Research

Expression and distribution of Toll-like receptors 11–13 in the brain during murine neurocysticercosis

Bibhuti B Mishra†, Uma Mahesh Gundra† and Judy M Teale*

Address: Department of Biology, South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, One UTSA Circle, San Antonio, Texas 78249-1644, USA

Email: Bibhuti B Mishra - bibhuti.mishra@utsa.edu; Uma Mahesh Gundra - Umamahesh.gundra@utsa.edu; Judy M Teale* - judy.teale@utsa.edu

* Corresponding author   †Equal contributors

Abstract

The functions of Toll-like receptors (TLRs) 11–13 in central nervous system (CNS) infections are currently unknown. Using a murine model of neurocysticercosis, we investigated the expression and distribution of TLRs 11–13 by using both gene specific real-time PCR analysis and *in situ* immunofluoresence microscopy in both control and neurocysticercosis brains. In the mock infected brain, mRNAs of TLRs 11–13 were constitutively expressed. Parasite infection caused an increase of both mRNAs and protein levels of all three TLRs by several fold. All three TLR proteins were present in both CNS and immune cell types. Among them TLR13 was expressed the most in terms of number of positive cells and brain areas expressing it, followed by TLR11 and TLR12 respectively. Among the nervous tissue cells, TLRs 11–13 protein levels appeared highest in neurons. However, TLR13 expression was also present in ependymal cells, endothelial cells of pial blood vessels, and astrocytes. In contrast, infiltrating CD11b and CD11c positive myeloid cells predominantly produced TLR11 protein, particularly early during infection at 1 wk post infection (~50% cells). TLRs 12 and 13 proteins were present on approximately 5% of infiltrating immune cells. The infiltrating cells positive for TLRs 11–13 were mostly of myeloid origin, CD11b+ cells. This report provides a comprehensive analysis of the expression of TLRs 11–13 in normal and parasite infected mouse brains and suggests a role for them in CNS infections.

Background

Neurocysticercosis (NCC) is the most common parasitic disease of the central nervous system (CNS) caused by the larvae of *Taenia solium* [1]. This disease is a public health problem in many third world and developing countries [1-3]. The symptomatic phase of the disease includes clinical signs such as epilepsy [2], increased intracranial (i.c.) pressure, obstructive hydroencephalus, stroke, and encephalitis [1,4]. Autopsy specimens of symptomatic patients reveal evidence of inflammation consisting of a chronic granulomatous reaction [4]. The observed inflammation probably is detrimental. Considering the CNS is devoid of a classically defined lymphatic system, the innate immune response might play a major role in this process.

Toll-like receptors are key host molecules in innate immune responses during infections [5]. To date, thirteen mammalian TLR paralogues have been identified (10 in humans and 12 in mice) [6]. These receptors are highly conserved proteins that recognize distinct mutation resistant molecular patterns common to pathogens, termed

pathogen-associated molecular patterns (PAMPs) [7,8]. Ligand recognition by TLRs culminates invariably in the manifestation of inflammatory responses and induction of adaptive immune responses [9,10]. Growing evidence suggests that TLRs 2, 3, 4, and 9 participate in host immune responses in a variety of CNS diseases [11-23]. Furthermore, recent studies have shown that several nervous tissue cells upregulate particular TLRs as a result of infection, trauma, or autoimmune disease [24-29]. However, little information is available for TLR functions in NCC; indeed, the expression profile of TLRs 11–13 is unknown in any CNS disease.

In an experimental murine model for NCC, mice receive intracranial (i.c.) inoculations of *Mesocestoides corti* (*M. corti*) metacestodes. The brain immune response in this model is associated with a predominant TH1 pathway of cytokine responses [30,31]. Analysis of the expressions and distributions of TLRs 1–9 suggested that *M. corti* parasite infection increased both gene expression and protein levels of each TLRs1–9 several fold except TLR5 where only the mRNA was upregulated [12]. In addition, these TLRs were differentially distributed among various CNS cell types and infiltrating leukocytes. In this study, we preformed gene specific Real-time polymerase chain reaction (RT-PCR) analysis to detect TLRs 11–13 at the mRNA level for both infected and mock-infected mice. *In situ* immunofluoresence (IF) microscopy, using antibodies specific for each of the TLRs in combination with antibodies for distinct cell surface markers, determined the expression of TLRs by particular cell types in infected and uninfected brains. The data obtained from these two approaches implicate TLRs 11–13 in host immune surveillance in the CNS, particularly in NCC.

Methods
Mice
Female Balb/c mice used in this study were purchased from the National Cancer Institute Animal Program (Bethesda, MD). Experiments were conducted under the guidelines of the IACUC, UTSA, University of Texas System, the US Department of Agriculture, and the National Institutes of Health.

Murine model of neurocysticercosis
In this study we used a well characterized mouse model of NCC developed in our laboratory [30,32]. Briefly, we maintained larvae of *M. corti* parasites by serial, intraperitoneal (i.p.) inoculations of 6–8 wk old female Balb/c mice. We harvested the larvae aseptically and induced murine NCC by intracranial injections of 50 μl of Hank's Buffered Salt Solution (HBSS) containing approximately 40 parasites into 3–5 week old mice under short term anesthesia. Mock infected control mice were injected by

the intracranial route with 50 μl sterile HBSS using the same protocol.

RNA isolation and real-time PCR analysis
To determine the CNS gene expression of TLRs 11–13 in murine NCC, brains were removed from infected and mock infected control mice at 1, 3, 6, and 10 wk post infection (p.i.) (n = 3 in each group). Animals were perfused prior to sacrifice to avoid RNA contamination from blood cells. Briefly, mice were anaesthetized with 100 μl of mouse cocktail containing 100 mg/ml of ketamine and 20 mg/ml of rompum (Laboratory animal resource, UTHSCSA, TX) and perfused with 10 ml of PBS, pH 7.4 through the left ventricle. Brains were immediately removed after perfusion and total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturers' instructions. Real-time PCR analysis of these samples, using SYBR green as the detection dye, measured the expression levels of TLR-specific mRNAs. One microgram of total RNA from either infected or mock infected mice was reverse transcribed into cDNA by using a high capacity cDNA reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, CA, USA). Transcript levels of the housekeeping ribosomal 18S and TLRs 11–13 were PCR amplified in each sample by using specific primers (Advanced Nucleic Acids Core Facility, UTHSCSA, TX). Sequences of the specific primers used for 18S and TLRs 11–13 are as follows: 18S (sense) 5'-CATGTGGTGTTGAGGAAAGCA-3' and (anti sense) 5'-GTCGTGGGTTCTGCATGATG-3'; TLR11 (sense) 5'-TCCTTCCTCTGATTAGCTGTCCTAA-3' and (antisense) 5'-TCCACATAATTTCCACCAACAAGT-3'; TLR12 (sense) 5'-GCCGCCATTCCAAGCTA TC-3' and (antisense) 5'-CTCCACAGTCCGAGGTACAACTT-3'; and TLR13 (sense) 5'-ATGGCACAAAACGGAGAAGAA-3' and (antisense) 5'-CTTTGTATACCCATGCCTCATCAG-3'. The target expression levels were normalized to levels of the house keeping 18S in the same sample. The normalized values give the relative abundance of mRNA for each TLR gene.

