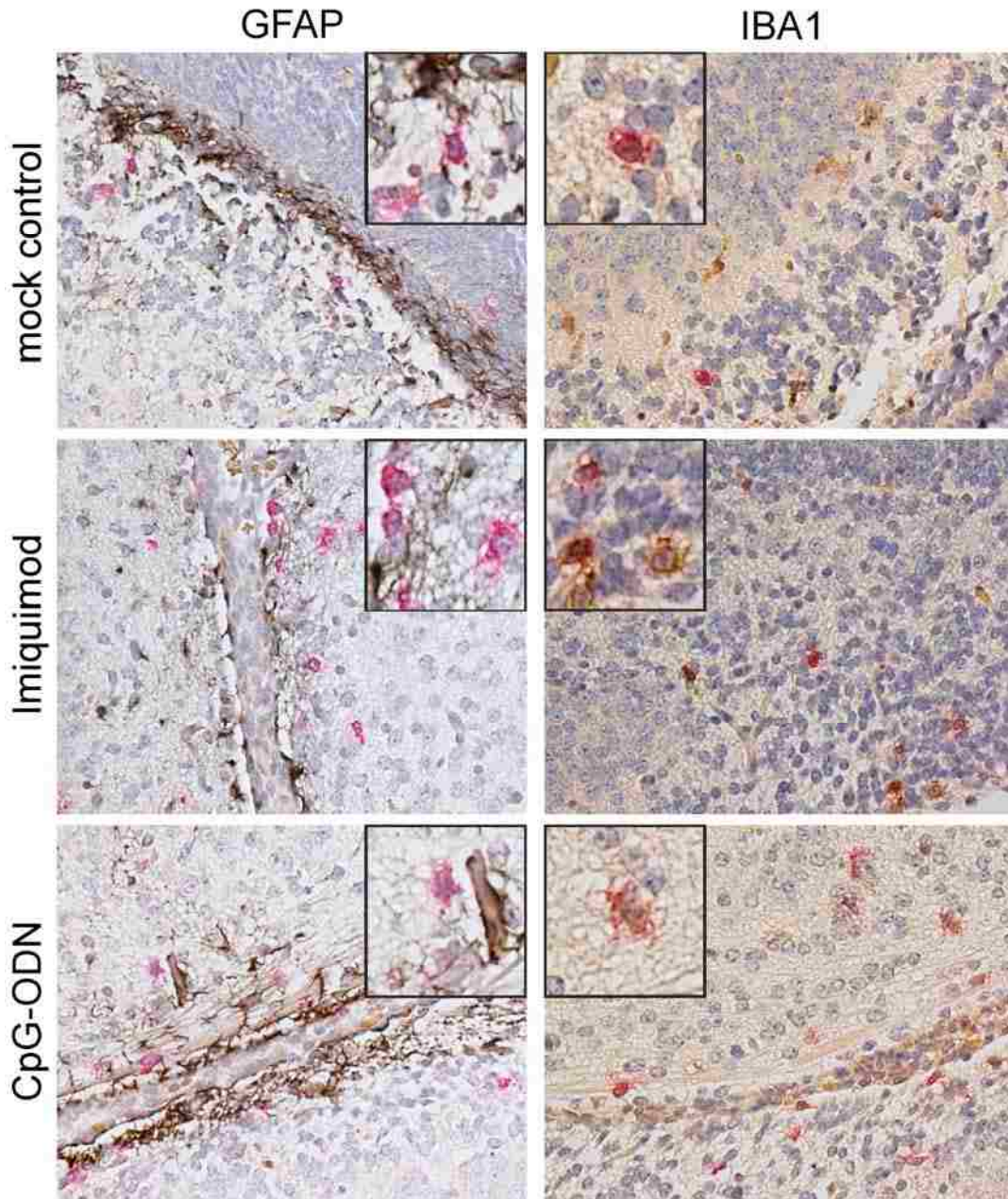
Dual staining with beta tubulin or NeuN was inconclusive as to the identity of these cells due to the intensity of caspase 3 staining and the decrease in marker expression on apoptotic cells. However, some of the apoptotic cells had long dendrites suggesting that some of the active caspase 3 positive cells were neurons (Fig. 4.15E). Interestingly, some active caspase 3 positive cells were detected in lateral and 3<sup>rd</sup> ventricle, where TNF expressing cells were detected. This difference in active caspase 3 positive cells was only detected at 12 hours post inoculation and was not observed at 48 hours (data not shown).





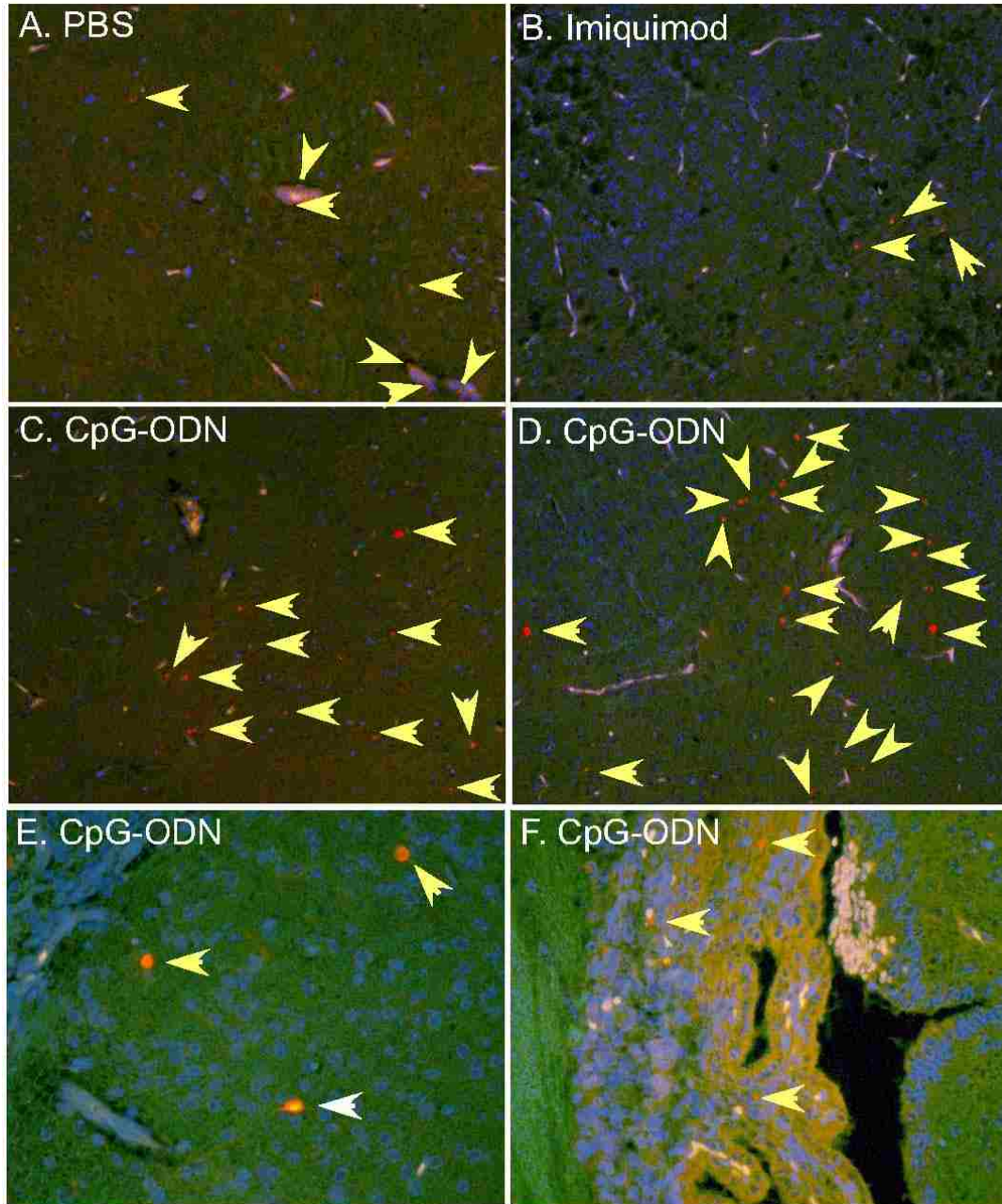
**Fig. 4.13. *In situ* Hybridization-Immunohistochemistry Analysis of Coronal Sections of the Brain Tissues from Mock, Imiquimod, and CpG-ODN Inoculated Mice at 12 hps.** Tissue sections were analyzed for *Tnf* mRNA expression using a DIG-labeled RNA probe and detected with Fast Red substrate (red color). Sections were also analyzed for GFAP expression by immunohistochemistry using a peroxidase antibody and DAB (brown black color). Shown are the lateral ventricle and 3rd ventricles regions, which were the primary regions showing *Tnf*-producing cells. All images are 400X and were taken with a digital camera attached to a Nikon scope. Images are representative of the tissue. Non-specific RNA probes and no-primary antibody controls were used as controls for all experiments.





**Fig. 4.14. Detection of CCL12 Expressing Microglia and/or Macrophages in the Brain Tissues from Mock, Imiquimod, and CpG-ODN Inoculated Mice at 12 hps.** Tissue sections were analyzed for *Ccl12* mRNA expression using a DIG-labeled RNA probe and detected with Fast Red substrate (red color). Sections were also analyzed for GFAP or IBA1 expression by immunohistochemistry using a peroxidase antibody and DAB (brown-black color). Inserts are shown to demonstrate dual staining for *Ccl12* mRNA and either GFAP or IBA1. White arrows indicate cells that are dual positive for *Ccl12* mRNA and IBA1. All sections are from the cortex region. All images are 200 X and were taken with a digital camera attached to a Nikon scope. Images are representative of the tissue. Non-specific RNA probes and no-primary antibody controls were used as controls for all experiments.





**Fig. 4.15. Detection of Apoptotic Cells Following Agonist Inoculation in the Brain.** Mice were inoculated with (A) PBS control, (B) 100 nmoles of imiquimod or (C-F) 80 pmoles of CpG-ODN. Brain tissues were removed at twelve hpi, cut in four coronal sections and processed for histology. Tissue sections were analyzed for active caspase 3 expressing apoptotic cells by staining with rabbit anti-active caspase 3, and either mouse monoclonal  $\beta$  tubulin or Neu N antibody for staining neurons. Following primary antibody, these sections were stained with either goat anti-rabbit AlexaFluor 555 or goat anti-mouse AlexaFluor 488 secondary antibodies. Image magnifications are (A-D) 200X, (E-F) 400X. Yellow arrows indicate active caspase 3 positive cells and white arrow indicate an active caspase 3 positive neuron.

### 4.3. DISCUSSION

The current study demonstrated that the TLR9 agonist, CpG-ODN, induced a more pronounced inflammatory response in the central nervous system compared to the TLR7 agonist, imiquimod. CpG-ODN inoculation induced more severe meningeal inflammation in the CNS (Fig. 4.2, Table. 4.1) as well as increased cellular infiltration (Fig. 4.6) and heightened production of proinflammatory cytokines and chemokines (Fig 4.9 and 4.11). This more pronounced inflammatory response induced by CpG-ODN inoculation may explain why CpG-ODN inoculation by the intracerebroventricular route is toxic to mice at nanomolar concentrations, while imiquimod is not lethal even at micromolar concentrations (Fig. 4.1). Understanding the differences in their neuroinflammatory capabilities will be important when utilizing the agonists for treatment of neurological diseases including neurovirulent virus infections.

The severe symptoms and/or death induced by i.c.v. inoculation of 1-20  $\mu$ g (155-3100 pmoles) of CpG-ODN (Fig. 4.1) may be due in part to the high levels of TNF, IL-6 and IL-1 $\beta$  that are induced following CpG-ODN inoculation (Fig. 4.11). High expression levels of all three proteins are associated with meningococcal meningitis as well as septic shock and TNF has been suggested to play a role in endothelial damage, vasodilatation and capillary leakage. Similar events may occur following the upregulation of these cytokines following CpG-ODN inoculation and lead to the death of the neonates observed in this study and in others (Pedras-Vasconcelos et al., 2006).

The difference in meningeal inflammation associated with CpG-ODN and imiquimod inoculation (Fig. 4.2, Table 4.1) suggested that these cells may be the cell type responsible for the difference in response between CpG-ODN and imiquimod. Analysis of meningeal cell

cultures stimulated with these agonists did not demonstrate any substantial differences in cytokine responses between CpG-ODN and imiquimod stimulated cells (Fig. 4.8). This suggests that the primary differences in cytokine responses are most likely mediated by other cell types. One difference that was significant between imiquimod and CpG-ODN stimulation of meningeal cells was *Icam1* mRNA expression (Fig. 4.12). *Icam1* mRNA was upregulated to a higher level by CpG-ODN stimulation compared to imiquimod stimulation, both in the meningeal cell cultures (Fig. 4.12) and in the whole brain tissue (Fig. 4.6). *Icam1* is an adhesion molecule, expressed by meningeal and endothelial cells in the brain, that is often upregulated in the brain following infection and is involved in the recruitment of inflammatory cells to the CNS (Drescher et al., 2002; Lopez et al., 1999; Wells et al., 2001). The more pronounced meningeal inflammation in CpG-ODN inoculated mice may be due, in part, to increased expression of *Icam1* by CpG-ODN stimulated meningeal cells and the subsequent recruitment of inflammatory cells to the brain.

mRNA levels for *Tnf* were comparable between imiquimod and CpG-ODN-treated animals (Fig. 4.9), but protein levels were not (Fig. 4.11). TNF is regulated at the translation level by AU-binding proteins, which bind *Tnf* mRNA and prevent translation until they are phosphorylated by the mitogen activated protein kinase (MAPK) pathway (Kumar et al., 2003). *Tnf* mRNA was primarily produced by ependymal cells in the brain tissue from both imiquimod and CpG-ODN inoculated mice, indicating that source of mRNA was the same in both groups (Fig. 4.13). However, since TNF protein was expressed at substantially higher levels in the CpG-ODN-inoculated mice, it is possible that CpG-ODN induced a substantially higher level of MAPK activation in the ependymal cells that allowed translation of the TNF protein. It is also possible that the ependymal cells in the third ventricle, which were positive for *Tnf* mRNA in

CpG-ODN inoculated mice, are responsible for the high levels of TNF protein found in the brain tissue of CpG-ODN inoculated mice.

Some of the differences in the cytokine production between imiquimod and CpG-ODN inoculated mice may also be explained by the higher level of infiltrating cells and meningitis in CpG-ODN inoculated mice. Analysis of *Ccl12* expressing cells demonstrated cytokine-producing infiltrating macrophages in both imiquimod and CpG-ODN inoculated mice, without a clear substantial difference between the two groups (Fig. 4.14). However, some of the other cytokines including CCL3, IL-6 and IL-12 p40 may be produced by inflammatory cells in the meningeal regions of the brain. Further *in situ* hybridization and immunohistochemistry analysis should provide a more substantial analysis of which cells are responsible for the individual cytokines or chemokines. However, the sensitivity of the *in situ* hybridization assay will limit the detection of many of these factors.

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## **CHAPTER 5**

### **EXAMINING THE RESPONSES OF GLIAL CELLS FOLLOWING TLR7 AND TLR9 STIMULATION\***

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## 5.1. INTRODUCTION

Astrocytes and microglia are two important cell types in the central nervous system (CNS) that have vital functions in regulating brain development and/or homeostasis. Astrocytes modulate the glutamate levels in the extracellular space contributing to functional neuronal synapses and preventing glutamate induced neurotoxicity (Parpura et al., 2004). Astrocytic end feet are located in close apposition to the capillary endothelium and maintain the structural and functional integrity of the blood-brain barrier (Wolburg and Lippoldt, 2002). Microglia play an important role in remodeling of the fetal brain by scavenging the dead cells in the neocortex during development (Voyvodic, 1996).

In addition to the roles in brain development and homeostasis, both astrocytes and microglia have important roles in innate immunity in the brain. The presence of blood-brain barrier and absence of lymphatic system in the brain restricts entry of immune cells from peripheral circulation (Barker and Billingham, 1977; Carson et al., 2006). In addition, resident dendritic cells are absent in the brain. Thus, astrocytes and microglia are the primary cell types in the CNS that can respond to pathogens invading the CNS. Activated astrocytes and microglia are associated with multiple neurological diseases including viral infections, bacterial infections and diseases of unknown etiologies (Kelder et al., 1998; Nau and Bruck, 2002; Rock et al., 2004; Vargas et al., 2005; Wilson and Hunter, 2004). Following activation, these cells undergo proliferation, morphological changes and release a wide array of proinflammatory cytokines and chemokines that act on and engender responses in target cells. Since microglia are bone-marrow derived, while astrocytes differentiate from neuroprogenitor cells, the response of these two cell types to similar stimulation may vary significantly. The types of cytokines and/or chemokines produced by these cell types in the CNS can affect the response of other cell types in the CNS, as well as influence neuronal damage or the recruitment of inflammatory cells in the CNS.