Antibodies
Anti-mouse antibodies were purchased from the following companies: TLR11 from Serotec (Raleigh, NC), TLRs 12–13 from Imgenex (San Diego, CA.), biotinylated or R-Phycoerythrin (PE) conjugated CD11b [myeloid cells] from Pharmingen (San Diego, CA.), and Biotinylated Neuronal nuclear protein [Neun] from US Biological (Swampscott, MA.). Anti-mouse glial fibrillary protein [GFAP] (Pharmingen) was conjugated to Alexa Fluor 488 (Molecular Probes) according to manufacturers' instructions. For indirect immunofluoresence, rabbit anti-TLRs 11, 12, and 13 followed by Alexa 488 conjugated (green) chicken anti-rabbit (Molecular Probes) or Rhodamine red-conjugated (red) goat anti-rabbit secondary antibodies (Jackson laboratory) were used. Biotinylated primary

antibodies were detected using Alexa Fluor 488-labeled streptavidin (Molecular Probes).

Immunofluorescence

We used *in situ* immunofluorescence (IF) to detect tissue expression and distribution of TLRs in the CNS. Parasite infected brains were analyzed at various times post infection (1 wk, 3 wk, and 6 wk) and compared to mock-infected brains from control animals. Brains were immediately removed after perfusion (as described above), embedded in optimal cutting temperature (OCT) medium, and snap frozen as previously described [12,33]. Serial horizontal cryosections, 10 μm in thickness, were placed on silane prep slides (Sigma Biosciences, St. Louis, Mo). One in every five slides was fixed in formalin for 12 min at room temperature and stained with hematoxylin and eosin. The remaining slides were air dried overnight and fixed in fresh acetone for 20s at room temperature. Acetone-fixed sections were wrapped in aluminum foil and stored at -80°C or processed immediately for IF.

IF analysis determined the location of individual TLRs in brain sections. All steps were carried out at room temperature. Sections were incubated with TLR-specific primary antibodies in PBS buffer with 3% host serum to prevent non-specific binding. After 1 hr, sections were washed seven times for 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20 and incubated with appropriate secondary antibodies for 30 min. Sections were then washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20. The above mentioned procedures were sequentially repeated for double IF staining. The sections were mounted using FluorSave reagent (Calbiochem, La Jolla, CA) containing 0.3 μM 4', 6'-diamidino-2-pheny-lidole (DAPI)-diacetate (Molecular Probes). Additional control staining was performed to rule out any nonspecific staining. In each case, sections were blocked with saturating concentrations of appropriate host serum antibodies to eliminate false positive staining due to FcR-mediated nonspecific binding. Staining in the absence of primary antibodies provided additional negative controls.

Quantification of TLRs 11–13 protein expression

Sections of brains from mock-infected and NCC mice were stained with specific anti-TLR antibodies followed by labeled secondary antibodies to quantify the differences in TLR 11–13 protein levels. Images at 20× magnification of a number of brain areas from the entire brain of each mock infected and NCC animal were taken using identical camera settings so that the number and intensity of pixels would reflect the differences in their protein expression. The atlas of the mouse brain and spinal cord book by Sidman RL [34] provided neuroanatomy guidance to select identical brain areas for analysis. Protein level of a particular TLR was determined by measuring the area (number

of pixels) and fluorescent intensity (average intensity of pixels) of the staining from twenty four images captured randomly in the CNS parenchyma from each mock and parasite infected brain exhibiting TLR protein staining on nervous tissue cells. The unit area of each image was 0.172 sq. mm. Analysis was performed by using the IP Lab 4.0 imaging software (BD Biosciences Bioimaging, Rockville MD). The relative TLR protein expression was calculated by multiplying the number of pixels (area) by the average intensity of pixels.

Statistical analysis

We used the Student's *t*-test for comparison of means of different groups [SIGMA PLOT 8.0 (Systat Software, San Jose, CA)]. A *P* value less than 0.05 was considered to be statistically significant.

Results

mRNA expression of TLRs 11–13 in normal and infected brain tissue

Quantitaive real-time PCR analysis was performed to determine the expression profile of TLRs 11–13 in mock infected and parasite infected brain. Constitutive mRNA expression was present for each TLR with a relatively
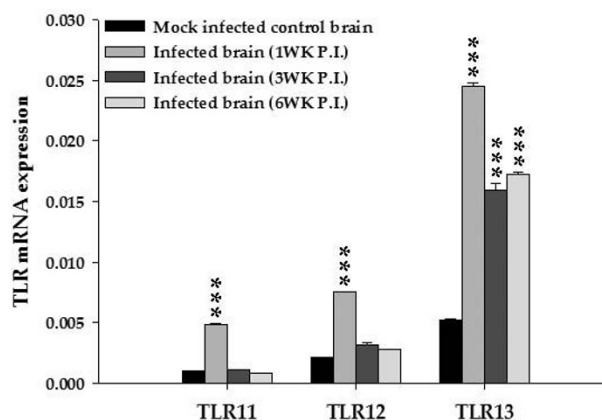
Figure 1
***M. corti* infection elevates mRNA expression of TLRs 11–13 in brain**. Total RNA was isolated from infected and mock infected control mouse brains at 1 wk, 3 wk, and 6 wk p.i. using Trizol reagent. Isolated RNA was reversed transcribed to cDNA by using random primers. Levels of TLR specific RNA and housekeeping gene 18S in these samples were measured by Real Time PCR analysis as described below using SYBR green as the detection dye. TLR specific mRNA levels were normalized to the mRNA level of the housekeeping gene 18S in the same sample and expressed in arbitrary units. The normalized values corresponding to the mRNA expression for each TLR gene are shown in the y-axis and represent the mean of three independent experiments. The data obtained were compared using a Student's *t*-test.

higher level of TLR13 gene expression. (Fig. 1). Upon infection, gene expression was significantly upregulated at 1 wk p.i. (P < 0.001). At 3 wk p.i., mRNAs of TLR11 and TLR12 almost returned to normal baseline levels and remained so at 6 wk p.i. (Fig. 1) and 10 wk p.i. (data not shown). In contrast, gene expression of TLR13 was significantly upregulated at all the post infection times tested. This upregulation was greatest at 1 wk p.i. with a relatively lower level at later stages (3 wk, 6 wk) (Fig. 1) and 10 wk (data not shown). Among TLRs 11–13, expression of TLR13 was the highest in both normal and parasitic infected brains and remained at an elevated level throughout infection (Fig. 1). These results indicate that TLR 11–13 mRNAs are expressed in normal, uninfected mouse brains and their levels of expression increase during parasite infection.

Expression and distribution of TLR 11–13 proteins among nervous tissue and infiltrating immune cell types

In situ IF microscopy analyses revealed the distribution of TLR 11–13 proteins in brain tissues from mock infected control mice and NCC mice. Brain tissue cells were determined by their characteristic morphology together with cell markers: microglia (CD11b), astrocytes (GFAP), and neurons (NeuN). Surface markers helped identify infiltrating cells: macrophages/microglia cells (CD11b), dendritic cells (CD11c), B-cells (CD19), and αβ T cells (TCR β chain). Brain tissues were analyzed at 1 wk, 3 wk, and 6 wk p.i. At least three mice were analyzed per time point with reproducible results. TLRs 11–13 staining was found to be upregulated in the CNS as a result of infection (Fig. 2, 3, 4).

TLR11

TLR11 IF staining was evident in brain tissue cells of both uninfected (Fig. 2A and 2E) and infected mice (Fig. 2B, C, D and 2F), and in infiltrating immune cells during parasite infection (Fig 2H). In the uninfected animals, TLR11 expression was detected at a low basal level mostly in neurons as shown in red color in the insert of a portion of the frame in Fig. 2E (colliculus posterior). The positive staining was present in the frontal cortex, anterior-posterior and external colliculi. In addition, TLR11 expression in neurons was visible in the stratum pyramidale hippocampi (CA1 and CA2), but not in CA3 neurons. At 1 wk p.i., TLR11 protein levels increased in the above mentioned brain areas (Fig. 2B, colliculus posterior), and further increased by 3 wk p.i (Fig. 2C). However, at 6 wk p.i., TLR11 protein levels decreased (Fig. 2D) to levels similar to 1 wk p.i. (Fig. 2B). Morphological, anatomical correlation, and colocalization of TLR11 and Neun staining strongly suggested that the upregulated TLR11 expression was mainly in neurons (Fig. 2F). We quantified protein expression by measuring the mean pixel intensity. Figure 2l shows upregulated TLR11 protein expression in parasite infected brain at 3 wk p.i. Staining, however, was undetected in the nervous tissue cells present in the cerebellum and periventricular and leptomeningeal (LM) areas in either uninfected or in parasite infected NCC mice.

The highest number of infiltrating cells expressed TLR11 in infected brain tissue. Immunofluorescent staining detected no TLR11 infiltrating cells in mock infected mouse brain (Fig. 2G). At 1 wk p.i. about half of the immune cells present in meninges and ventricles were positive for TLR11 protein (Fig. 2H, third ventricle). However, as the length of time p.i. increased, the number of TLR11 positive leukocytes progressively declined. At 3 wk p.i. only a small number of infiltrating cells (~5%) expressed TLR11 protein. At 6 wk p.i., TLR11 positive infiltrating cells were barely present. This is in contrast to the maximal staining observed for TLR11 in nervous tissue cells at 3 wk in NCC mice. Double IF analysis with anti-TLR11 and antibodies to cell surface markers specific for various immune cells indicated that the majority of TLR11 positive cells were CD11b positive myeloid cells (~90%). A few CD11c positive dendritic cells (approximately 5%) were also present (data not shown). However, as microglia also express both CD11b and CD11c, it is possible that some of these TLR11 positive cells detected in the extraparencymal areas of CNS can be migrating microglia as well.

TLR12

TLR12 expression was evident in brain tissue cells of both uninfected (Fig. 3A and 3E) and infected mice (Fig. 3B, C, D and 3F). The expression in nervous tissue was generally similar to TLR11 expression with respect to cell types and their location. However, TLR12 protein levels in brain areas as well as the number of positive cells in a particular area appeared to be less than TLR11. TLR12 positive cells in normal or mock-infected animals were fewer in number and displayed only low levels of protein (Fig. 3A &3E). The number of TLR12 positive nervous tissue cells increased at 1 wk p.i. (Fig. 3B). These induced effects were even more pronounced and appeared to reach a maximal expression level at 3 wk p.i. (Fig. 3C and Fig. 3F, yellow/orange). At 6 wk p.i. TLR12 protein levels, measured by both intensity of staining and number of positive cells, substantially decreased (Fig. 3D). Measurement of mean pixel intensity of TLR12 staining confirmed that parasite infection increased expression of TLR12 protein at 3 wk p.i. in the whole brain (Fig. 3G) as compared to the controls.