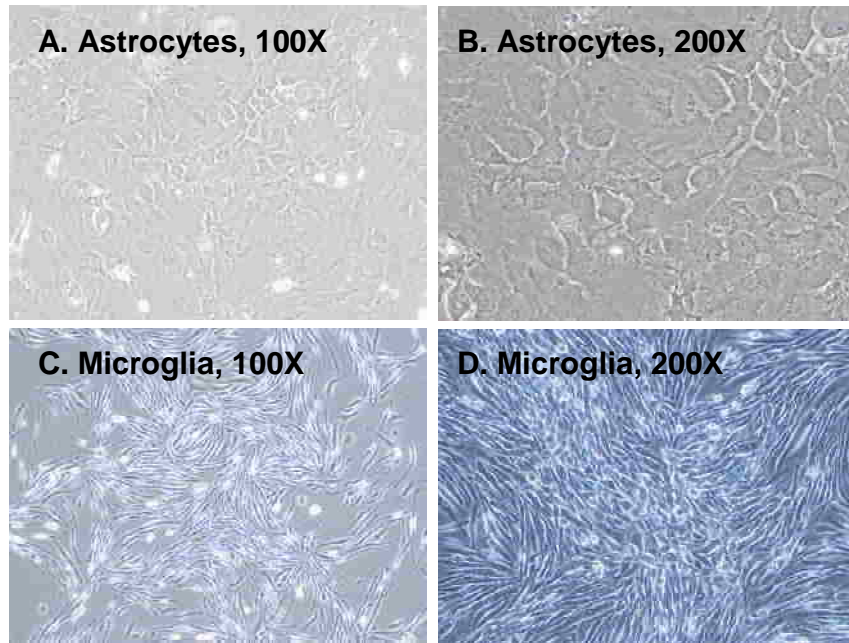
Recent studies demonstrated that toll-like receptor (TLR) 7, which recognizes viral single-stranded RNA, and TLR9, which recognizes unmethylated DNA with CpG motifs generated by certain viruses and bacteria, can play an important role in both the activation of innate immune responses and in viral pathogenesis (Lewis et al., 2008; Sorensen et al., 2008; Town et al., 2009). In addition, agonists of both TLR7 and TLR9 are also being investigated for the potential use in treating CNS-related diseases (Butchi et al., 2008; El Andaloussi A. et al., 2006; Pedras-Vasconcelos et al., 2006; Pedras-Vasconcelos et al., 2008; Prins et al., 2006). Both murine astrocytes and microglia express *Tlr7* and *Tlr9* mRNA (Carpentier et al., 2005; McKimmie and Fazakerley, 2005). Furthermore, both astrocytes and microglia can respond to TLR7 or TLR9 agonist stimulation *in vitro* (Bowman et al., 2003; Dalpke et al., 2002; Gurley et al., 2008; Hosoi et al., 2004; Iliev et al., 2004; Zhang et al., 2005). However, an in depth comparison of the innate immune responses elicited by these cell types in response to TLR7 or TLR9 stimulation has not been completed.

In the present study, we analyzed the response of astrocytes and microglia following TLR7 and/or TLR9 agonist stimulation. Primary astrocytes and microglia from neonatal mice were cultured *in vitro* and stimulated with TLR7 agonist, imiquimod and/or TLR9 agonist, CpG-ODN 1826. The expression of genes involved in TLR7/TLR9 signaling and production of proinflammatory cytokines and chemokines by astrocytes and microglia were compared. The effect of TLR7/TLR9 agonists and supernatants from TLR7/TLR9 stimulated astrocytes and microglia on neuronal survival was also analyzed.

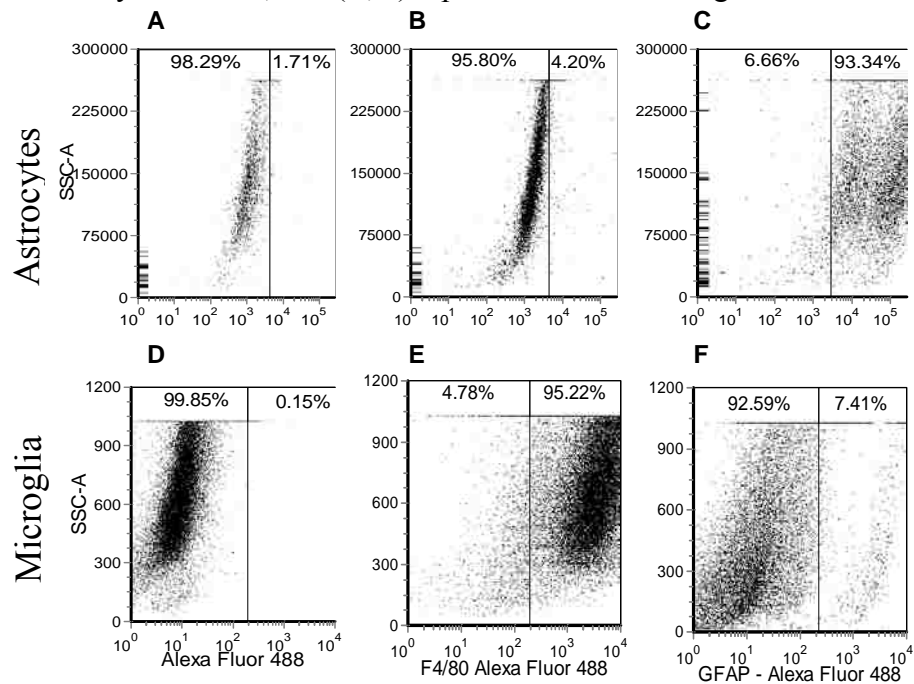
## **5.2. RESULTS**

### **5.2.1. TLR7 and TLR9 Expression in Primary Astrocytes and Microglia**

Primary astrocyte and microglia cultures were generated from brain tissue of 1-2 day old mice. Microglia were separated from astrocytes by percoll gradient centrifugation as described in

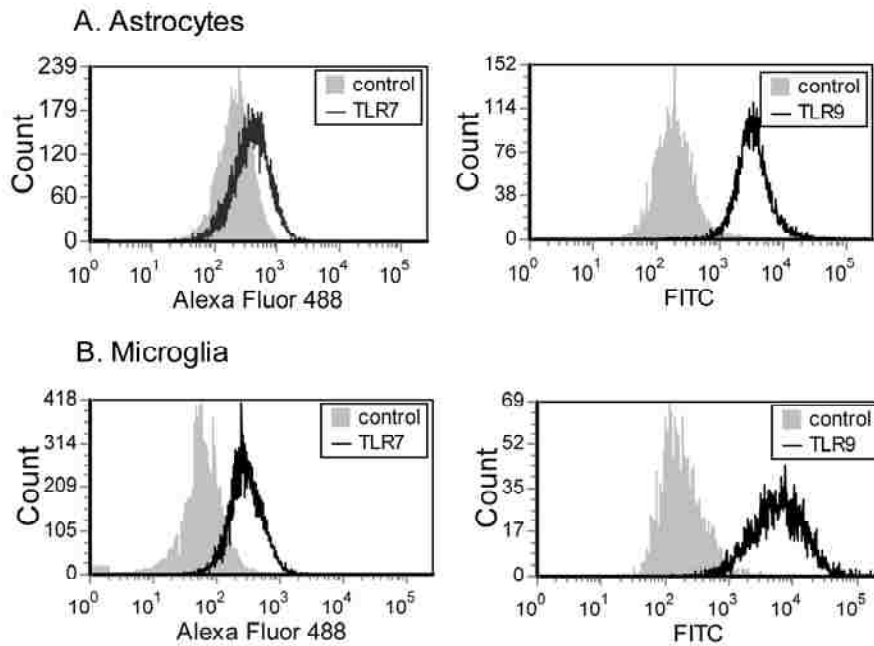


**Fig. 5.1. Primary Astrocytes and Microglia in Culture.** Astrocytes and microglia from brain cortex of 1-2 day old mice were cultured as described in materials and methods. (A,B) representative of astrocyte cultures, and (C,D) representative of microglia cultures.



**Fig. 5.2. Purity of Astrocytes and Microglia Cultures.** (A-C) Astrocyte and (D-F) microglia cultures were analyzed for expression of the (B, E) microglia/macrophage marker F4/80 or (C, F) astrocyte marker GFAP by intracellular flow cytometry analysis. The percentages of cells stained with the cell specific markers were shown at the top of each panel. Data were collected on a FACSaria and analyzed with FCS Express V3 software (De Novo).

methods. Following initial plating and 7-10 days in culture, astrocytes were further purified by orbital shaking overnight at 250 RPM to remove contaminating oligodendrocytes and remaining microglia. Astrocytes and microglia displayed morphological characteristics associated with both cell types in culture (Fig 5.1).



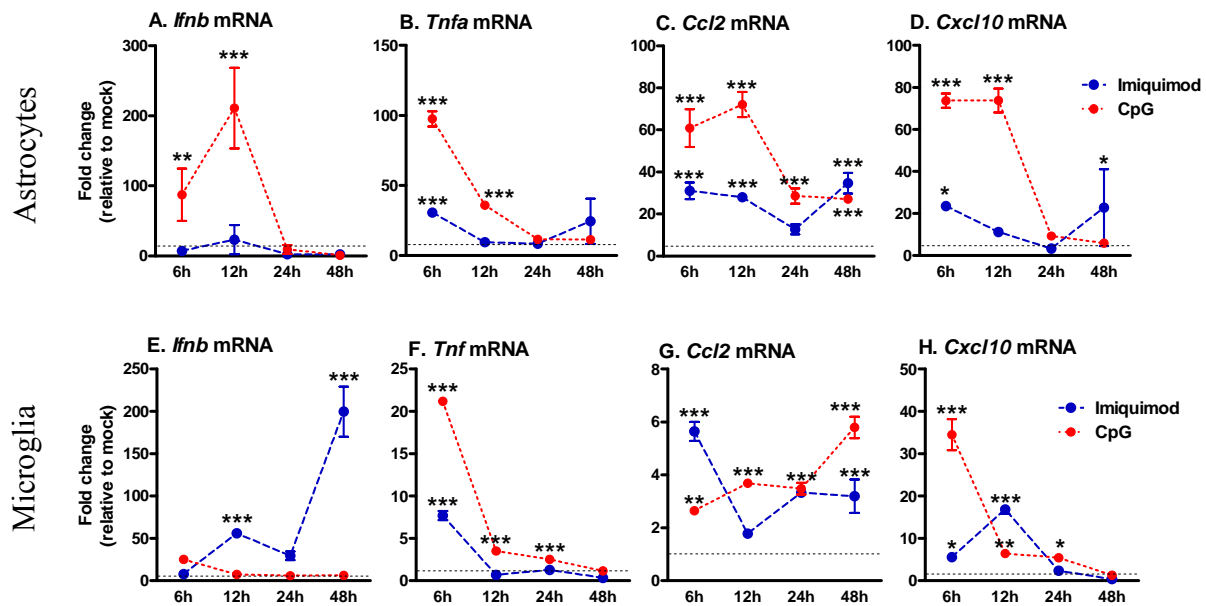
**Fig. 5.3. Expression of TLR7 and TLR9 on Primary Astrocytes and Microglia.** (A) astrocyte and (B) microglia cultures were analyzed for expression of TLR7 and TLR9 by flow cytometry. Data are shown as a histogram with fluorescence intensity on X-axis and counts/cell numbers on Y-axis. For detection of TLR7, cells were incubated with Alexa Fluor 488 conjugated goat anti-rabbit, either with rabbit anti-TLR7 (TLR7) or with no primary antibody (control). The mean fluorescence intensity (MFI) was substantially higher for TLR7-stained astrocytes (470, 349) than for the no primary antibody controls (269, 211) demonstrating that astrocytes expressed TLR7 protein, albeit at low levels. For detection of TLR9, cells were stained with a FITC- conjugated mouse-IgG (control) or with mouse anti-TLR9 and FITC-conjugated anti-mouse IgG (TLR9). Data were collected on a FACSAria and analyzed with FCS Express V3 software (De Novo). Data are representative of two replicate experiments.

Purity of astrocyte and microglia cultures were analyzed by intracellular flow cytometry, which consistently revealed the presence of >93% GFAP positive cells and >95% F4/80 positive cells respectively (Fig. 5.2). Expression of TLR7 and TLR9 was analyzed on these cells, as some studies reported expression of *Tlr7* and *Tlr9* mRNA in astrocytes, while other studies did not

detect these receptors (Carpentier et al., 2005; Jack et al., 2005; McKimmie and Fazakerley, 2005). In the present study, TLR7 and TLR9 protein expression was observed in both cell types with higher expression levels of both proteins on microglia compared to astrocytes (Fig. 5.3).

### 5.2.2. TLR7 and TLR9 Stimulation in Astrocytes and Microglia Induces Most Cytokines and Chemokines in 6-12 Hours Post Stimulation

To examine if TLR7 and TLR9 agonists stimulate astrocytes and/or microglia cells, we stimulated primary cultures of each cell type with either 50  $\mu$ M of the TLR7 agonist, imiquimod or 80 nM of the TLR9 agonist, CpG-ODN 1826. Concentrations used were based on optimal concentrations for the stimulation of the astrocyte cell line, C8D1A. Gene expression of

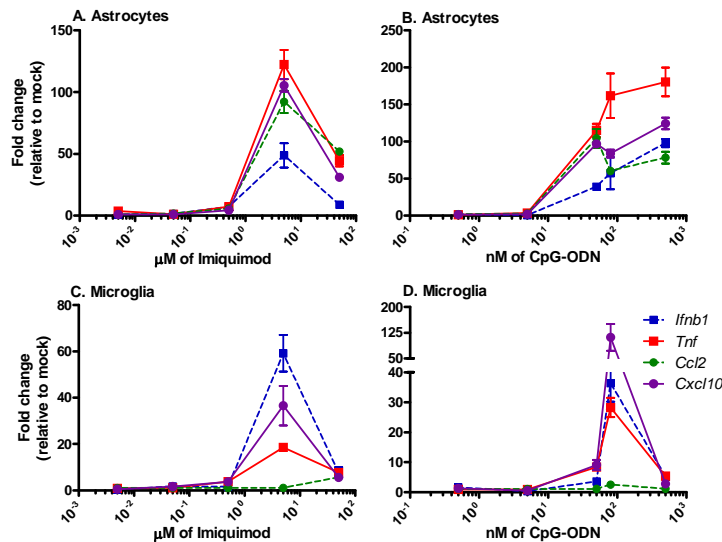


**Fig. 5.4. Kinetic Analysis of Cytokine and Chemokine Gene Expression by Cultured Astrocytes and Microglia Following Stimulation with Imiquimod or CpG-ODN.** Astrocytes and microglia were cultured in 12 well plates as described in materials and methods, and stimulated with mock or 50  $\mu$ M imiquimod or 80 nM CpG-ODN. At 6, 12, 24 and 48 hps, RNA was isolated from the cells and supernatants were frozen in aliquots at  $-80^{\circ}\text{C}$  for future use. RNA samples were processed for real-time quantitative RT-PCR analysis and values were calculated relative to expression of *Gapdh* controls. Data are presented as the fold induction of each gene of interest relative to mock controls. Mock expression levels were calculated as the mean of 3 well per time point. Data represent the standard mean error per group per time point and represent one of the two independent experiments. Statistical analysis was completed by two-way ANOVA using Bonferroni post-test. \*,  $P < 0.05$ , \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$ .

cytokines and chemokines were analyzed by real-time RT PCR at 6, 12, 24 and 48 hps (hours post stimulation). Stimulation of TLR7 or TLR9 in astrocytes induced mRNA for *Tnf*, *Ccl2* and *Cxcl10* that peaked at 6 hps, whereas mRNA expression of *Ifnb1* peaked at 12 hps (Fig. 5.4.A-D). Stimulation of microglia with TLR7 or TLR9 agonists also induced cytokine and chemokines mRNA expression that generally peaked at 6 hps although peak expression of *Cxcl10* and *Ifnb1* was observed at later time points (Fig. 5.4.E-H). Thus, kinetic analysis of gene expression was observed at later time points (Fig. 5.4.E-H). Thus, kinetic analysis of gene expression indicated peak expression of proinflammatory cytokines and chemokines at 6-12 hours post stimulation (hps), with most cytokine mRNA expression diminishing by 48 hps.

### 5.2.3. Dose Response of Astrocytes and Microglia to TLR7 and TLR9 Agonists

The concentration of imiquimod or CpG-ODN 1826 used for stimulation could influence the response of each cell type. We first identified the optimal stimulatory concentration of TLR7 and TLR9 agonists for both microglia and astrocytes using a range of 5 nM to 50  $\mu$ M of



**Fig. 5.5. Cytokine Response to Imiquimod or CpG-ODN Stimulation in Astrocytes and Microglia Is Concentration Dependent.** Cultured Astrocytes and microglia at 80-90% confluency were stimulated using varying concentrations of imiquimod or CpG-ODN. At 6 hps, cells were lysed, RNA was isolated and analyzed by Reverse transcription followed by real-time PCR. Samples were analyzed as described in Fig. 5.4. Data represent the mean  $\pm$  standard error of three samples per group. Data are representative of one of two replicate experiments.

imiquimod or 0.5 nM to 500 nM of CpG-ODN 1826. Optimal cytokine production in both cell types was induced with 5  $\mu$ M (1.25  $\mu$ g/ml) of imiquimod and 80 nM (0.5  $\mu$ g/ml) of CpG-ODN 1826, with induction of high levels of interferon beta 1 (*Ifnb1*), tumor necrosis factor (*Tnf*), monocyte chemo attractant protein 1 (*Ccl2/MCP1*) and interferon- $\gamma$ -inducible protein 10 (*Cxcl10/IP-10*) mRNA expression (Fig. 5.5). Concentrations greater than 500 nM of CpG-ODN 1826 were toxic to both astrocytes and microglia cultures. For all further studies, 5  $\mu$ M imiquimod and/or 80 nM CpG-ODN 1826 were used (except where noted) and the gene expression was analyzed at 6 hps, and cytokine production was analyzed at 12 hps.