Similar to TLR11, among nervous tissue cells, TLR12 staining was abundant in neurons (Fig. 3E, red in the insert e' &3F), but not in astrocytes and ependymal cells (data not shown). In addition, TLR12 staining was present
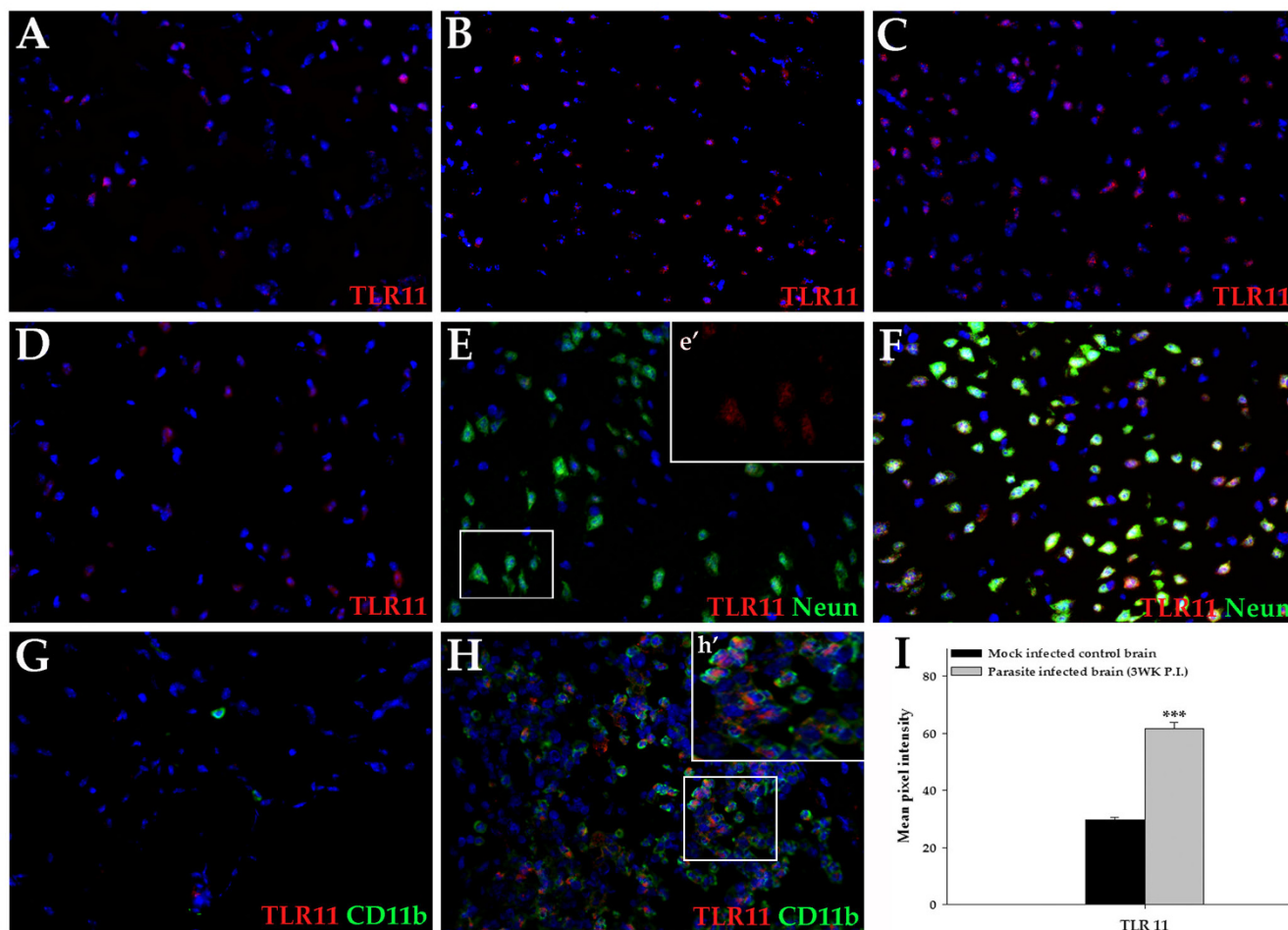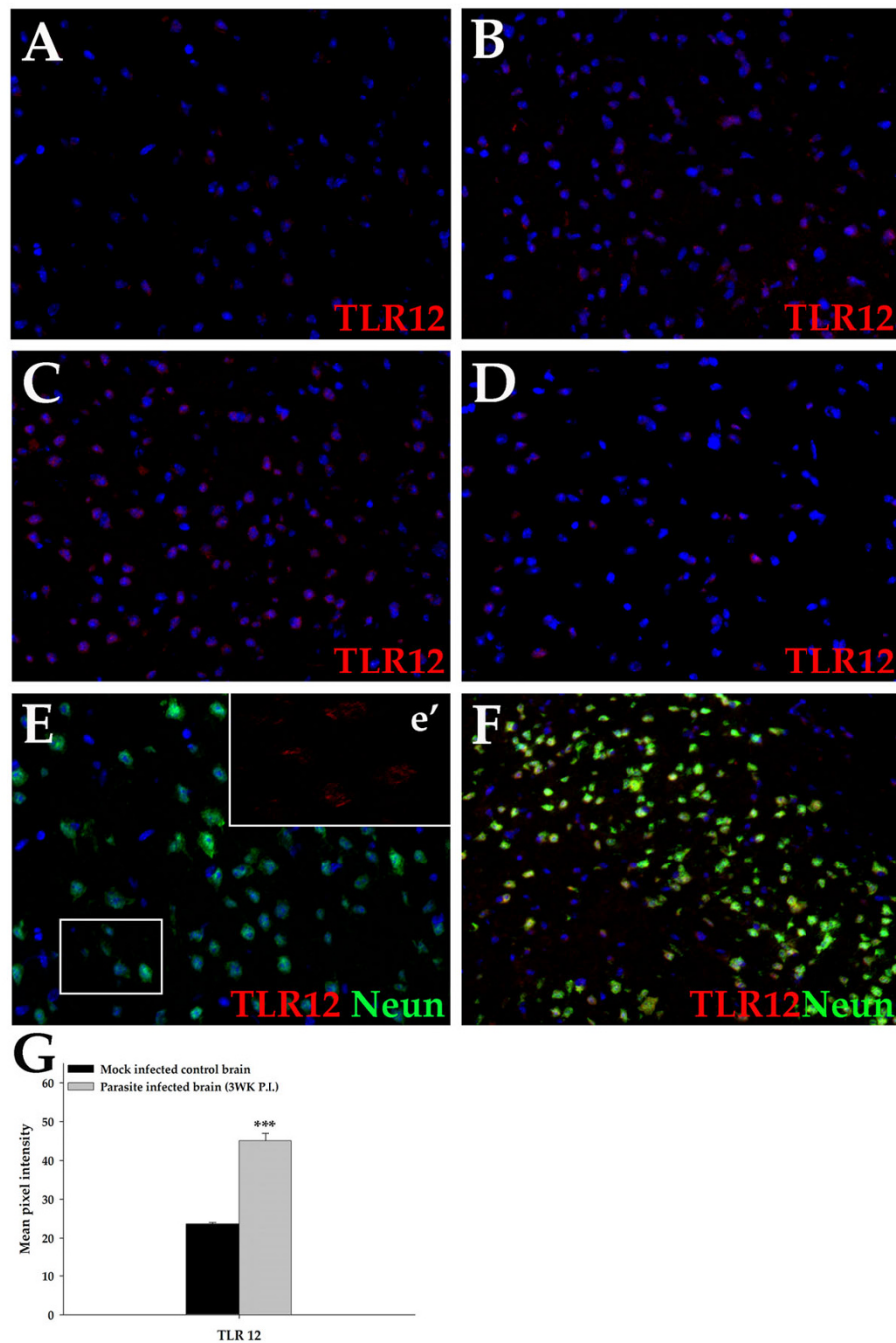
Figure 2
Expression of TLR11 in brains of mock infected control and NCC mice analyzed by immunofluorescence microscopy. Balb/c mice were infected intracranially with *M. corti* and sacrificed at various times p.i. Brain crysections were analyzed for expression of TLR11 and cell specific markers using fluorochrome conjugated antibodies. Nuclear staining DAPI is blue. (A) Mock infected control stained with TLR11 specific antibody (400×). (B) *M. corti* infection at 1 wk showing TLR11 positive staining in neurons (400×). (C) *M. corti* infection at 3 wk when maximum TLR11 expression was observed (400×). (D) *M. corti* infection at 6 wk showing TLR11 positive staining (400×). (E) Mock infected control mice stained with TLR11 and neuron specific antibody (Neun) (400×). The insert e' represents a 2× magnification of a selected area depicting TLR11 expression (Red) on neurons. (F) *M. corti* infection at 3 wk showing TLR11 positive neurons, merged image (yellow/orange; 400×). (G) Mock control mice stained with anti-TLR11 and anti-CD11b antibody (400×). (H) *M. corti* infection at 1 wk showing TLR11 and CD11b double positive cells, merged image (yellow/orange; 400×). Insert h' is a 2× magnification of a selected area to better illustrate TLR11 and CD11b double positive staining (I) The relative levels of TLR11 expression in mock and *M. corti* infected animals was calculated as described in experimental procedures.

in brain areas such as the anterior-posterior and external colliculi and pyramidal neurons of the hippocampus (CA1 and CA2). However, their pattern of expression was different in infiltrating immune cells where only a few immune cells (approximately 5%) were positive for TLR12. The TLR12 positive infiltrating cells were mostly CD11b positive myeloid cells (data not shown). Taken together, the constitutive expression of TLR12 in normal brain and its increased expression during NCC suggest a possible role in CNS function.

TLR13
TLR13 expression was evident in brain tissue cells of both uninfected (Fig. 4A and 4E) and infected mice (Fig. 4B, C, D, 4F and 4G). TLR13 staining was predominantly in neuronal cells of both uninfected and infected mice (Fig. 4A–G). In mock infected/normal mouse brain, TLR13 was present in some astrocytes in periventricular areas and in proximity to the meninges (data not shown). However, a much greater number of neurons expressed TLR13 in these brains (Fig. 4A and 4E, red in the insert e'). In nor-

Figure 3
Expression of TLR12 in brains of mock infected control and NCC mice analyzed by immunofluorescence microscopy. Balb/c mice were infected intracranially with *M. corti* and sacrificed at various times p.i. Brain cryosections were analyzed for expression of TLR12 and cell specific markers using fluorochrome conjugated antibodies. Nuclear staining DAPI is blue. (A) Mock infected control stained with TLR12 specific antibody (400×). (B) *M. corti* infection at 1 wk showing TLR12 positive staining (400×). (C) *M. corti* infection at 3 wk when maximum TLR12 expression was observed (400×). (D) *M. corti* infection at 6 wk showing TLR12 positive staining (400×). (E) Mock control mice stained with TLR12 and neuron specific antibody (Neun) (400×). The insert e' represents a 2× magnification of a selected area depicting TLR12 expression (Red) on neurons. (F) *M. corti* infection at 3 wk showing TLR12 positive neurons, merged image (yellow/orange; 400×). (G) The relative levels of TLR12 expression in mock and *M. corti* infected animals was calculated as described in experimental procedures.
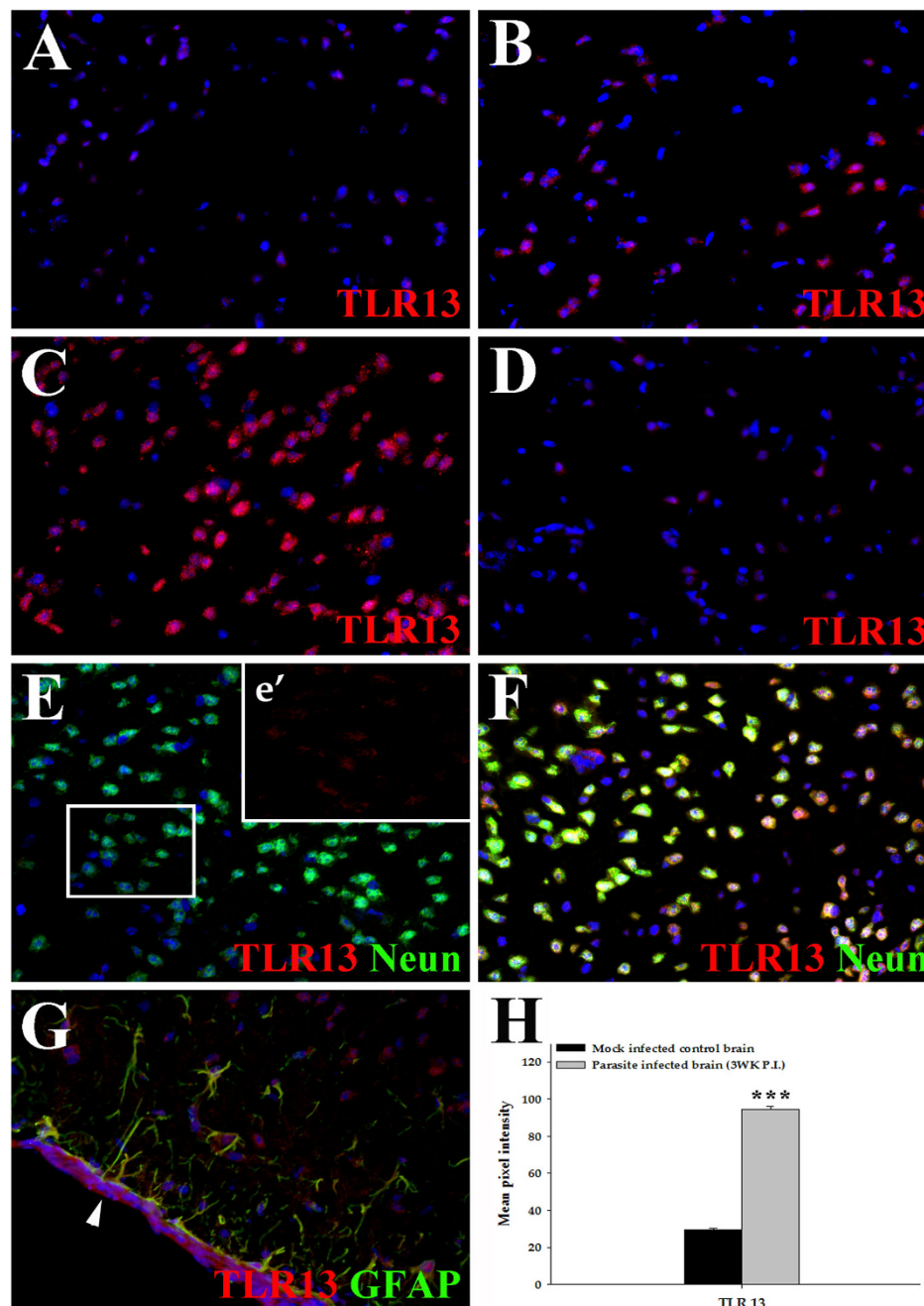
Figure 4
Expression of TLR13 in brains of mock infected control and NCC mice analyzed by immunofluorescence microscopy. Balb/c mice were infected intracranially with *M. corti* and sacrificed at various times p.i. Brain cryosections were analyzed for expression of TLR13 and cell specific markers using fluorochrome conjugated antibodies. Nuclear staining DAPI is blue. (A) Mock infected control stained with TLR13 specific antibody (400×). (B) *M. corti* infection at 1 wk showing TLR13 positive staining in neurons (400×). (C) *M. corti* infection at 3 wk when maximum TLR13 expression was observed (400×). (D) *M. corti* infection at 6 wk showing TLR13 positive staining (400×). (E) Mock control mice stained with TLR13 and neuron specific antibody (Neun) (400×). The insert e' represents a 2× magnification of a selected area depicting TLR13 expression (Red) on neurons. (F) *M. corti* infection at 3 wk showing TLR13 positive neurons, merged image (yellow/orange; 400×). (G) *M. corti* infection at 3 wk showing TLR13 positive astrocytes and its processes, merged image (yellow/orange; 400×). The arrow points to the pial vessel expressing TLR13. (H) The relative levels of TLR13 expression in mock and *M. corti* infected animals was calculated as described in experimental procedures.