#### **5.2.4. mRNA Expression of Signal Transduction Genes Following TLR7/9 Stimulation.**

The signaling cascade induced by CpG-ODN stimulation differs between macrophages and dendritic cells and results in different cytokine production by each cell type (Schroder et al., 2007). This may also be true for astrocytes and microglia, as these cells have distinct origins and functions in the CNS. We analyzed the influence of TLR7 and TLR9 stimulation on mRNA expression of 84 genes associated with toll-like receptor pathway, using a TLR pathway focused cDNA real-time PCR array. Although the level of gene upregulation did vary between imiquimod and CpG-ODN stimulated samples, the same genes were upregulated by both stimuli (Table 5.1). There was some variation in mRNA upregulation between astrocytes and microglia following TLR activation, with astrocytes increasing mRNA expression of more signal transduction related genes than microglia (Table 5.1, Fig. 5.6).

Both cell types upregulated expression of a number of proinflammatory cytokine and chemokine genes as well as a few TLRs. Surprisingly, *Tlr9* mRNA expression was not upregulated by either imiquimod or CpG stimulation, while *Tlr7* mRNA expression was only upregulated in microglia. Instead, mRNAs for other TLRs, including *Tlr1* and *Tlr2* were upregulated by stimulation, while *Tlr4* and *Tlr5* mRNAs were downregulated (Fig. 5.6,

**Table 5.1. TLR7 and TLR9 Stimulation in Astrocytes and Microglia Induces the Expression of Multiple mRNAs – Toll-like Receptors, Adaptors, Effectors and TLR Interacting Proteins**

	Astrocytes						Microglia					
	Imiquimod <sup>a</sup>		CpG <sup>b</sup>		Imiquimod+CpG <sup>c</sup>		Imiquimod		CpG		Imiquimod+CpG	
	Mean <sup>d</sup>	SD <sup>e</sup>	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b><u>Toll Like Receptors</u></b>												
<i>Tlr1</i>	<b>12.6</b>	3.7	<b>12.4</b>	2.3	<b>10.4</b>	5.8	<b>6.6</b>	0.7	<b>4.8</b>	1.1	<b>7.2</b>	1.7
<i>Tlr2</i>	<b>15.2</b>	0.3	<b>15.3</b>	2.3	<b>14.3</b>	1.3	<b>9.5</b>	0.4	<b>10.8</b>	0.4	<b>8.0</b>	0.7
<i>Tlr3</i>	<b>3.2</b>	1.3	<b>2.2</b>	0.6	<b>2.3</b>	1.1	-1.4	0.1	1.7	0.4	-0.9	1.6
<i>Tlr4</i>	0.6	1.5	0.5	1.5	-0.5	1.6	-5.0	1.5	-2.7	1.2	-5.1	2.5
<i>Tlr5</i>	-3.1	1.9	-2.7	1.6	-1.8	0.5	-226.3	157.8	-12.1	8.3	-31.4	14.0
<i>Tlr7</i>	-0.3	1.3	1.4	0.4	0.1	2.2	<b>9.0</b>	<b>1.1</b>	<b>14.1</b>	<b>6.6</b>	<b>10.8</b>	3.1
<b><u>Adaptors, Effectors and TLR Interacting Proteins</u></b>												
<i>Cd14</i>	<b>202.3</b>	9.0	<b>181.8</b>	6.3	<b>166.7</b>	19.7	<b>5.1</b>	0.3	<b>2.8</b>	0.1	<b>4.4</b>	0.4
<i>Hspa1a</i>	-1.2	0.1	-1.2	0.0	-1.4	0.1	<b>2.9</b>	0.4	<b>4.4</b>	0.6	<b>2.6</b>	0.3
<i>Myd88</i>	<b>5.0</b>	0.7	<b>4.5</b>	0.1	<b>4.6</b>	0.1	1.9	0.2	<b>2.2</b>	0.1	<b>2.2</b>	0.3
<i>Ripk2</i>	<b>10.4</b>	1.6	<b>10.2</b>	2.5	<b>8.8</b>	2.5	<b>2.7</b>	0.4	<b>3.1</b>	0.5	<b>2.3</b>	0.5
<i>Ticam2</i>	<b>3.8</b>	0.1	<b>2.9</b>	1.0	<b>3.3</b>	0.4	1.3	0.2	0.5	1.3	1.5	0.3
<i>Irak2</i>	<b>3.2</b>	0.4	<b>3.5</b>	0.1	<b>2.7</b>	0.1	1.3	0.2	1.9	0.1	1.1	0.1

a: imiquimod at 5 µM,

b: CpG0ODN at 80 pM,

c: imiquimod at 5 µM and CpG-ODN at 80 pM,

d: fold change:  $2^{-\Delta Ct}$  of 3 treatment samples /  $2^{-\Delta Ct}$  of 3 mock samples,  $\Delta Ct = Ct \text{ Gene of interest} -$

Average Ct House keeping genes,

e: standard deviation of 3 samples per group

<b>Table 5.2. TLR7 and TLR9 Stimulation in Astrocytes and Microglia Induces the Expression of Multiple mRNAs – Downstream Signaling Molecules</b>												
	<b>Astrocytes</b>						<b>Microglia</b>					
	<b>Imiquimod<sup>a</sup></b>		<b>CpG<sup>b</sup></b>		<b>Imiquimod+CpG<sup>c</sup></b>		<b>Imiquimod</b>		<b>CpG</b>		<b>Imiquimod+CpG</b>	
	<b>Mean<sup>d</sup></b>	<b>SD<sup>e</sup></b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>
<b><u>Downstream Signaling Molecules</u></b>												
<i>Chuk</i>	<b>2.7</b>	0.4	<b>3.0</b>	0.3	<b>2.6</b>	0.8	-0.4	1.3	1.2	0.3	-0.1	1.8
<i>Cebpb</i>	<b>2.9</b>	0.2	<b>2.7</b>	0.4	<b>2.6</b>	0.4	<b>3.9</b>	0.2	<b>2.7</b>	0.2	<b>3.8</b>	0.2
<i>Clec4e</i>	<b>52.8</b>	8.1	<b>36.9</b>	24.1	<b>33.0</b>	11.1	<b>31.7</b>	6.8	<b>23.4</b>	4.8	<b>34.4</b>	8.6
<i>Hrb</i>	<b>12.9</b>	1.4	<b>11.8</b>	2.3	<b>11.4</b>	1.2	-1.2	0.1	-0.4	1.3	-1.1	0.0
<i>Irf1</i>	<b>12.3</b>	1.0	<b>11.5</b>	2.3	<b>12.9</b>	1.1	<b>4.9</b>	0.4	<b>11.9</b>	1.2	<b>4.8</b>	0.4
<i>Map2k3</i>	<b>18.5</b>	0.8	<b>15.8</b>	4.4	<b>16.7</b>	0.6	0.3	1.4	-0.5	1.3	-0.4	1.4
<i>Nfkb1</i>	<b>5.6</b>	0.3	<b>5.8</b>	0.7	<b>5.7</b>	0.3	<b>5.2</b>	0.5	<b>4.2</b>	0.5	<b>4.4</b>	0.8
<i>Nfkb2</i>	<b>6.8</b>	0.8	<b>7.5</b>	1.0	<b>7.6</b>	0.9	<b>16.3</b>	3.4	<b>5.6</b>	0.6	<b>12.8</b>	1.2
<i>Nfkbia</i>	<b>12.5</b>	1.0	<b>13.5</b>	4.0	<b>12.2</b>	4.1	<b>10.1</b>	0.6	<b>7.4</b>	1.1	<b>11.0</b>	2.1
<i>Nfkbib</i>	<b>2.7</b>	0.4	<b>3.1</b>	0.6	<b>2.7</b>	0.6	<b>2.0</b>	0.3	-1.1	0.0	1.5	0.1
<i>Ptgs2</i>	<b>11.3</b>	2.3	<b>10.4</b>	3.6	<b>8.7</b>	2.6	<b>40.5</b>	2.3	<b>15.6</b>	2.0	<b>28.3</b>	3.6
<i>Rel</i>	<b>4.0</b>	0.7	<b>3.7</b>	0.5	<b>3.2</b>	0.9	<b>2.1</b>	0.2	<b>2.2</b>	0.2	1.9	0.2
<i>Rela</i>	<b>2.3</b>	0.3	<b>2.7</b>	0.1	<b>2.3</b>	0.3	1.4	0.1	1.2	0.1	1.3	0.0
<i>Tnfaip3</i>	<b>19.4</b>	1.7	<b>18.1</b>	8.7	<b>14.9</b>	5.2	<b>7.0</b>	1.3	<b>5.9</b>	0.4	<b>7.5</b>	1.3

a: imiquimod at 5  $\mu$ M,

b: CpG0ODN at 80 pM,

c: imiquimod at 5  $\mu$ M and CpG-ODN at 80 pM,

d: fold change:  $2^{-\Delta Ct}$  of 3 treatment samples /  $2^{-\Delta Ct}$  of 3 mock samples,  $\Delta Ct = Ct$  Gene of interest – Average Ct House keeping genes,

e: standard deviation of 3 samples per group



**Table 5.3. TLR7 and TLR9 stimulation in astrocytes and microglia induces the expression of multiple mRNAs – Cytokines, chemokines and co-stimulatory molecules**

	Astrocytes						Microglia					
	Imiquimod <sup>a</sup>		CpG <sup>b</sup>		Imiquimod+CpG <sup>c</sup>		Imiquimod		CpG		Imiquimod+CpG	
	Mean <sup>d</sup>	SD <sup>e</sup>	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b><u>Cytokines and Chemokines</u></b>												
<i>Ccl2</i>	82.5	2.8	81.9	25.2	76.6	28.3	0.7	1.6	2.8	1.2	0.7	1.7
<i>Csf2</i>	178.0	11.0	221.9	48.8	131.7	17.5	8.1	3.1	25.2	8.8	7.7	2.6
<i>Csf3</i>	33.3	3.7	20.9	2.5	24.4	3.5	252.7	52.2	70.9	13.7	175.7	49.1
<i>Cxcl10</i>	159.2	44.3	124.9	17.5	130.8	42.7	69.8	45.6	343.2	216.6	49.3	33.9
<i>Ifnb1</i>	29.8	9.2	13.4	2.1	17.5	4.3	53.2	9.7	35.5	7.0	30.2	12.4
<i>Il1a</i>	580.0	88.9	464.8	168.9	354.9	155.8	267.5	22.0	47.7	8.7	187.8	26.1
<i>Il1b</i>	2,005.8	196.3	1,904.8	602.9	1,503.7	711.1	3,629.1	322.6	712.3	147.2	2,644.7	218.7
<i>Il6</i>	54.2	5.6	66.2	30.0	43.1	17.5	47.9	5.5	84.4	30.0	42.2	3.6
<i>Il10</i>	54.5	4.0	83.2	42.6	119.4	61.2	115.4	34.9	19.5	3.0	59.6	15.3
<i>Il12a</i>	6.1	1.5	3.7	1.7	5.5	0.8	2.8	4.1	3.9	1.8	5.2	2.7
<i>Lta (Tnfb)</i>	2.5	3.9	3.7	0.9	4.5	3.5	10.1	4.2	7.2	1.1	8.4	3.1
<i>Tnf</i>	323.1	8.0	485.0	39.7	390.4	65.6	19.8	4.5	34.0	13.0	21.8	8.3
<b><u>Co-stimulatory Molecules</u></b>												
<i>Cd80</i>	2.4	0.2	2.4	0.2	2.3	0.1	-2.2	0.2	0.4	1.3	-1.7	0.0

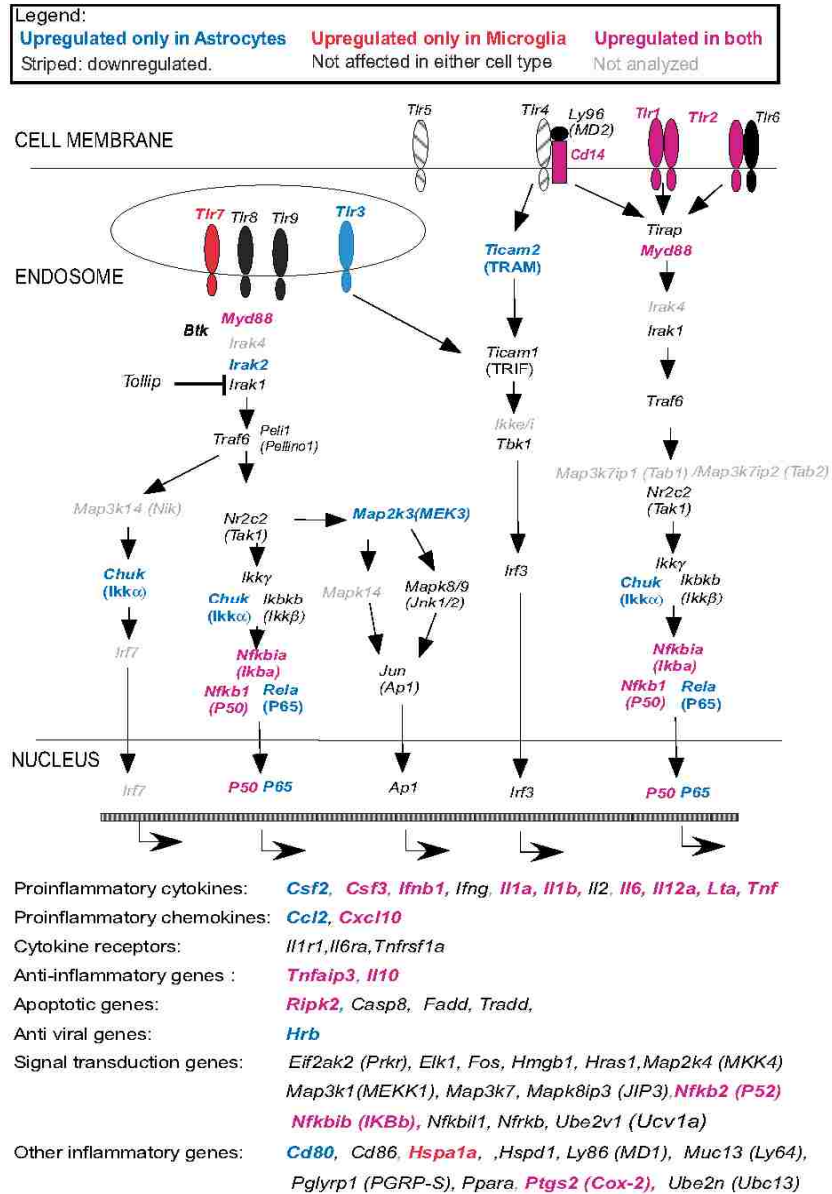
a: imiquimod at 5 µM,

b: CpG0ODN at 80 pM,

c: imiquimod at 5 µM and CpG-ODN at 80 pM,

d: fold change:  $2^{-\Delta Ct}$  of 3 treatment samples /  $2^{-\Delta Ct}$  of 3 mock samples,  $\Delta Ct = Ct \text{ Gene of interest} - \text{Average Ct House keeping genes}$ ,

e: standard deviation of 3 samples per group



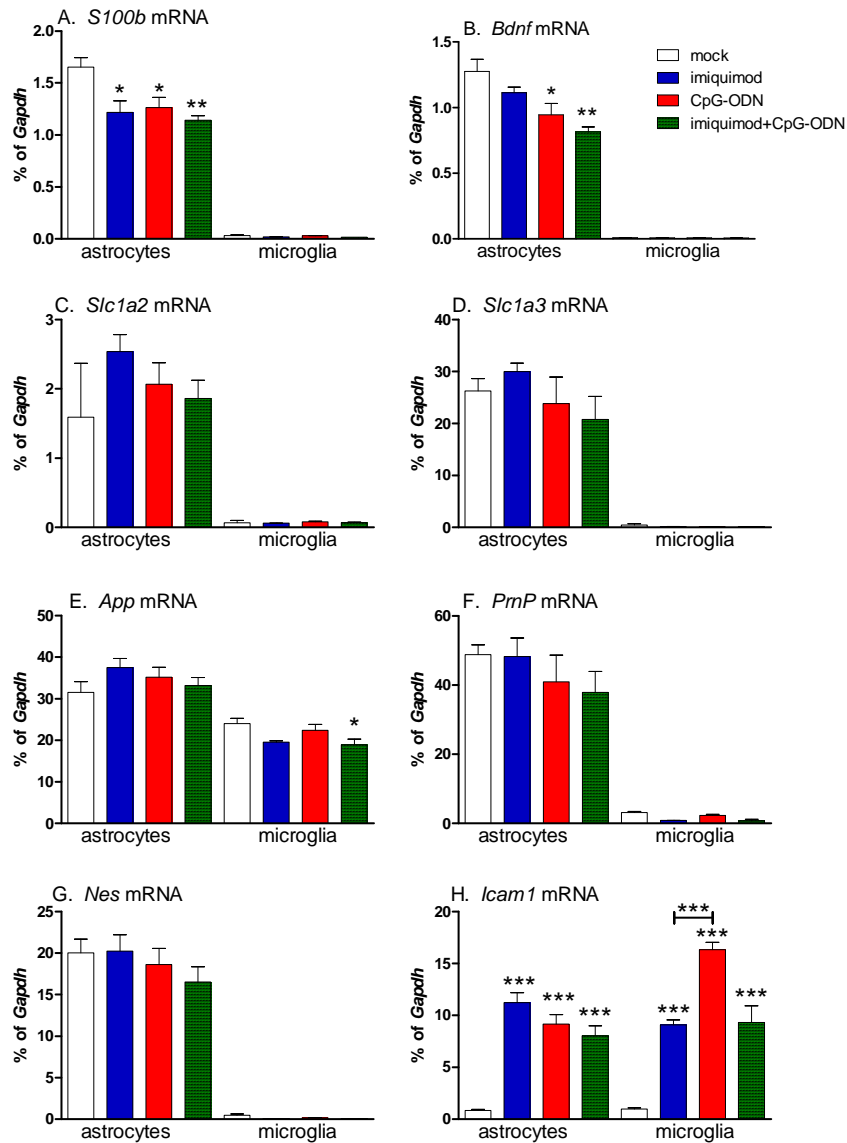
**Fig. 5.6. Change in mRNA Expression Following TLR7 and/or TLR9 Agonist Stimulation in Astrocytes and Microglia.** Diagram of gene expression based on Table 5.1-3. Cultured astrocytes and microglia were stimulated with 5  $\mu$ M imiquimod or 80 nM CpG-ODN 1826 or both. RNA was isolated at 6 hps, reverse transcribed and cDNA analyzed for mRNA expression by quantitative real-time PCR analysis using a TLR-gene related super array. Genes were presented as a schematic of their involvement in the TLR signaling cascade or as genes induced by the signaling cascade. Increased mRNA expression of these genes in both microglia and astrocytes is indicated by bold purple lettering, in astrocytes only by bold blue lettering and in microglia by bold red lettering. *Tlr4* mRNA was downregulated in microglia, while *Tlr5* mRNA was downregulated in both microglia and astrocytes. Genes indicated in black lettering were not altered in either cell type and in grey lettering were not analyzed for mRNA expression.

Table 5.1). Thus, TLR stimulation does not appear to automatically induce self upregulation of mRNA expression, suggesting a more complex regulation of TLR gene expression.

*Cd14* mRNA expression was substantially upregulated on astrocytes, but not microglia, following TLR agonist stimulation. Since microglia respond more strongly to CD14 stimulation than astrocytes, we verified *Cd14* mRNA expression using quantitative real-time RT-PCR with different sets of primers. *Cd14* mRNA was substantially upregulated by TLR stimulation in astrocytes by real-time RT-PCR; however, this increase in *Cd14* mRNA expression by astrocytes was still lower than the basal level of *Cd14* mRNA expression by microglia cells (data not shown).

#### **5.2.5. mRNA Expression of Inflammatory Genes Following TLR7/9 Stimulation.**

Since astrocytes function as support cells for neurons, we examined whether TLR7 agonist or TLR9 agonist stimulation altered the mRNA expression of non-immune genes that can affect neuropathogenesis. A minor difference was noted in the expression of *S100b* and/or brain derived neurotrophic factor (*Bdnf*) mRNA following TLR7 or TLR9 agonist stimulation of astrocytes (Fig. 5.7). However, the expression of other genes including the glutamate scavenging receptors, *Slc1a2* and *Slc1a3*, were not altered by TLR agonist stimulation. Expression of genes whose products are involved in protein-aggregation-related diseases such as the amyloid beta precursor protein (*App*) or prion protein (*Prnp*) were also not altered by TLR agonist stimulation (Fig. 5.7 E, F). Thus TLR7 and TLR9 agonist stimulation did not appear to substantially alter the mRNA expression of neuronal support-related genes in astrocytes. In contrast, mRNA for *Icam1*, an adhesion molecule, was upregulated by both TLR agonists (Fig. 5.7 H).

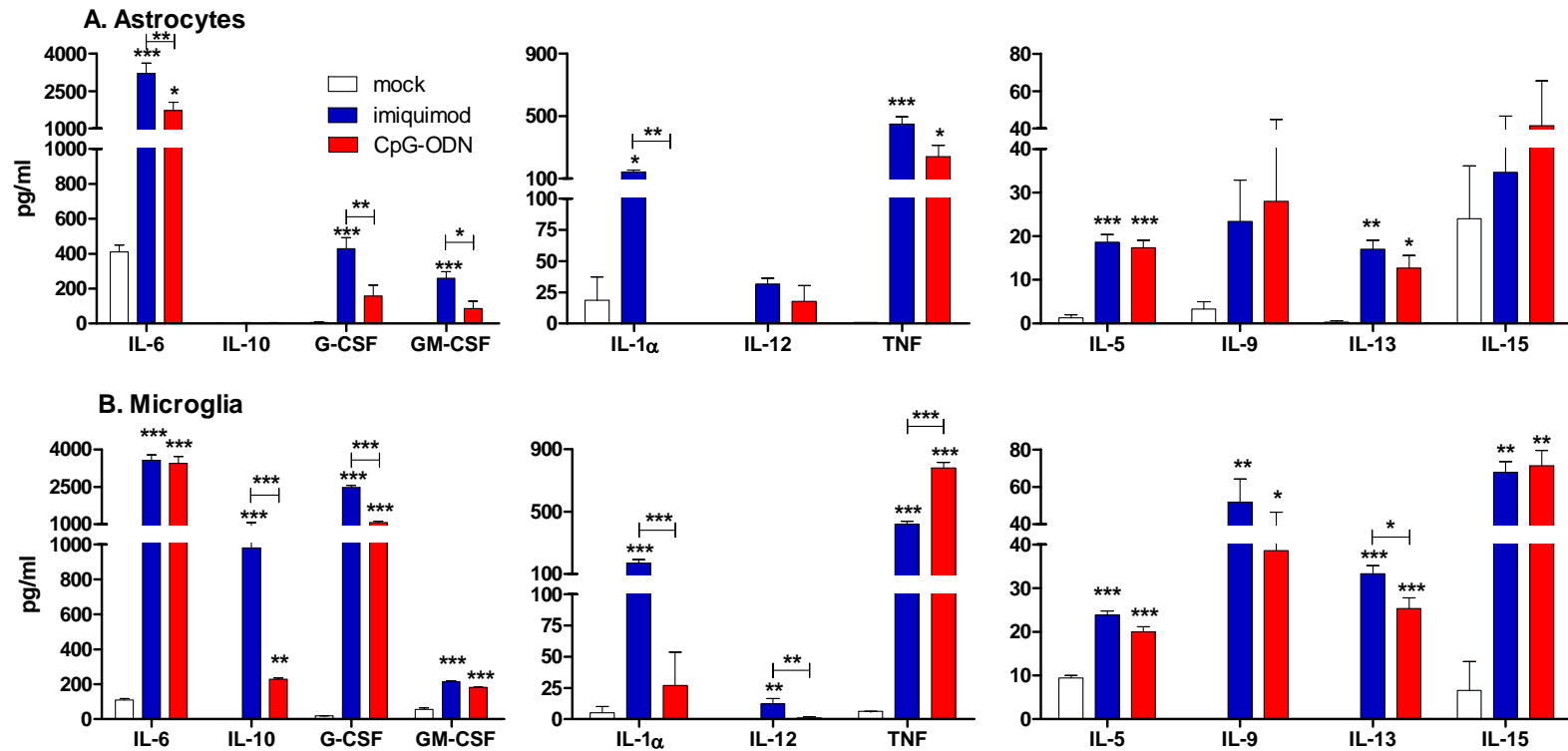


**Fig. 5.7. Influence of Imiquimod, CpG-ODN, or Co-Stimulation in Astrocytes and Microglia on the mRNA Expression of Inflammatory Genes.** (A) *S100b*, (B) Brain-derived neurotrophic factor (*Bdnf*), glutamate transporters (C) *Slc1a2*, (D) *Slc1a3*, (E) Amyloid precursor protein (*App*), (F) Prion protein (*Prnp*), (G) Nestin (*Nes*) and (H) Adhesion molecule (*Icam1*). Cultured astrocytes and microglia were stimulated with 5  $\mu$ M imiquimod, 80 nM CpG-ODN 1826, or both. At 6 hps, RNA was isolated, processed and analyzed by realtime RT-PCR. Values were calculated as gene expression as a percentage of *Gapdh* mRNA expression per sample. Data represent the mean  $\pm$  SEM of three samples per group. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.

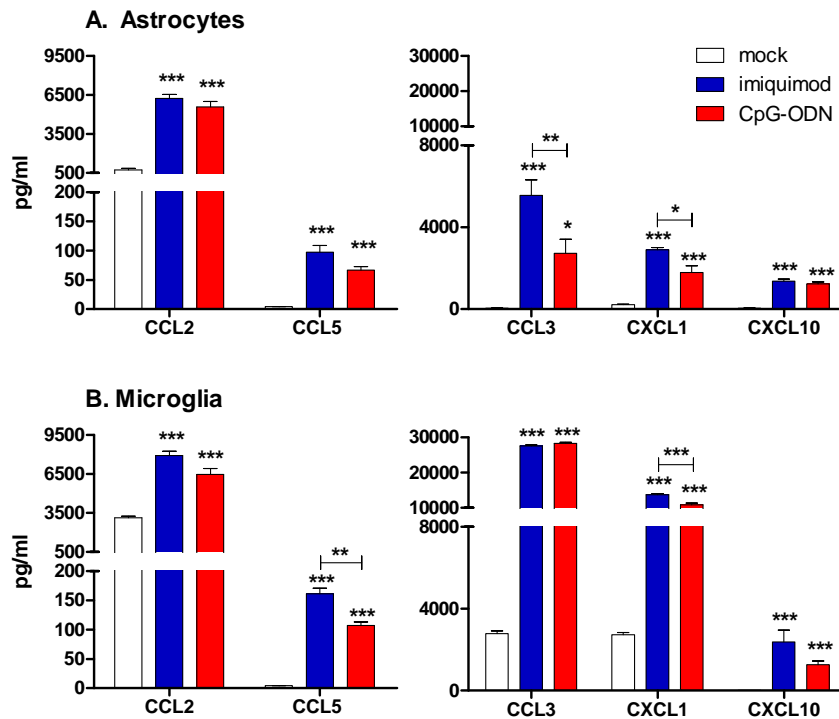
### **5.2.6. Cytokine and Chemokine Production by Astrocytes and Microglia Following TLR7/9 Stimulation.**

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. Analysis of supernatants from astrocytes and microglia stimulated with either imiquimod or CpG-ODN demonstrated that TLR7 or TLR9 activation of glial cells induced a pronounced upregulation of proinflammatory cytokines including cytokines normally associated with virus infections in the CNS such as IL-6 and TNF (Fig. 5.8). Interestingly, microglia also produced high levels of granulocyte colony stimulating factor (G-CSF) and IL-9, two cytokines that have anti-apoptotic, neuroprotective properties (Fontaine et al., 2008; Pitzer et al., 2008), as well as IL-15, which induces glial activation (Gomez-Nicola et al., 2008), and IL-10, an anti-inflammatory cytokine. This demonstrates a difference in the cytokine response of microglia and astrocytes to the same stimuli, with microglia producing high levels of both proinflammatory and antiinflammatory / neuroprotective cytokines, while astrocytes produced primarily proinflammatory cytokines.

Chemokine production by astrocytes and microglia plays an important role in regulating the recruitment of inflammatory cells to the CNS following infection or injury. Both astrocytes and microglia produced a number of chemokines following stimulation with either imiquimod or CpG-ODN (Fig. 5.9). Microglia, which had higher basal level production of most chemokines, were induced to produce higher levels of chemokine production than astrocytes. For cytokine and chemokine production, stimulation of either cell type with the TLR7 agonist appeared to induce a slightly higher level of protein production than stimulation with the TLR9 agonist (Fig. 5.8, 5.9).



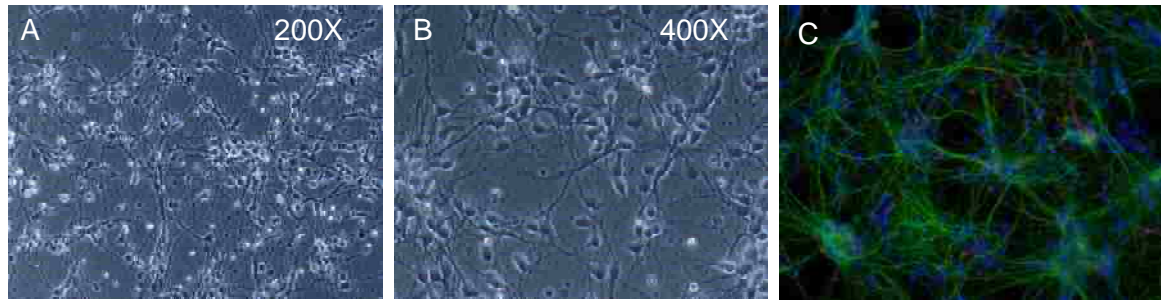
**Fig. 5.8. Comparison of Cytokine Protein Production by (A) Astrocytes and (B) Microglia Stimulated with Imiquimod or CpG-ODN.** Cultured astrocytes and microglia were stimulated with mock or 5  $\mu$ M imiquimod or 80 nM CpG-ODN 1826 for 12 hours. Supernatants were analyzed for cytokine protein production by multiplex bead array or by ELISA assay. Samples were calculated as pg/ml using a standard curve from in-plate standards. Data represent the mean  $\pm$  SEM of 3 samples per group. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.



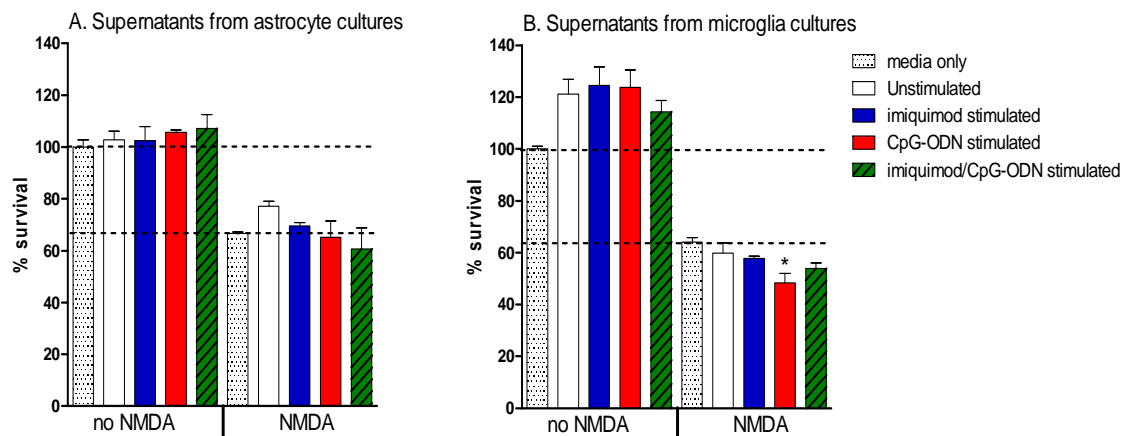
**Fig. 5.9. Comparison of Chemokine Protein Production by (A) Astrocytes and (B) Microglia Stimulated with Imiquimod or CpG-ODN.** Cultured astrocytes and microglia were stimulated with 5  $\mu$ M imiquimod or 80 nM CpG-ODN 1826 for 12 hours and supernatants were analyzed as described in Fig 5.8. Data represent the mean  $\pm$  SEM of 3 samples per group. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.

### 5.2.7. TLR7/9 Stimulated Astrocyte and Microglia Supernatants Have No Effect on Neuronal Survival

Neuronal cultures were generated from cortices of embryonic mice (Fig. 5.10-A,B) and contained only a small percent of astrocytes as detected by GFAP (Fig. 5.10C). To examine whether the secreted proteins produced by microglia or astrocytes had any effect on neuronal survival, supernatants from TLR7 and TLR9 stimulated astrocytes and microglia were overlaid on primary cortical neurons either in the presence or absence of NMDA. Neuronal survival was measured by MTT assay at 72 hours post stimulation.



**Fig. 5.10. Primary Cortical Neurons in Culture.** Cortical neurons from E16-E18 pregnant mice were cultured as described in materials and methods. (A,B) Representative of neuron cultures, (C) Immunofluorescence staining for  $\beta$ -tubulin (green) and GFAP (red).



**Fig. 5.11. Effect of TLR7 and TLR9 Stimulated Supernatants from Astrocyte and Microglia Cultures on Primary Cortical Neuron Cultures.** Cortical neurons were cultured for 4 hours following isolation and then cultured with media containing a 1:1 ratio of neurobasal media, containing all neuron growth factors, and supernatants from either (A) astrocyte or (B) microglia cultures in the presence or absence of NMDA. Neuron cultures were incubated at 37°C at 5% CO<sub>2</sub> for 72 h and the cell survival was measured by MTT assay. Data represent the mean  $\pm$  SEM of three to four samples per group. Statistical analysis was completed by one-way ANOVA with Dunnett's multiple comparison test with mock controls. \* P<0.05. Asterisks above bars indicated a significant upregulation compared to mock-treated controls.