mal uninfected animals, a few blood vessels, microglia and ependymal cells were positive for TLR13 (data not shown). The positively stained neurons were in the cerebral cortex adjoining fissura longitudinales, frontal cortex, and anterior-posterior and external colliculi. Astrocytes for TLR13 were solely in the periventricular, and pial and subpial areas. In comparison to TLR11 and 12, TLR13 expression was present in additional areas including periventricular areas, fissure longitudinalis cerebri and CA3 pyramidal neurons of the hippocampus that were negative for TLR11 and TLR12 staining.

Parasite infection produced higher levels of TLR13 protein by several fold (Fig. 4B, C, D, 4F, G and 4H). After reaching maximal expression at 3 wk p.i. (Fig. 3C), levels of TLR13 progressively decreased at 6 wk p.i. (Fig. 4D), but always exhibited elevated levels irrespective of the length of time post infection, even at 10 wk p.i (data not shown). The increased expression of TLR13 at 3 wk p.i. was compared with mock infected mice and expressed as mean pixel intensity (Fig. 4H). TLR13 staining was most evident in nervous tissue cells of infected mice, predominantly in neuronal cells (Fig. 4F). A few infiltrating CD11b myeloid cells were also detected positive for TLR13 (data not shown). In addition to neurons, increased TLR13 expression was present in other CNS cells including astrocytes (Fig 4G), Purkinje cells, ependymal cells and endothelial cells of pial blood vessels (Fig 4G, arrow points to positive pial vessel). A striking increase in TLR13 expression was apparent in terms of both intensity and numbers of astrocytes expressing them. The increased staining in astrocytes was most obvious in periventricular and leptomeningeal white matter areas and in the cerebellar white matter of the brain. In contrast, astrocytes present in parenchymal gray matter areas were not positive for TLR13 staining. The maximum numbers of neurons and astrocytes expressing TLR13 was observed at 3 wk p.i. (Fig. 4G, yellow). Nevertheless, similar to TLR12, TLR13 expression was predominantly in neurons (Fig. 4E and 4F, yellow/orange color in merged image).

Discussion

Historically, the CNS has been regarded as an immune-privileged organ [35]. However, we now recognize that the CNS possesses active immune processes and control mechanisms [36]. Pattern recognition receptors (PRRs) that constitute the recognition system may be involved in altered brain homeostasis and several CNS diseases with diverse causes such as experimental brain injury caused by stereotactic transection of axons in the entorhinal cortex [37], ischemia [38-42], and autoimmune diseases [43]. Signaling through PRRs appears to elicit innate immune responses that eventually culminate in specific adaptive immune responses [44] and could conceivably generate neuroprotective benefits [27]. In this report we studied

the expression and distribution of TLRs 11–13 in the brain under normal physiological conditions and during murine NCC. Previously only one study reported mRNA expression of TLR 13 in the CNS after Semliki Forest Virus (SFV) infection [45]. This is the first report demonstrating the expression of TLRs 11–13 in the CNS following infection.

This study and our previous findings [12] indicate that TLRs 1–13 exhibit differential expression and regulation in normal and NCC mouse brains. TLR2 and TLR13 were expressed at high levels in almost all CNS cell types both in the uninfected and parasite infected brain. However, TLR13 seems to be highly expressed in neurons while TLR2 is more abundant in astrocytes. Nevertheless, high upregulation following infection of both TLR2 and TLR13 in astrocytes and their foot processes that terminate at blood vessels may provide necessary signals for initiation of infiltration of immune cells to the CNS and the subsequent adaptive immune response [46]. This observation could be important in understanding the role of particular TLRs in pathological processes unique to brain tissue, specifically during NCC.