Neurons cultured with the media used for either microglia or astrocyte cultures were used as controls. Supernatants from TLR7 or TLR9 stimulated astrocytes and microglia had no substantial effect compared to supernatants from unstimulated cells (Fig. 5.11),



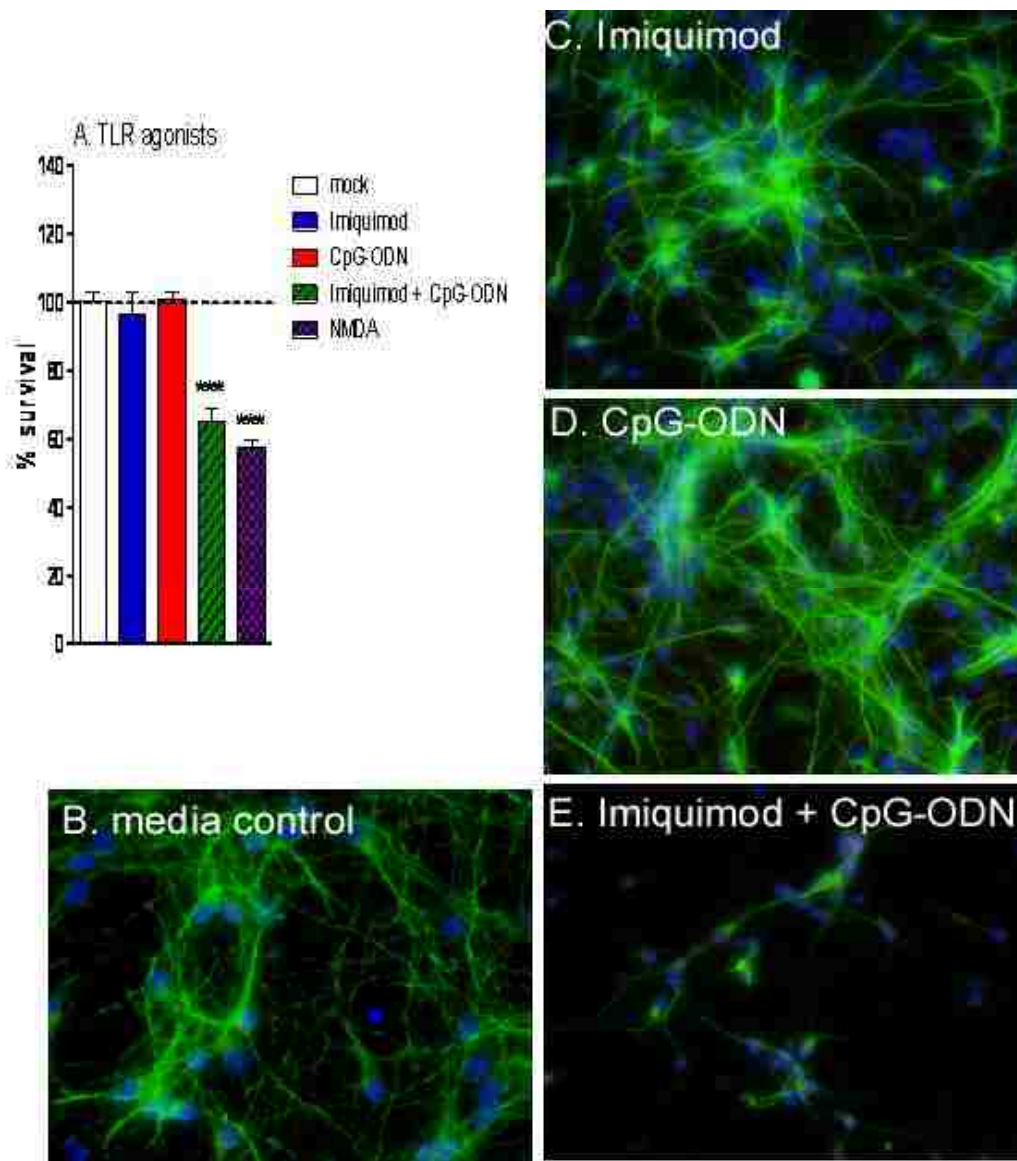
although there was a slight decrease in neuronal survival with neurons cultured with NMDA (N-methyl-D-aspartic acid) and supernatant from TLR9 stimulated neurons (Fig. 5.11B). Supernatants from cells stimulated with both TLR7 and TLR9 agonists also had no substantial effect on neuronal survival.

#### **5.2.8. Direct Stimulation of Neurons with TLR7/9 Agonists**

To rule out any possible affect of the agonists present in the cultured supernatant from stimulated microglia and astrocytes, we also stimulated neuronal cultures directly with 5  $\mu$ M of Imiquimod and/or 80 nM of CpG-ODN (Fig. 5.12A). Interestingly, direct co-stimulation of neurons with imiquimod and CpG-ODN induced neuronal cell death similar to NMDA-induced death. This suggests a synergistic affect of TLR7 and TLR9 agonist stimulation on neurons (Fig. 5.12A, E). This was surprising as co-stimulation with TLR7 and TLR9 agonists did not induce cell death in either astrocytes or microglia cultures.

### **5.3. DISCUSSION**

Multiple immune cell types, including plasmacytoid dendritic cells (pDC's), macrophages and B cells, express both TLR7 and TLR9 (Hornung et al., 2002; Kadowaki et al., 2001; Krug et al., 2001). In the current study, astrocytes and microglia expressed both TLR7 and TLR9, and produced a functional response to TLR7 and TLR9 agonist stimulation. Interestingly, the cytokine profile produced by agonist stimulation was very similar between the type of TLR stimulation, but varied between cell types. Microglia, but not astrocytes produced anti-inflammatory and anti-apoptotic cytokines in addition to pro-inflammatory cytokines (Fig. 5.8). In contrast, astrocytes upregulated mRNA expression of a greater number of innate immune response genes than microglia



**Fig. 5.12. Effect of TLR7 and TLR9 Agonists on Primary Cortical Neuron Cultures.** Cortical neurons were cultured for 4 hours following isolation and then stimulated with 5  $\mu$ M imiquimod or 80 nM CpG-ODN 1826 and/or NMDA as described in methods. Neuron cultures were incubated at 37°C at 5% CO<sub>2</sub> for 72 h and the cell survival was measured by (C) MTT assay or (D-G) immunofluorescence staining for B-tubulin. Data represent the mean  $\pm$  SEM of three to four samples per group. Statistical analysis was completed by one-way ANOVA with Dunnett's multiple comparison test with mock controls. \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls.

including mRNA expression for genes whose proteins are involved in signal transduction responses (Fig. 5.6). Since astrocytes are not derived from an immune cell lineage, these cells may upregulate the expression of signal transduction proteins when they become activated in order to allow them to respond more strongly to innate immune stimuli. The strong differences in the response between astrocytes and microglia also indicate that the astrocytic response is not due to a contamination of microglia cells. The kinetics of gene expression may also vary between microglia and astrocytes. Although similar kinetics were observed in gene expression between microglia and astrocytes for *Ccl2*, *Cxcl10* and *Tnf* (Fig. 5.4), the expression of other genes in the innate immune response may differ. The current study only analyzed a single time point and it is possible that there are fluctuations in other gene expression at earlier or later time points.

TNF, at concentrations higher than 200 pg/ml can be neurotoxic (Gelbard et al., 1993; Westmoreland et al., 1996). Surprisingly, neither supernatants from activated microglia nor activated astrocytes altered neuronal survival, despite high levels of TNF (Fig. 5.11). Previous studies have demonstrated that CpG-ODN activation of co-cultured microglia and neurons can induce neuronal toxicity, mediated in part by TNF (Iliev et al., 2004). The inability of supernatants from CpG-ODN activated microglia to induce neurotoxicity suggests that cell to cell interactions may also be a necessary component of microglia-induced neuronal cell death and that other cytokines induced by TLR activation may counteract the neurotoxic effects of TNF.

A few discrepancies were observed between mRNA expression and protein production in astrocytes and microglia in this study. For example, *Il10* mRNA was upregulated by TLR7/9 agonist stimulation in both astrocytes and microglia; however,

IL-10 protein was only detected in culture supernatants from microglia. *Tnf* mRNA levels were substantially higher in astrocytes, but protein levels were either similar or higher in microglia. Both IL-10 and TNF undergo post-transcriptional or post-translational modification that may influence the secretion of the protein in one cell type versus another (Pauli, 1994; Powell et al., 2000). Possibly, microglia may be able to translate cytokine and chemokine mRNA into functional proteins at a higher rate than astrocytes, resulting in higher protein production despite lower mRNA expression.

The combination of TLR7 and TLR9 agonists had a synergistic effect on neuronal death (Fig. 5.12). Neither TLR7 nor TLR9 agonists alone affected neuronal survival, which is similar to previous reports (Iliev et al., 2004; Ma et al., 2006). However, the combination of TLR7 and TLR9 agonists together was neurotoxic as determined by MTT assay as well as  $\beta$ -tubulin staining (Fig. 5.12). Primary mouse neurons do not express TLR7 (Ma et al., 2006). One possible explanation is that the combination of TLR7 and TLR9 agonists altered the binding of CpG-ODN to the TLR9 receptor and induced an altered response that lead to cell death. Alternatively, the combination of CpG-ODN and imiquimod may have activated TLR8, as TLR8 activation can induce caspase 3-induced death in neurons (Ma et al., 2006). Oligonucleotides have been shown to enhance binding of TLR7/8 agonists to the TLR8 receptor, while inhibiting signaling to TLR7 in HEK cells (Gorden et al., 2006a; Gorden et al., 2006b).

TLR7 and TLR9 agonists are being explored for their potential use as immune activators or immune response modifiers to enhance the vaccine efficacy and to treat infectious diseases, allergic diseases, and in cancer therapy (Pedras-Vasconcelos et al., 2006; Pedras-Vasconcelos et al., 2008, El et al., 2006; Prins et al., 2006). It is important to

understand how these agonists affect glial cells, both to understand the mechanisms by which these agonists can be used as therapeutics and to examine how TLR7/TLR9 activation of glial cells could alter the CNS microenvironment. In the present study, TLR7/TLR9 agonists induced multiple proinflammatory cytokines and chemokines at varying levels by astrocytes and microglia (Figs 5.6, 5.8, 5.9), but did not alter the mRNA expression of several non-inflammatory genes that are important to astrocytic functions (Fig. 5.7). This suggests that TLR7/TLR9 agonists may not alter the primary function of astrocytes, but still induce a strong proinflammatory response that may be limiting to virus infection and/or tumor growth. However, it will be important to assess if repeated stimulation of glial cells with the same agonist alters the functional responses of astrocytes and microglia, or reduces their ability to respond to TLR stimulation.

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## **CHAPTER 6**

### **INTERACTIONS BETWEEN TLR7 AND TLR9 AGONISTS AND RECEPTORS IN INDUCING INNATE IMMUNE RESPONSES IN THE GLIAL CELLS AND THE CENTRAL NERVOUS SYSTEM\***

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## 6.1. INTRODUCTION

Synergistic co-activation among multiple TLRs has been reported. For example, synergistic response have been observed between TLR3 and TLR7 (Gautier et al., 2005; Napolitani et al., 2005; Roelofs et al., 2005; Warger et al., 2006), TLR4 and TLR7 (Gautier et al., 2005; Napolitani et al., 2005; Roelofs et al., 2005), TLR3 and TLR9 (Whitmore et al., 2004) and TLR2/4 and TLR9 (Agren et al., 2006). In contrast to induce synergistic co-activation, the TLR9 family receptors may inhibit each other signaling. Natural and synthetic TLR7 agonists were reported to inhibit CpG-ODN induced IFN $\alpha$  production from plasmacytoid dendritic cells and B cells following TLR7/TLR9 co-stimulation (Berghofer et al., 2007a; Marshall et al., 2007b). This may have important implications during pathogen infections *in vivo* since during virus infections, such as cytomegalovirus, HSV-1, HIV, both TLR7 and TLR9 pathways can be activated (Beignon et al., 2005; Mandl et al., 2008; Zucchini et al., 2008). Additionally, DNA and RNA derived from bacterial pathogens as well as necrotic or apoptotic cells can simultaneously stimulate TLR7 and TLR9 (Kariko et al., 2005). Understanding the interaction of TLR7/TLR9 agonists in the CNS is important for understanding pathogenesis.

In addition to the agonist interactions, the level of receptor expression may also impact TLR7/TLR9 signaling. Co-expression of either TLR8 or TLR9 in TLR7 transfected HEK cells inhibited the response of these cells to TLR7 agonist stimulation, but not TLR8 or TLR9 agonist stimulation. Co-transfection of TLR8, but not TLR7, in TLR9 transfected human embryonic kidney (HEK) cells inhibited TLR9 agonist-induced responses, while neither TLR7 nor TLR9 expression altered TLR8 agonist-induced responses in HEK cells (Wang et al., 2006). The inhibitory effects between the receptors may be due to the mechanisms by which these TLRs are transported to endosomes.

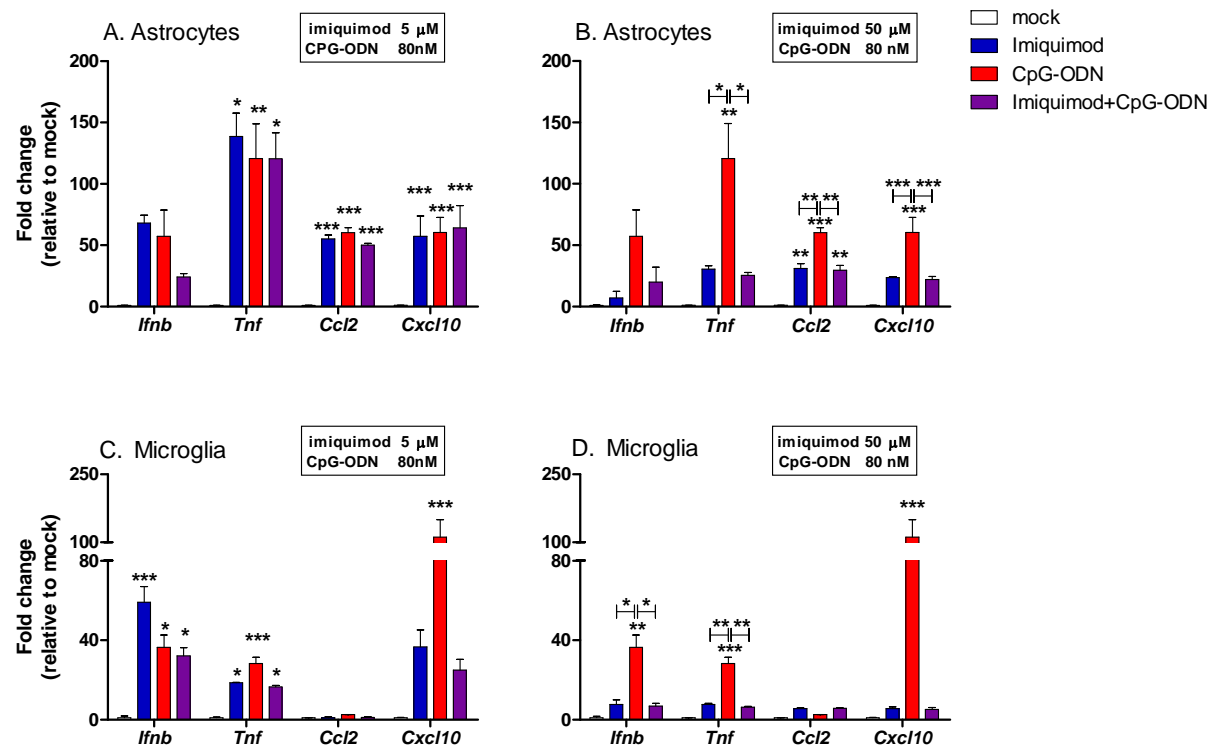
TLR7 and TLR9 trafficking from ER to endolysosomes is controlled by a multi transmembrane protein called Unc93B1, which reside in the endoplasmic reticulum. Once in the endosome, TLR7 and TLR9 are proteolytically cleaved to generate functionally competent receptors (Ewald et al., 2008; Park et al., 2008). Recent studies indicated that Unc93B1 inversely links TLR7 and TLR9 responses, with bias towards TLR9 and against TLR7. Thus, the presence or absence of one receptor may alter the responses of other receptor.

In the present study, we analyzed the interaction between TLR7 and TLR9 agonists, as well as their receptors, in regulating innate immune responses both *in vitro* and *in vivo*. We utilized wild-type mice and mice deficient in TLR7, and glial cells cultured from TLR7 deficient mice.

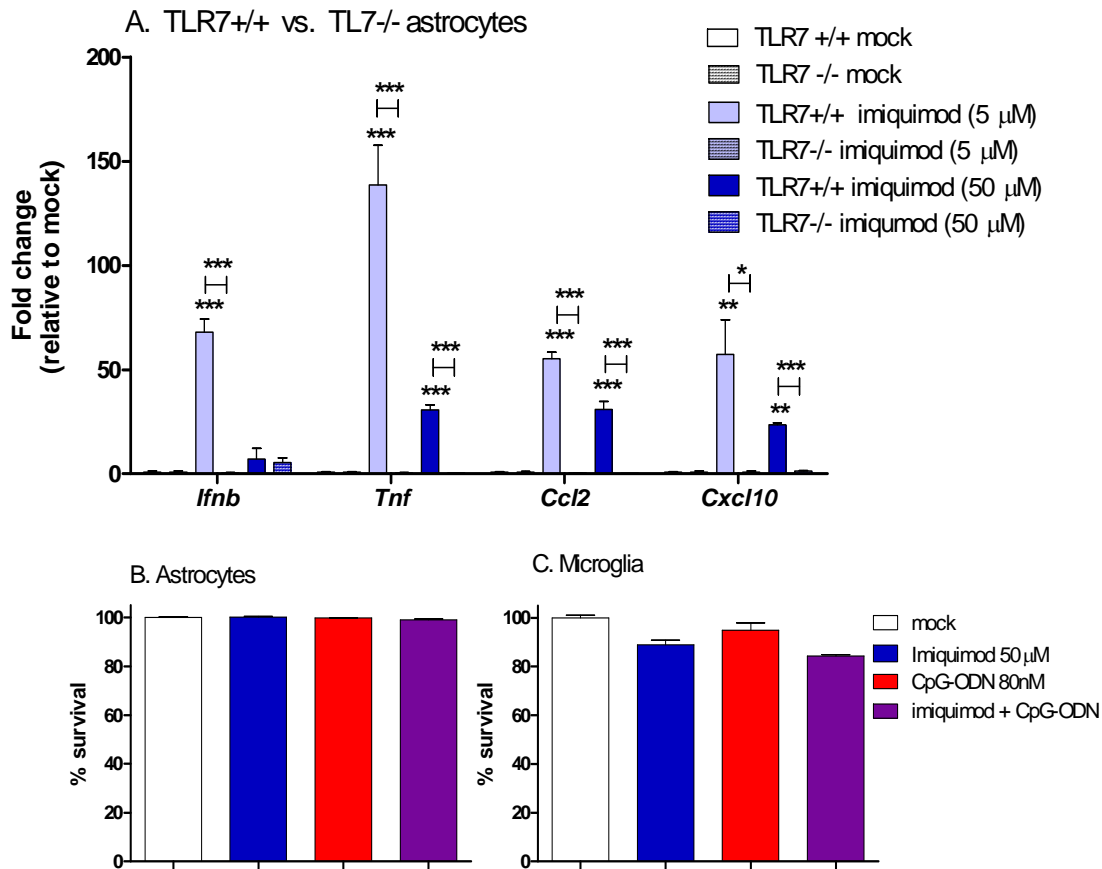
## **6.2. RESULTS**

### **6.2.1. TLR7 Agonist Can Inhibit TLR9 Agonist Stimulation in A Concentration Dependent Manner in Astrocytes and Microglia.**

Natural and synthetic TLR7 ligands were reported to inhibit CpG-ODN induced IFN $\alpha$  production from plasmacytoid dendritic cells and B cells following TLR7/TLR9 co-stimulation (Berghofer et al., 2007a; Marshall et al., 2007b). Comparison of the fold increase in mRNA expression of innate immune response genes in astrocytes and microglia demonstrated only minimal suppression by imiquimod on CpG-ODN-induced responses when both agonists were added together (Table 5.1, Fig.6.1 A,C). To examine if a higher concentration of imiquimod was inhibitory to CpG-ODN induced responses, 50  $\mu$ M imiquimod was used in a co-stimulation experiment. The high dose of 50  $\mu$ M was inhibitory to CpG-ODN induced cytokine and chemokine production (Fig. 6.1 B,D). This inhibition was not due to cell death as all cultures had comparable numbers of live cells as determined by an MTT cell viability assay (Fig. 6.2 B,C).



**Fig. 6.1. Co-stimulation with TLR7/TLR9 Agonists Inhibits TLR9-Induced Cytokine and Chemokine mRNA Expression in Astrocytes and Microglia.** Cultured (A,B) astrocytes and (C,D) microglia were stimulated with either (A,C) 5  $\mu$ M imiquimod or (B,D) 50  $\mu$ M imiquimod and/or (A-D) 80 nM CpG-ODN 1826. At 6 hps, RNA was isolated from all samples and processed for real-time quantitative RT-PCR analysis and values were calculated relative to expression of *Gapdh* controls. Data are presented as the fold induction of each gene of interest relative to mock controls. Mock expression levels were calculated as the mean of 3 wells per group. Data represent the mean  $\pm$  SEM of 6-9 samples per group and present the combined data from two independent experiments. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.



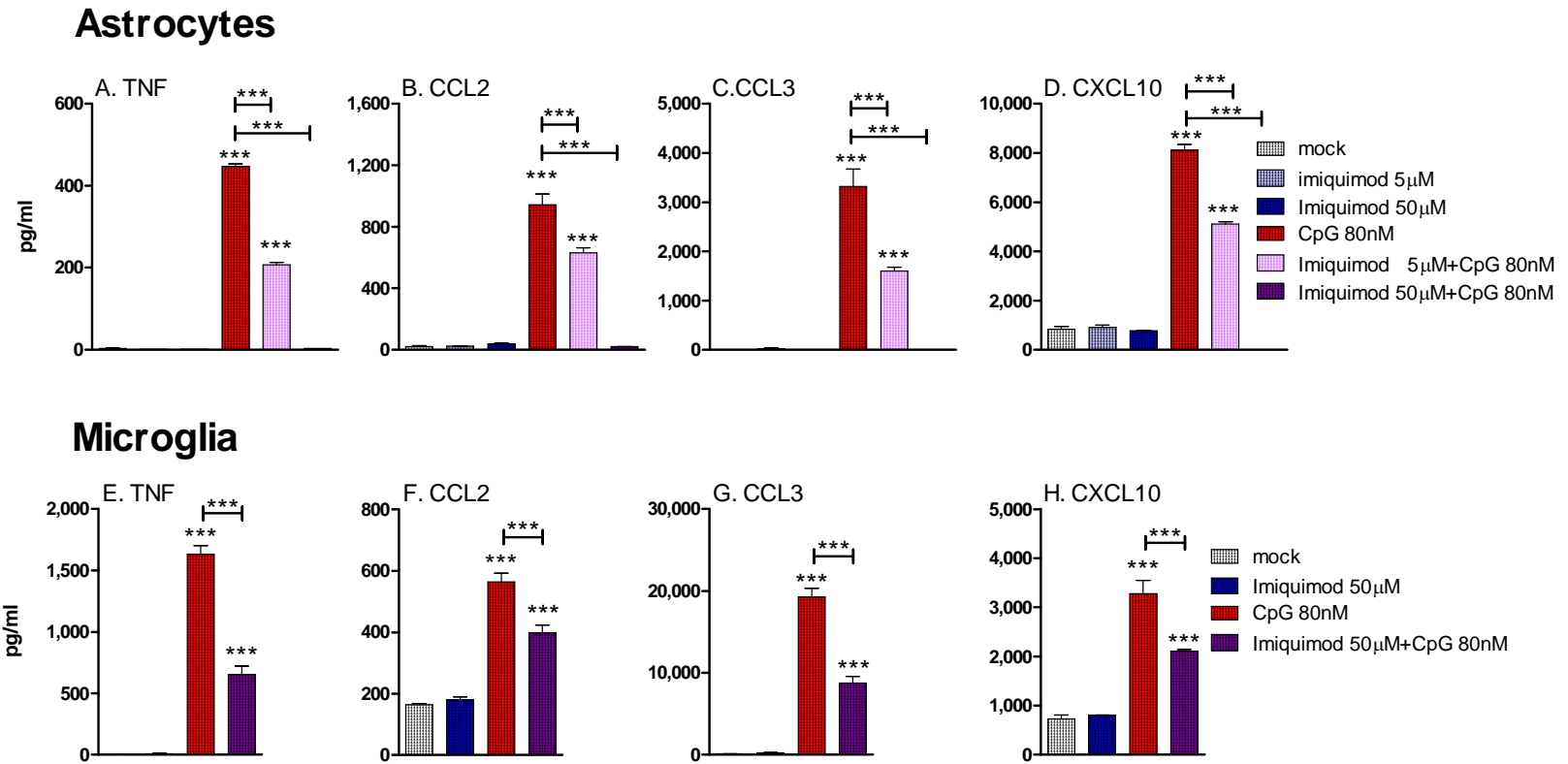
**Fig. 6.2. Specificity of Both Concentrations of Imiquimod for TLR7.** Cultured (A,B) astrocytes or (C) microglia were stimulated with either (A) 5 μM imiquimod or (A,B,C) 50 μM imiquimod and/or (B,C) 80 nM CpG-ODN 1826. (A) Stimulation of astrocytes generated from wild-type (TLR7<sup>+/+</sup>) and TLR7 deficient (TLR7<sup>-/-</sup>) mice to verify specificity of both concentrations of imiquimod. At 6 hps, RNA was isolated from all samples and processed for quantitative realtime RT-PCR. Samples were analyzed as described in Fig. 6.1. (B,C) Cell survival was measured by MTT assay at 6 h post stimulation. Data represent the mean +/- SEM of 3-6 samples per group and present the combined data from two independent experiments. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \* P<0.05, \*\* p<0.01 and \*\*\* p< 0.001. Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.

The stimulatory capability of certain TLR agonists can decline when used at high concentrations (Gorden et al., 2005; Marshall et al., 2007a). Since the 50  $\mu$ M concentration of imiquimod induced lower cytokine and chemokine responses than the 5  $\mu$ M concentration of imiquimod (Fig.6.1), we verified that both concentrations induced cytokine production through a TLR7 dependent mechanism. TLR7 was necessary for cytokine and chemokine responses at both 5  $\mu$ M and 50  $\mu$ M concentrations in astrocyte cultures (Fig. 6.2 A). Specificity of the response in microglia was not analyzed since the 50  $\mu$ M concentration of imiquimod did not induce a significant response in these cells.

### **6.2.2. TLR7 Agonist Inhibition of TLR9 Agonist-Induced Responses is Not TLR7 Dependent.**

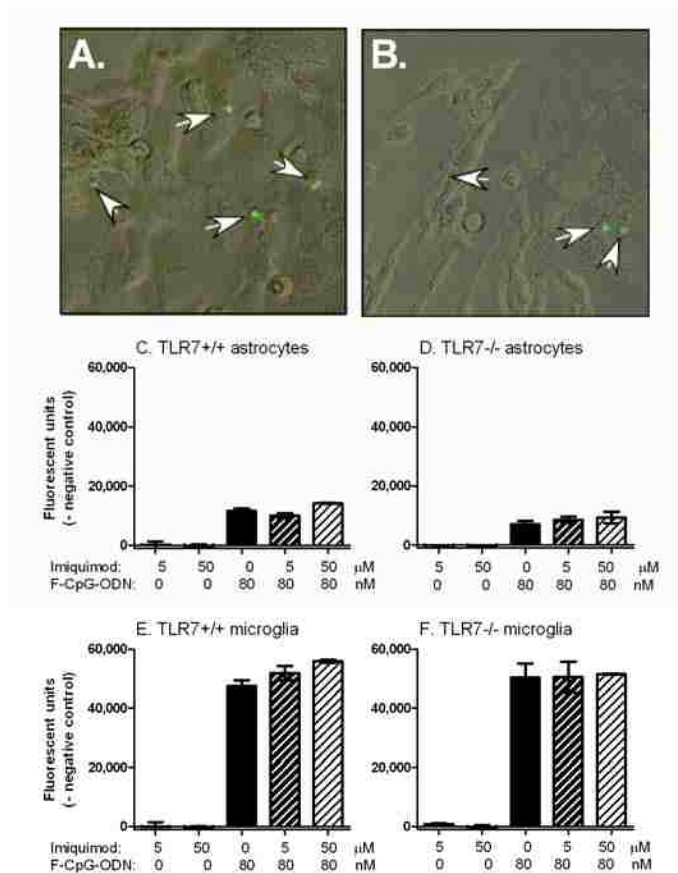
To examine the mechanism by which high concentrations of imiquimod suppressed CpG-ODN responses, we first analyzed whether TLR7 signaling was required for this inhibition. Interestingly, imiquimod suppressed CpG-ODN induced cytokine and chemokine production in glial cells from TLR7 deficient mice (Fig. 6.3). Thus, imiquimod suppression of CpG-ODN responses was not mediated by signaling of TLR7. We next examined whether imiquimod could be inhibiting the uptake of CpG-ODN by astrocytes or microglia.

Cell entry analysis using FITC-labeled CpG-ODN demonstrated that CpG-ODN was taken up by both astrocytes and microglia at similar levels in the presence or absence of imiquimod (Fig. 6.4 A-F). Thus, imiquimod inhibits CpG-ODN by a mechanism independent of either TLR7 signaling or cell entry. Instead, this suppression could be mediated by other mechanisms, including the possibility that high concentrations of imiquimod may interfere with CpG-ODN binding to TLR9.



**Fig. 6.3. TLR7 Is Not Necessary for TLR7 Agonist Inhibition of TLR9-Induced Cytokine Responses in (A-D) Astrocytes or (E-H) Microglia Cultures.** Astrocyte and microglia cultures from TLR7 deficient mice were stimulated with TLR7 and/or TLR9 agonists for 12h as shown in the figure and the supernatants were analyzed for cytokine protein production by multiplex bead array. Samples were calculated as pg/ml using a standard curve from in-plate standards. Data represent the mean  $\pm$  SEM of 3 samples per group. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Asterisks above bars indicated a significant upregulation compared to mock-treated controls. Lines with asterisks above the lines indicate the difference between the indicated groups.



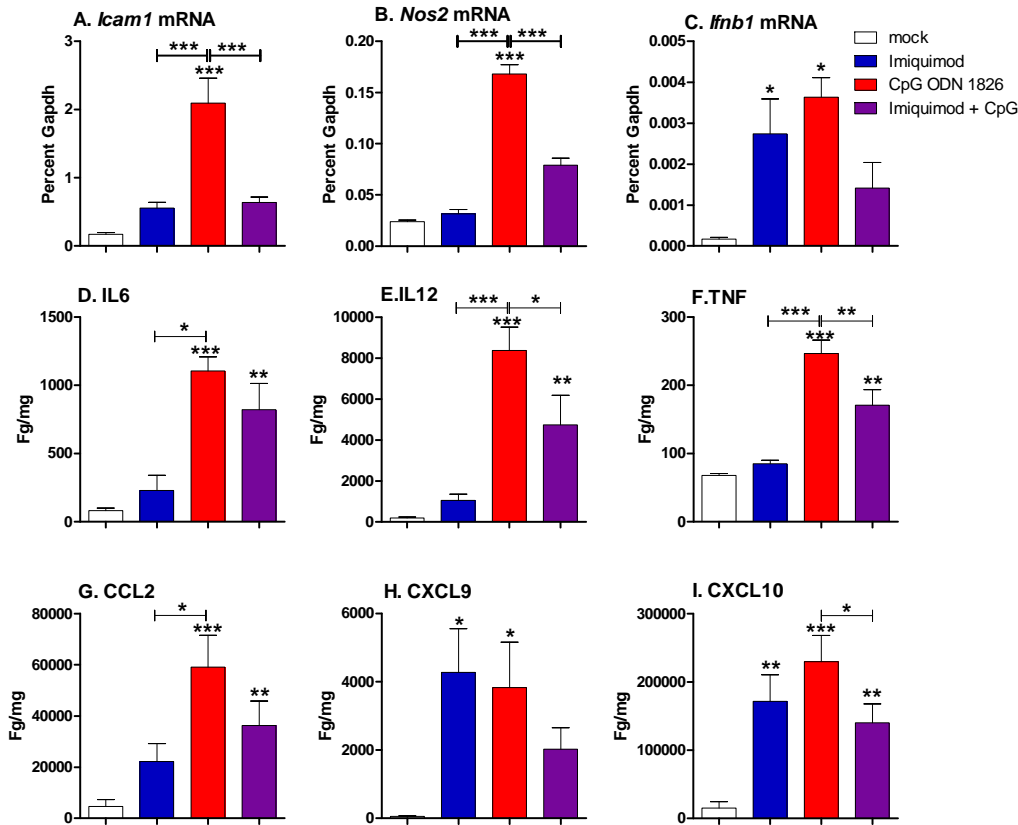


**Fig. 6.4. TLR7 Agonists Do Not Inhibit the Endocytosis of FITC Labeled CpG-ODN Into Astrocytes or Microglia.** Astrocyte and microglia cultures at near confluency were incubated with 80 nM of FITC-labeled CpG-ODN and/or 5 to 50 μM of imiquimod for 30 min, and then washed extensively with PBS to remove unbound or non-internalized FITC-CpG. FITC was detected in the cells of both (A) astrocytes and (B) microglia. Cells were then lysed and the level of fluorescence measured using a microplate reader (Polar star Omega, BMG labtech). Fluorescence levels in unstimulated astrocytes and microglia were used as a baseline for each culture. Data represent the average of 3 wells per group for (C-D) astrocytes or (E-F) microglia cultures generated from (A-C,E) wild-type or (D,F) TLR7 deficient mice. Data is representative of one of the two replicate experiments.