Vertebrate TLRs can be divided into six subfamilies based on the similarity of amino acid composition, extracellular leucine rich repeat (LLR) length and phylogenetic analysis. The TLR2 subfamily includes TLR1, 2, and 6; TLR9 subfamily: TLR7, 8, and 9; TLR11 subfamily: TLR11, 12, and 13 [47]. TLR3, TLR4 and TLR5 subfamilies each consists of a single member [7,47]. Among all the TLRs, very little data detail the functions and mechanisms of action of TLRs 11–13. One major hindrance is the absence of knowledge about their ligands. TLR11 apparently recognizes uropathogenic bacteria [48]. Usually TLRs belonging to a particular family recognize a particular class of ligands, PAMPs, presumably due to selective pressure for specific PAMP recognition. For example, the TLR2 subfamily is specific for lipopeptide, the TLR3 family for dsRNA, the TLR4 family for LPS, the TLR5 family for flagellin, and the TLR7–9 subfamilies for nucleic acid and heme motifs [8,49-52]. A recent report indicated a profilin-like molecule from the protozoan parasite *Toxoplasma gondii* interacts with TLR11 generating a potent interleukin-12 (IL-12) response in murine dendritic cells that is dependent on myeloid differentiation factor 88 [53]. Ligands for the other TLRs suggest that PAMPs for TLRs 11–13 are likely to be protein components. Other studies from our laboratory indicate that *M. corti* metacestodes release carbohydrate containing molecules directly upon invasion whereas other glycoconjugates are secreted throughout the infection process [54-56]. These molecules may potentially act as specific PAMPs that have not yet been described for TLRs. It may be also possible that infections induce cytokines indirectly to stimulate the

production of particular TLR proteins [55-57]. In this light, it is of interest to observe that TLRs 11–13 were detected at a lower level at 6 wk p.i. as compared to 1 wk and 3 wk p.i. In the NCC model, acute infection with *M. corti* is associated with a TH1 response early during infection (1–5 wk). However, after 5 wk p.i., a mixed TH1/2 immune responses along with expression of IL-10 are detected [30]. Such anti-inflammatory responses may contribute to the decreased expression of TLRs 11–13 later in infection helping to diminish inflammation induced tissue damage.

TLRs have a major role in host inflammatory responses. Increasing evidence also indicates that TLRs have a major role in several inflammatory CNS pathologies [58-62]. The critical question is whether TLRs have other roles besides inflammation that may be important in diseases and other CNS functions. In this light, constitutive expression and infection-induced upregulation of particular TLR 11–13 proteins in distinct nervous tissue cell types, especially in neurons is of interest. One possibility is that neurons are able to recognize some forms of infection. Previous studies indicated that neurons express TLR2 [12,63] and TLR3 [45,64]. However, in the latter study, unlike glial cells, neuroblastoma cells could not synthesize IFN-α or pro-inflammatory cytokines upon stimulation. Alternatively, or in addition, perhaps activation of neurons, astrocytes and even endothelial cells of blood vessels [27,64-66], via TLRs 11–13 could augment the neuroprotective functions. Indeed, activation of TLR3 is correlated with the production of neuroprotective, angiogenic and migratory factors by astrocytes [24]. TLR signaling is even involved in adult neurogenesis [67,68].

Although the vertebrate TLRs are not fast-evolving genes, TLRs 11–13 are most divergent compared to other TLRs [47] suggesting possible alternate functions other than recognition of PAMPs. Emerging evidence suggests that TLRs recognize several host molecules during pathological conditions. It is reasonable to expect that TLR recognition and interaction with host molecules might participate in normal homeostatic processes as well. Understanding TLR 11–13 functions in specific CNS cell types and the ligands they recognize should provide insight into their roles in the CNS under normal physiological condition as well as during infection.

Conclusion
In the present study, we showed that TLRs 11–13 are expressed in the CNS and their expression was significantly upregulated during murine NCC. TLR 11–13 proteins were abundantly expressed in neurons of both normal and parasite infected mouse brains. An enhanced understanding of TLR 11–13 functions in specific CNS cell types, particularly in neurons, should provide insight

into their role in initiation of immune reactions and deleterious sequelae or perhaps possible neuroprotective roles in chronic infections, particularly in CNS parasitic infection.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the experiments: BM JT. Performed the experiments: BM UG. Analyzed the data: BM UG JT. Contributed reagents/materials/analysis tools: JT. Wrote the paper: BM UG JT. All authors have read and approved the final manuscript.

Acknowledgements

References
1. White AC: **Neurocysticercosis: Updates on Epidemiology, Pathogenesis, Diagnosis, and Management.** *Annual Review of Medicine* 2000, **51**:187-206.
2. Roman G, Sotelo J, Del Brutto O, Flisser A, Dumas M, Wadia N, Botero D, Cruz M, Garcia H, de Bittencourt PR, Trelles L, Arriagada C, Lorenzana P, Nash TE, Spina-França A: **A proposal to declare neurocysticercosis an international reportable disease.** *Bull World Health Organ* 2000, **78**:399-406.
3. Ostrosky-Zeichner L, Garcia-Mendoza E, Rios C, Sotelo J: **Humoral and cellular immune response within the subarachnoid space of patients with neurocysticercosis.** *Arch Med Res* 1996, **27**:513-517.
4. White AC: **Neurocysticercosis: a major cause of neurological disease worldwide.** *Clinical Infectious Diseases* 1997, **24**:101-113.
5. Akira S, Uematsu S, Takeuchi O: **Pathogen recognition and innate immunity.** *Cell* 2006, **124**:783-801.
6. Beutler B: **Innate immunity: an overview.** *Mol Immunol* 2004, **40**:845-859.
7. Takeda K, Kaisho T, Akira S: **Toll-like receptors.** *Annu Rev Immunol* 2003, **21**:335-376.
8. Aderem A, Ulevitch RJ: **Toll-like receptors in the induction of the innate immune response.** *Nature* 2000, **406**:782-787.
9. Iwasaki A, Medzhitov R: **Toll-like receptor control of the adaptive immune responses.** *Nat Immunol* 2004, **5**:987-995.
10. Takeda K, Akira S: **Toll-like receptors in innate immunity.** *Int Immunol* 2005, **17**:1-14.
11. Glezer I, Simard AR, Rivest S: **Neuroprotective role of the innate immune system by microglia.** *Neuroscience* 2007, **147**:867-883.
12. Mishra BB, Mishra PK, Teale JM: **Expression and distribution of Toll-like receptors in the brain during murine neurocysticercosis.** *J Neuroimmunol* 2006, **181**:46-56.
13. Herrmann I, Kellert M, Schmidt H, Mildner A, Hanisch UK, Bruck W, Prinz M, Nau R: **Streptococcus pneumoniae Infection aggravates experimental autoimmune encephalomyelitis via Toll-like receptor 2.** *Infect Immun* 2006, **74**:4841-4848.
14. Kielian T, Haney A, Mayes PM, Garg S, Esen N: **Toll-like receptor 2 modulates the proinflammatory milieu in Staphylococcus aureus-induced brain abscess.** *Infect Immun* 2005, **73**:7428-7435.
15. Kielian T: **Toll-like receptors in central nervous system glial inflammation and homeostasis.** *J Neurosci Res* 2006, **83**:711-730.
16. Carpentier PA, Duncan DAS, Miller SD: **Glial toll-like receptor signaling in central nervous system infection and autoimmunity.** *Brain, Behavior, and Immunity* 2008, **22**:140-147.
17. Kerfoot SM, Long EM, Hickey MJ, Andonegui G, Lapointe BM, Zanardo RC, Bonder C, James WG, Robbins SM, Kubes P: **TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease.** *J Immunol* 2004, **173**:7070-7077.