### 6.2.3. TLR7 Agonist Can Inhibit TLR9 Agonist Stimulation *in vivo*, Which Is Independent of TLR7.

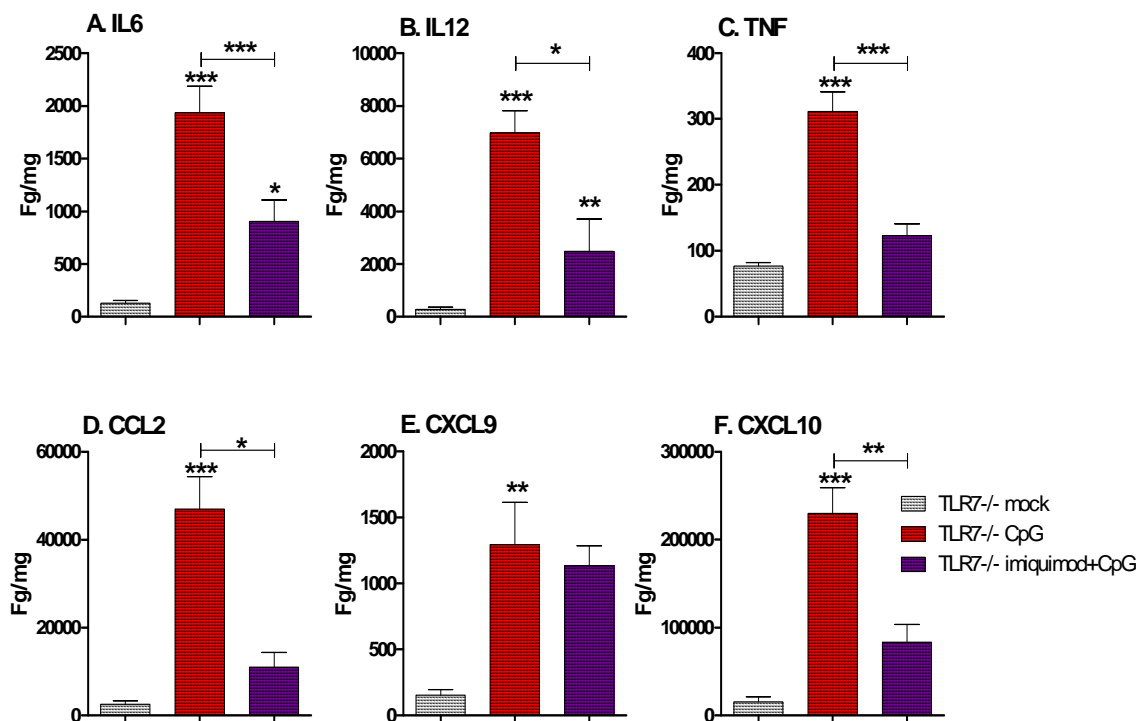
To determine if the suppressive effect of imiquimod on CpG-ODN-induced cytokine production *in vitro* was also observed *in vivo*, we completed co-stimulation studies in the neonatal brain using optimal dose of agonists. Co-administration of

imiquimod and CpG-ODN *in vivo* suppressed CpG-ODN-induced expression of *Icam1* and *Nos2* mRNA expression, and production of cytokines IL12, TNF, CCL2 and CXCL10 to varying degrees (Fig 6.5).



**Fig. 6.5. Co-stimulation with TLR7/TLR9 Agonists Inhibits TLR9-Induced Cytokine and Chemokine mRNA and Protein Expression in the Neonatal Brain.** Mice at 48 h of age were inoculated by intracerebroventricular injection with 100 nmoles of imiquimod, 80 pmoles of CpG-ODN, or both, or vehicle control (0.2% trypan blue in PBS). Brain tissues were removed at 12 hps and snap frozen for (A-C) RNA or (D-I) protein analysis as described. (A-C) RNA samples were processed for real-time quantitative RT-PCR analysis and values were calculated relative to expression of Gapdh controls. Mock expression levels were calculated as the mean of 3 animals. Data represent the mean  $\pm$  standard deviation of 3-4 mice per group. (D-I) One half of sagittally divided brain was homogenized in lysis buffer containing protease inhibitors and analyzed for protein expression using a Biosource 20-plex bead array on a Bioplex Luminex system. Samples were calculated as pg/ml using a standard curve from in-plate standards and subsequently converted to fg/mg brain tissue. Data represent the mean  $\pm$  SEM of 4-9 mice per group. Statistical analysis was completed by one way ANOVA with Newman-keuls post-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

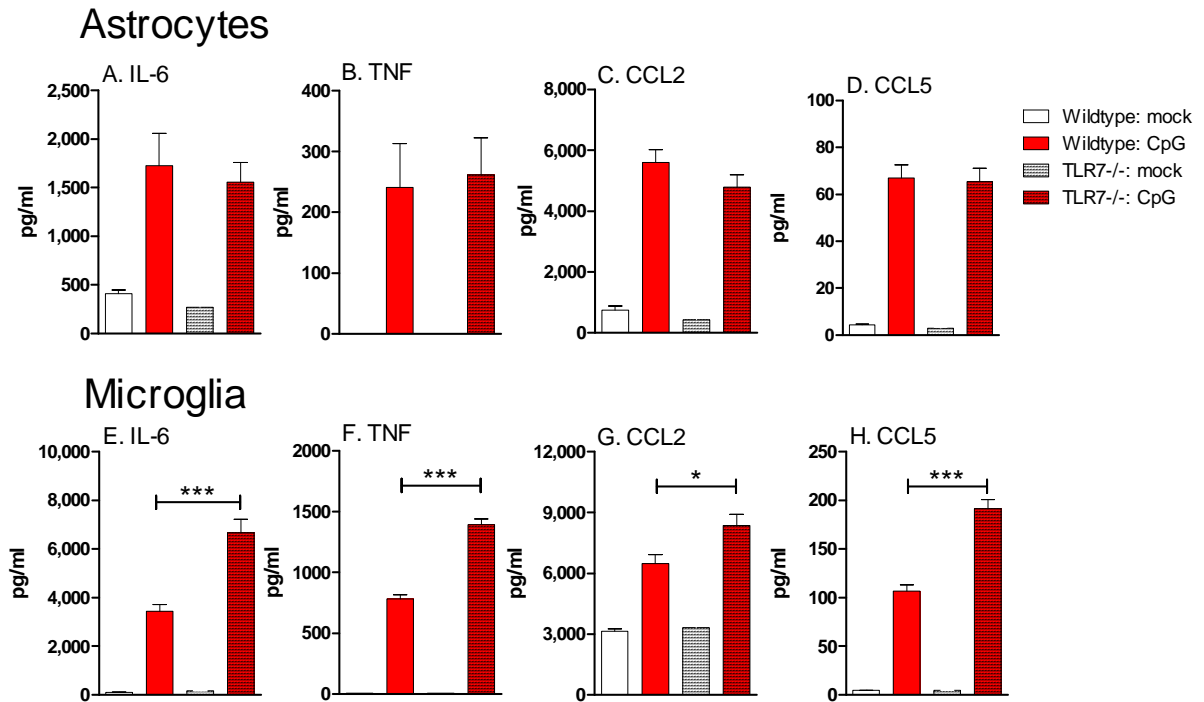
As the inhibitory effect of imiquimod on CpG-ODN-induced cytokine responses in astrocytes and microglia was independent of TLR7 signaling (Fig 6.3), we analyzed if TLR7 signaling was necessary for inhibition in the brain. Similar to the *in vitro* data, imiquimod suppressed cytokine production by CpG-ODN inoculation in the brain, in the absence of TLR7 (Fig 6.6). Thus, imiquimod inhibits CpG-ODN by a mechanism independent of TLR7 signaling both *in vitro* and *in vivo*.



**Fig. 6.6. TLR7 Is Not Necessary for TLR7 Agonist Inhibition of TLR9-Induced Cytokine Response in the Neonatal Brain.** TLR7 deficient mice were inoculated with vehicle control or 80 pmoles of CpG-ODN  $\pm$  100 nmoles of imiquimod by i.c.v. inoculation and tissues were removed at 12 hpi. One half of sagittally divided brain was homogenized in lysis buffer containing protease inhibitors and analyzed for protein expression using a Biosource 20-plex bead array on a Bioplex Luminex system. Samples were calculated as pg/ml using a standard curve from in-plate standards and subsequently converted to fg/mg brain tissue. Data represent the mean  $\pm$  SEM of 4-7 mice per group. Statistical analysis was completed by one way ANOVA with Bonferonni post-test. \*P<0.05, \*\*P<0.01. \*\*\*P<0.001.

### 6.2.4. Negative Influence of TLR7 on CpG-ODN-Induced Responses in Microglia.

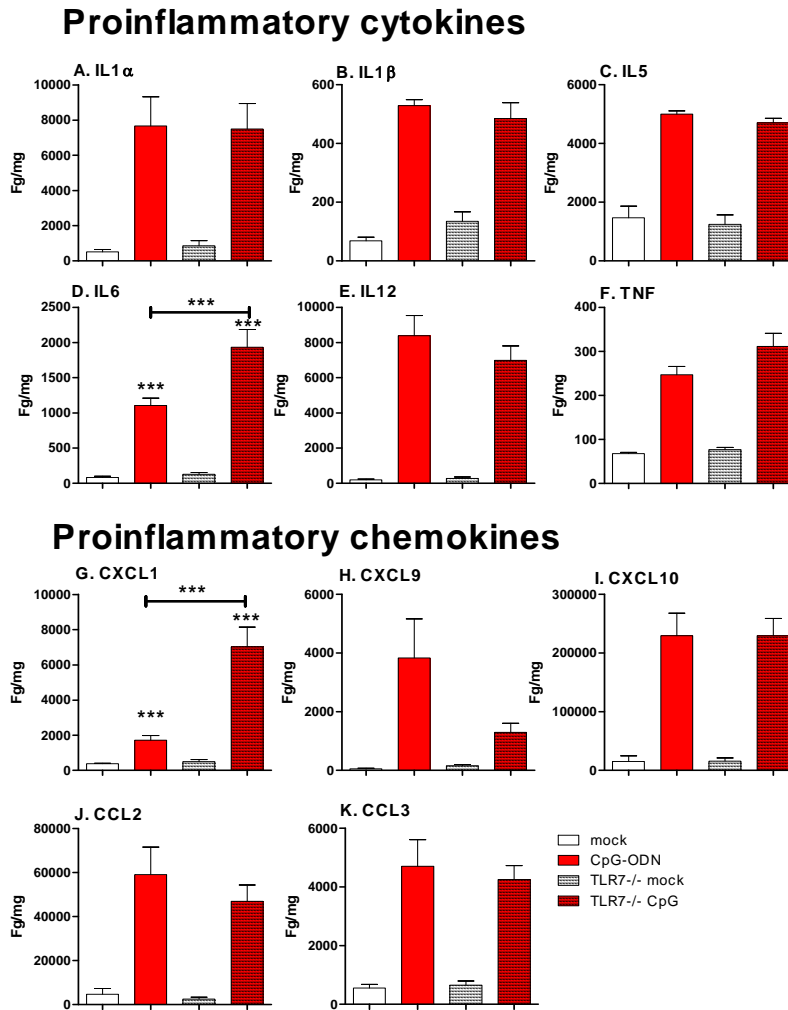
To examine whether TLR7 itself had any impact on CpG-ODN stimulation, we examined the influence of TLR7 deficiency on CpG-ODN stimulation of glial cells. TLR7 deficiency had no effect on CpG-ODN induced cytokine stimulation by astrocytes (Fig. 6.7 A-D). However, an increase in cytokine production was observed in TLR7-deficient microglia stimulated with CpG-ODN compared to wild-type microglia (Fig. 6.7 E-H). Thus, both TLR7 agonists and TLR7 itself, have a suppressive effect on CpG-ODN induced cytokine production by microglia.



**Fig. 6.7. Effect of TLR7 Deficiency on TLR9-Induced Cytokine and Chemokine Production in Astrocytes and Microglia.** (A-D) Astrocyte and (E-F) microglia cultures from wild type and TLR7 deficient mice were stimulated with mock control or 80 nM of CpG-ODN 1826 for 12h and the supernatants were analyzed as described in Fig 6.2. Data represent the mean  $\pm$  SEM of 3 samples per group. Statistical analysis was completed by one-way ANOVA with Bonferroni post-test. \*  $P < 0.05$ , \*\*\*  $p < 0.001$ . Asterisks above the lines indicate the difference between the indicated groups.

### 6.2.5. Influence of TLR7 on CpG-ODN-Induced Responses *in vivo*

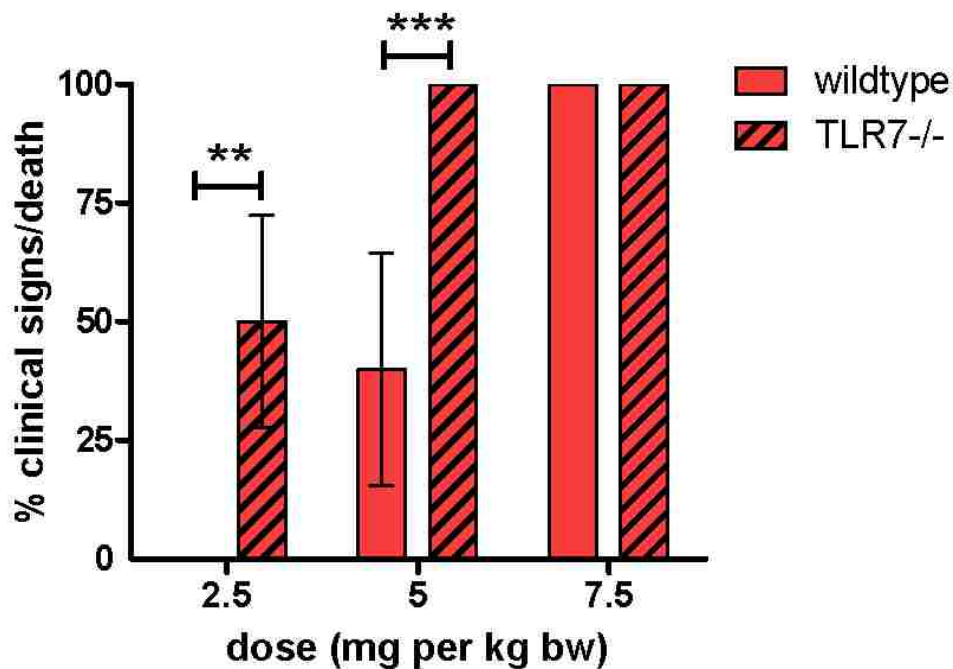
To see if a similar phenomenon was *observed in vivo*, both wild-type and TLR7 deficient mice were inoculated i.c.v. with CpG-ODN and examined the induction of cytokine and chemokine induction. TLR7 deficiency had no effect on most of the cytokines and chemokine production by CpG-ODN inoculation, with the exception of IL6 and CXCL1 (Fig 6.8).



**Fig. 6.8. Effect of TLR7 Deficiency on TLR9-Induced Cytokine and Chemokine Production in the CNS.** Wild-type TLR7 deficient mice were inoculated with vehicle control or 80 pmoles of CpG-ODN by i.c.v. inoculation and brain tissues were removed at 12 hpi, processed analyzed as described in Fig. 6.5. Data represent the mean  $\pm$  SEM of 4-7 mice per group. Statistical analysis was completed by one way ANOVA with Bonferonni post-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### 6.2.6. Analysis of Effect of TLR7 Deficiency on CpG-ODN-Induced Clinical Signs.

As the TLR7 agonists and TLR7 itself have a suppressive effect on CpG-ODN-induced proinflammatory cytokine production in microglia, we analyzed if this suppressive ability of TLR7 would protect the mice from TLR9-induced clinical signs and/or death. We utilized the TLR7 deficient mice and stimulated with CpG-ODN with a dose that induced severe clinical signs of respiratory distress and unresponsiveness. Interestingly, TLR7 deficiency induced severe clinical signs/death at lower dose of CpG-ODN (Fig. 6.9) compared to wild type mice. Thus, the presence of TLR7 appears to regulate TLR9 response and protect mice from TLR9-agonist induced clinical signs and/or death.



**Fig. 6.9. Comparison of CpG-ODN-Induced Clinical Signs between Wild Type and TLR7 Deficient Mice.** Wild type and mice deficient in TLR7 were inoculated with 2.5  $\mu$ g, 5  $\mu$ g or 7.5  $\mu$ g per mg bodyweight and analyzed for clinical signs of respiratory distress and unresponsiveness. Statistical analysis was completed by two-way ANOVA with Bonferonni post-test. \*\*P<0.01. \*\*\*P<0.001. 5-12 mice were used per dose and per strain.

### 6.3. DISCUSSION

In the present study, TLR7 agonist, imiquimod, could inhibit TLR9 agonist-induced innate immune responses, in both astrocytes and microglia in a concentration dependent manner. This was also observed *in vivo*, with TLR7 agonist inhibiting the TLR9 agonist induced responses. Surprisingly, this inhibition was not mediated by TLR7, as deficiency in TLR7 did not alter suppression of the TLR9 agonist-induced responses, either *in vitro* or *in vivo*. The suppression of innate immune responses was also not due to an inhibition of TLR9 agonist uptake, as demonstrated in both astrocytes and microglia. An antagonistic relationship was also observed between the two receptors in microglia, but not in astrocytes, with TLR7 deficiency resulting in enhanced cytokine responses to CpG-ODN stimulation. Furthermore, deficiency of TLR7 induced more severe clinical signs or death in mice inoculated with CpG-ODN compared to wild type mice. Thus, both TLR7 and its agonist can have inhibitory effects on TLR9 agonist -induced cytokine responses in the CNS.

High concentrations of imiquimod inhibited CpG-ODN induced responses in both microglia and astrocyte cultures (Fig. 6.1). Suppression of TLR9 agonist-induced interferon responses by both natural and synthetic agonists have previously been shown for both plasmacytoid dendritic cells and B cells (Berghofer et al., 2007b; Marshall et al., 2007a). However, the mechanism behind this suppression was not known. Our current results indicate that the mechanism behind this suppression is not due to a feedback mechanism by TLR7 signaling (Fig. 6.3). It is possible that the mechanism of suppression is due to an interaction between TLR7 and TLR9 agonists, with imiquimod interacting with CpG-ODN and preventing binding of CpG-ODN to TLR9. Alternatively,

high concentrations of imiquimod may allow imiquimod to interact directly with TLR9, thus inhibiting the binding of CpG-ODN to TLR9. In both scenarios, the inhibition would be concentration dependent and would explain why 50  $\mu$ M concentrations of imiquimod suppress CpG-ODN stimulation to a greater extent than 5  $\mu$ M (Figs. 6.1 & 6.3).

Inhibition of TLR9 agonist-induced responses by TLR7 agonists was also observed in the neonatal brain, similar to glial cells. This inhibition was observed at the optimal concentration, in contrast to the glial cells where only higher concentrations of TLR7 agonist inhibited TLR9 agonist-induced responses. This could be because both TLR7 and TLR9 agonists induced same level of cytokine and chemokine production *in vitro* (Fig. 5.8), and imiquimod does not appear to inhibit CpG-ODN responses at optimal concentration (Fig 6.1). However, TLR9 agonist induced substantially higher cytokine production in the neonatal brain compared to TLR7 agonist (Fig. 4.7), and the inhibition of TLR9 agonist-induced responses were easily observed following co-stimulation.

Although the TLR7 receptor did not have a direct role in imiquimod-mediated inhibition of CpG-ODN-induced responses in glial cells, TLR7 does appear to have a direct suppressive effect on TLR9 responses. Microglia, but not astrocytes, from TLR7 deficient mice had higher levels of cytokine production following CpG-ODN stimulation compared to microglia from wild type mice (Fig. 6.7). This suppressive effect was not observed with all cell types as astrocytes responded similarly to CpG-ODN in presence or absence of TLR7 (Fig. 6.7). Furthermore, the presence of TLR7 reduces the TLR9 agonist-induced clinical signs in mice probably by limiting TLR9 activation and suppressing the proinflammatory cytokines and chemokine production from microglia, ependymal cells and other cells responsible for the high levels of TNF, IL1 $\alpha$ , IL1 $\beta$ , and



IL6. Possibly, there is a distinct interaction between TLR7 and TLR9 regulation in different cell types. TLR7 and TLR9 compete for binding of the Ubiquitin protein, Unc93b1, which regulates the migration of these receptors from the endoplasmic reticulum to the endolysosomes (Fukui et al., 2009). The relative ratio of TLR7, TLR9, Unc93b, or MyD88, the signal transduction molecule associated with both TLR7 and TLR9, in the endosomes of different cell types may influence receptor signaling and cytokine production, and regulate the interactions between these two receptors.

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**CHAPTER 7**  
**SUMMARY AND FUTURE DIRECTIONS**

## 7.1. SUMMARY AND FUTURE DIRECTIONS

Toll-like receptors 7 (TLR7) and 9 (TLR9) are important mediators of innate immune responses (Barchet et al., 2005; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2000; Hornung et al., 2004). Numerous studies have demonstrated a clear role for these receptors in the activation of dendritic cells and B cells, as well as their role in a number of disease related conditions from autoimmunity to viral and bacterial infections (Aravalli et al., 2007; Kirton et al., 2005; Kristensson, 2006; Mishra et al., 2006; Tardieu et al., 2000). However, there is a lack of basic understanding of how these receptors function in specific responses, including systems that lack dendritic cells such as the CNS. The present study compared the neuroinflammatory responses to both TLR7 and TLR9 agonists, examined the differences in the responses and the interaction between receptors and agonists in regulating innate immune responses.

Intracerebroventricular inoculation of TLR9 agonists induced a more pronounced inflammatory response than TLR7 agonists and at higher concentrations induced death in mice. TLR9 agonist-induced inflammatory response was associated with high levels of *Icam1* expression and heightened levels of TNF, IL-1 $\beta$  and IL-6. The difference between TLR7 and TLR9 agonists does not appear to be due to specific activation of astrocytes or microglia as these cell types responded similarly *in vitro*. Instead, other cell types such as ependymal cells and endothelial cells may be responsible for the heightened immune response. In addition, this study identified an interaction between TLR7 and TLR9 agonists and receptors in regulating neuroinflammatory responses.

Intracerebroventricular inoculation of TLR9 agonist induced severe clinical signs and/or death in newborn mice depending on concentration (Fig. 4.1) (Pedras-Vasconcelos et al., 2006; Pedras-Vasconcelos et al., 2008). Potentially, the severity of symptoms following TLR9 agonist inoculation could be attributed to the high production of TNF or other cytokines following TLR9

stimulation, not seen with TLR7 stimulation (Fig. 4.1). High levels of TNF expression are associated with meningococcal meningitis as well as septic shock (Pedras-Vasconcelos et al., 2006). TNF has been suggested to play a role in endothelial damage, vasodilatation and capillary leakage (Pedras-Vasconcelos et al., 2006). Similar events may occur following the upregulation of TNF following CpG-ODN inoculation which lead to the death of the neonatal mice.

The proinflammatory cytokines IL6, IL1 $\beta$ , and IL1 $\alpha$  were also produced at higher levels following CpG-ODN inoculation in the brain. High expression levels of IL6, IL1 $\beta$  are also associated with meningococcal meningitis as well as septic shock (Pedras-Vasconcelos et al., 2006). High expression of IL6 is also associated with streptococcal meningitis (Marby et al., 2001). IL1 $\alpha$  expression is increased in neonatal sepsis and meningitis (Fida et al., 2006). The role of these cytokines as well as TNF in CpG-ODN induced meningitis and death can be studied by inoculating CpG-ODN, at a concentration that induced clinical signs/death in the wild-type mice, intracerebroventricularly in mice deficient in TNF or other cytokines, and analyze whether the absence of these cytokines protects these mice from TLR9 agonist inoculation.

One possible reason for the differences between imiquimod and CpG-ODN-induced neuroinflammation could be the expression of adhesion molecules that could lead to infiltration of peripheral immune cells into the brain. *Icam1* mRNA was upregulated to a higher level by CpG-ODN stimulation compared to imiquimod stimulation in the whole brain tissue (Fig. 4.6D) and also in the meningeal (Fig. 4.12) and microglial cell cultures (Fig. 5.7). *Icam1* is often upregulated in the brain following infection and is involved in the recruitment of inflammatory cells to the CNS (Drescher et al., 2002; Lopez et al., 1999; Wells et al., 2001). The proinflammatory cytokines, such as TNF and IL-1 $\beta$  were shown to markedly upregulate *Icam1* expression on the luminal surface of endothelial cells and increases the blood-brain barrier

permeability (Brabers and Nottet, 2006). The higher levels of proinflammatory cytokines secreted by CpG-ODN stimulation in the brain might have increased *Icam1* expression in the meninges and endothelial cells leading to cellular infiltration into the brain, causing more pronounced meningeal inflammation.

Although there were major differences between imiquimod and CpG-ODN stimulation in the neonatal brain, pronounced differences were not observed between imiquimod and CpG-ODN stimulation in astrocyte, microglia or meningeal cultures (Fig 5.8, 5.9, 4.12). Similar results were also observed in B cells, with no differences between TLR7 and TLR9 stimulation (Hanten et al., 2008). The cells in culture might be in an activated state and respond to these agonists similarly. One other possible reason is that the agonists may not be in direct contact with all different cell types in the brain and the spread of agonists is area specific, whereas all the cells in culture will be activated simultaneously. Additionally, the direct interactions between different cell types in the brain might alter responses between the agonist stimulations, or the cytokines secreted by one cell type might alter the responses of other cell types. The effect of one cell type on the other cell types, by direct contact or by secretion of cytokines, need to be investigated in co-culture studies. Although there were no major differences between two agonist stimulations, notable differences were observed between different cell types with astrocytes producing only proinflammatory cytokines, whereas the microglia producing both proinflammatory and anti inflammatory cytokines. The difference in cytokine production by astrocytes and microglia might have practical implications for disease. The anti-inflammatory cytokines produced by microglia might keep the balance in proinflammatory responses induced by both astrocytes and microglia, and prevent damage in the brain during pathogen infections.

Imiquimod inhibited CpG-ODN-induced innate immune responses both *in vitro* and *in vivo*. This supports previous studies demonstrating inhibition of CpG-ODN- induced IFN $\alpha$

production from plasmacytoid dendritic cells and B cells, following co-stimulation with natural and synthetic TLR7 ligands (Berghofer et al., 2007; Marshall et al., 2007). However, the mechanism behind this suppression was not known. In the current study, this inhibition was surprisingly not mediated by TLR7, as deficiency in TLR7 did not alter suppression of the TLR9 agonist-induced responses both in the glial cells and also in the neonatal brain. Our current results also indicate that the mechanism behind this suppression is not due to the inhibition of TLR9 agonist uptake. Possibly, imiquimod may directly interact with CpG-ODN and prevent its binding to TLR9, or imiquimod may directly interact with TLR9 and block the receptor signaling, thus inhibiting the binding of CpG-ODN to TLR9. This possible mechanism might be studied by over-expressing TLR9 in the cells and analyzing the inhibitory effects of imiquimod on CpG-ODN induced cytokine production. If the imiquimod directly binds to CpG-ODN and inhibit CpG-ODN induced responses, then the over-expression of TLR9 should not enhance the CpG-ODN induced responses. However, if imiquimod blocks TLR9 receptor, over-expression of TLR9 should reverse the inhibitory effects of imiquimod, bringing the CpG-ODN induced responses to the level in wild-type cells.

Co-stimulation of neurons with TLR7 and TLR9 agonists killed the neurons but not by individual agonists. Neither TLR7 nor TLR9 agonist alone affected neuronal survival, which is similar to previous reports (Iliev et al., 2004; Ma et al., 2006). Neuronal death following co-stimulation was surprising as co-stimulation with TLR7 and TLR9 agonists did not induce cell death in either astrocytes or microglia. These results are very interesting and need further investigation. The combination of TLR7 and TLR9 agonists might have altered the binding of CpG-ODN to the TLR9 receptor and induced an altered response that lead to cell death. Alternatively, the combination of CpG-ODN and imiquimod may have activated TLR8, as TLR8 activation can induce caspase 3-induced death in neurons. Oligonucleotides have been shown to enhance binding of

TLR7/8 agonists to the TLR8 receptor, while inhibiting signaling to TLR7 in HEK cells. These potential alterations in receptor binding can be studied utilizing neurons from TLR7, TLR9 and Unc93b1 knockout mice to examine which receptors mediate this response.

The present study also determined the cross-regulatory capabilities of the receptors *in vitro* and *in vivo*. An increase in cytokine production was observed in TLR7-deficient microglia stimulated with CpG-ODN compared to wild-type microglia. An antagonistic relationship between TLR7 and TLR9 was observed with only few cytokines in the neonatal brain, including IL6 and CXCL1. Possibly, there is a distinct interaction between TLR7 and TLR9 regulation in different cell types. One of the predicted mechanism for these interactions is the generation of heterodimers between TLR7 and TLR9 that could inhibit or enhance MyD88 binding and the signal transduction cascade (O'Neill and Bowie, 2007). However, it is difficult to demonstrate whether TLR7 and TLR9 can directly interact each other and form heterodimers at molecular level in astrocytes and microglia in their native state as these receptors are expressed at very low level. Direct interaction between these receptors might be studied by either co-immunoprecipitation and western blot studies, or mass spectrometry analysis of co-immunoprecipitates. However, the lack of good antibodies that do not cross-react with other proteins makes it difficult to analyze if these two receptors directly interact with each other. This can potentially be overcome by expressing HA tagged or FLAG tagged TLR7 and TLR9 in astrocytes and microglia and study the interactions by co-immunoprecipitation or mass spectrometry analysis utilizing anti-HA antibodies.

It is possible that these receptors might compete for some of the downstream signaling molecules or other proteins, rather than direct receptor interactions. Recent studies demonstrated that both TLR7 and TLR9 require the Ubiquitin protein, Unc93b1, for their transportation from endoplasmic reticulum to endolysosomes and compete for binding to Unc93b1 (Fukui et al.,



2009). Possibly, in the absence of TLR7, more TLR9 might bind to Unc93b1 and be transported to the endolysosomes. MyD88, the signal transduction molecule associated with both TLR7 and TLR9, is another possible protein for which these two receptors may compete. In the absence of either TLR7 or TLR9, Unc93b1 or MyD88 might be more accessible by single receptor and thus enhance downstream signaling. It would be interesting to study if the expression level of Unc93b1 or MyD88 differ between cell types. This would potentially explain why there is a difference between astrocytes and microglia in TLR7 regulation of TLR9-induced responses.

The present study has clinical relevance due to the current focus of TLR7 and TLR9 agonists as immunomodulatory compounds for treatment of a wide variety of illnesses from cancer to viral infections (Murad et al., 2007; Wang et al., 2005; Wille-Reece et al., 2005; Zaks et al., 2006). TLR7 agonists induced substantial pro-inflammatory responses in the CNS in terms of cellular activation and production of proinflammatory cytokines and chemokines, without inducing overt damage. TLR9 agonists induced substantially higher pro-inflammatory responses in the CNS compared to TLR7 agonists in a dose dependent manner. Co-stimulation of TLR9 with TLR7 agonists reduced the responses in the CNS. Utilizing TLR agonists as immune response modifiers in the CNS is a double edged sword, as the higher induction of neuroinflammation can cause neuronal damage and death. Utilizing a combination of TLR7 and TLR9 agonists as immune response modifiers might help in keeping the pro-inflammatory responses in the CNS in balance. These studies further need investigation in terms of infection or immunity against specific pathogens or conditions affecting brain.

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**APPENDIX-A  
COMMONLY USED ABBREVIATION**

ANOVA	Analysis of Variance
BBB	Blood-brain barrier
CCL2	Chemokine (C-C motif) ligand 2 / MCP-1 (monocyte chemo attractant protein1)
CCL3	Chemokine (C-C motif) ligand 3 / MIP-1a (Macrophage Inflammatory Protein 1 alpha)
CCL4	Chemokine (C-C motif) ligand 4 / MIP-1b (Macrophage Inflammatory Protein 1 beta)
CCL5	Chemokine (C-C motif) ligand 5 / RANTES
CCL12	Chemokine (C-C motif) ligand 12 / MCP-5 (Monocyte chemo attractant protein5)
cDNA	Complementary DNA
Ct	Cycle threshold
CXCL1	Chemokine (C-X-C motif) ligand 1 / KC
CXCL9	Chemokine (C-X-C motif) ligand 1 / MIG (Monokine induced by gamma interferon)
CXCL10	Chemokine (C-X-C motif) ligand 1 / IP10 (Interferon gamma inducible protein -10)
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
ELISA	Enzyme-linked immunosorbent assay
g	grams or radial centrifugal force
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte monocyte colony stimulating factor

h	Hours(s)
HEK	Human embryonic kidney
hpi	Hours post-inoculation
hps	Hours post-stimulation
IBA-1	Ionized binding calcium adaptor molecule-1
ICAM1	Intercellular adhesion molecule 1
i.c.v.	Intracerebroventricular
IFN $\beta$	Interferon beta
IL	Interleukin
IP	Intraperitoneal
IRW	Inbred Rocky mountain White
KO	Knock out
LPS	Lipopolysaccharide
mg	milligrams
NK	Natural killer cells
ODN	Oligo deoxynucleotides
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Reverse transcription
SEM	Standard error of mean

SD	Standard deviation
ssRNA	Single stranded ribonucleic acid
dsRNA	Double stranded ribonucleic acid
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

**APPENDIX-B  
LETTERS OF PERMISSION**

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Oxford OX4 2DQ  
UK  
Tel: +44 (0) 1865 476149  
Fax: +44 (0) 1865 471158  
Email: [katie.wade@wiley.com](mailto:katie.wade@wiley.com)

## VITA

Niranjan B Butchi was born to Mr. Narayana Butchi and Mrs. Jhansi Lakshmi Bai Butchi in Guntur, Andhra Pradesh, India, in 1977. Niranjan graduated with Bachelor of Veterinary Science and Animal Husbandry (B.V.Sc. & A.H.) degree in 2001 from College of Veterinary Science, Acharya N.G. Ranga Agricultural University, Hyderabad, India. He then went to the College of Veterinary Science, Tirupati, India, where he earned a Master of Veterinary Science (M.V.Sc.) degree in veterinary microbiology in 2003. In August 2006, He graduated with Master of Science from the Diagnostic Medicine and Pathobiology, School of Veterinary Medicine, Kansas State University, Manhattan, Kansas. He was accepted into the doctoral program in the Department of Pathobiological Sciences, School of Veterinary Medicine at Louisiana State University, Baton Rouge, Louisiana, under the mentorship of Assistant Professor Dr. Karin E Peterson, who later moved to Rocky Mountain Laboratories, LPVD, NIAID, National Institute of health, Hamilton, Montana, as a chair of Neuroimmunology unit. Niranjan completed his research project for the doctoral degree at Rocky Mountain Laboratories with Dr. Peterson. He will receive his Doctor of Philosophy degree (Ph.D.) in veterinary medical sciences in the Fall commencement, 2009. After graduation, Niranjan will pursue a post-doctoral fellowship in immunology field with the future goal of establishing his own independent research program.