18. Lehnardt S, Lehmann S, Kaul D, Tschimmel K, Hoffmann O, Cho S, Krueger C, Nitsch R, Meisel A, Weber JR: **Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia.** *J Neuroimmunol* 2007, **190:**28-33.

19. Stenzel W, Soltek S, Sanchez-Ruiz M, Akira S, Miletic H, Schluter D, Deckert M: **Both TLR2 and TLR4 are required for the effective immune response in Staphylococcus aureus-induced experimental murine brain abscess.** *Am J Pathol* 2008, **172:**132-145.

20. Tanga FY, Nutile-McMenemy N, DeLeo JA: **The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy.** *Proc Natl Acad Sci USA* 2005, **102:**5856-5861.

21. Touil T, Fitzgerald D, Zhang GX, Rostami A, Gran B: **Cutting Edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta.** *J Immunol* 2006, **177:**7505-7509.

22. Turrin NP, Rivest S: **Innate immune reaction in response to seizures: implications for the neuropathology associated with epilepsy.** *Neurobiol Dis* 2004, **16:**321-334.

23. Konat GW, Kielian T, Marriott I: **The role of Toll-like receptors in CNS response to microbial challenge.** *Journal of Neurochemistry* 2006, **99:**1-12.

24. Bsibsi M, Ravid R, Gveric D, van Noort JM: **Broad expression of Toll-like receptors in the human central nervous system.** *J Neuropathol Exp Neurol* 2002, **61:**1013-1021.

25. Crack PJ, Bray PJ: **Toll-like receptors in the brain and their potential roles in neuropathology.** *Immunol Cell Biol* 2007, **85:**476-480.

26. Zekki H, Feinstein DL, Rivest S: **The clinical course of experimental autoimmune encephalomyelitis is associated with a profound and sustained transcriptional activation of the genes encoding toll-like receptor 2 and CD14 in the mouse CNS.** *Brain Pathol* 2002, **12:**308-319.

27. Hanisch UK, Johnson TV, Kipnis J: **Toll-like receptors: roles in neuroprotection?** *Trends Neurosci* 2008, **31:**176-182.

28. Glezer I, Rivest S: **Glucocorticoids: protectors of the brain during innate immune responses.** *Neuroscientist* 2004, **10:**538-552.

29. Sterka D Jr, Rati DM, Marriott I: **Functional expression of NOD2, a novel pattern recognition receptor for bacterial motifs, in primary murine astrocytes.** *Glia* 2006, **53:**322-330.

30. Cardona AE, Restrepo BI, Jaramillo JM, Teale JM: **Development of an animal model for neurocysticercosis: immune response in the central nervous system is characterized by a predominance of gamma delta T cells.** *J Immunol* 1999, **162:**995-1002.

31. Cardona AE, Teale JM: **Gamma/delta T cell-deficient mice exhibit reduced disease severity and decreased inflammatory response in the brain in murine neurocysticercosis.** *J Immunol* 2002, **169:**3163-3171.

32. Cardona AE, Gonzalez PA, Teale JM: **CC chemokines mediate leukocyte trafficking into the central nervous system during murine neurocysticercosis: role of gamma delta T cells in amplification of the host immune response.** *Infect Immun* 2003, **71:**2634-2642.

33. Alvarez JI, Teale JM: **Evidence for differential changes of junctional complex proteins in murine neurocysticercosis dependent upon CNS vasculature.** *Brain Res* 2007, **1169:**98-111.

34. Sidman RL, Angevine JB, Pierce ET: *Atlas of the mouse brain and spinal cord* Cambridge, Mass.,: Harvard University Press; 1971.

35. Ransohoff RM, Kivisakk P, Kidd G: **Three or more routes for leukocyte migration into the central nervous system.** *Nat Rev Immunol* 2003, **3:**569-581.

36. Galea I, Bechmann I, Perry VH: **What is immune privilege (not)?** *Trends Immunol* 2007, **28:**12-18.

37. Babcock AA, Wirenfeldt M, Holm T, Nielsen HH, Dissing-Olesen L, Toft-Hansen H, Millward JM, Landmann R, Rivest S, Finsen B, Owens T: **Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation.** *J Neurosci* 2006, **26:**12826-12837.

38. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ: **Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice.** *Biochem Biophys Res Commun* 2007, **353:**509-514.

39. Lehnardt S, Wennekamp J, Freyer D, Liedtke C, Krueger C, Nitsch R, Bechmann I, Weber JR, Henneke P: **TLR2 and caspase-8 are essential for group B Streptococcus-induced apoptosis in microglia.** *J Immunol* 2007, **179:**6134-6143.

40. Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG, Lathia JD, Siler DA, Chigurupati S, Ouyang X, Magnus T, Camandola S, Mattson MP: **Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits.** *Proc Natl Acad Sci USA* 2007, **104:**13798-13803.

41. Ziegler G, Harhausen D, Schepers C, Hoffmann O, Rohr C, Prinz V, Konig J, Lehrach H, Nietfeld W, Trendelenburg G: **TLR2 has a detrimental role in mouse transient focal cerebral ischemia.** *Biochem Biophys Res Commun* 2007, **359:**574-579.

42. Hua F, Ma J, Ha T, Xia Y, Kelley J, Williams DL, Kao RL, Browder IW, Schweitzer JB, Kalbfleisch JH, Li C: **Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion.** *J Neuroimmunol* 2007, **190:**101-111.

43. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K: **Role of toll-like receptor signalling in Abeta uptake and clearance.** *Brain* 2006, **129:**3006-3019.

44. Larsen PH, Holm TH, Owens T: **Toll-like receptors in brain development and homeostasis.** *Sci STKE* 2007, **2007:**pe47.

45. McKimmie CS, Johnson N, Fooks AR, Fazakerley JK: **Viruses selectively upregulate Toll-like receptors in the central nervous system.** *Biochem Biophys Res Commun* 2005, **336:**925-933.

46. Abbott NJ: **Astrocyte-endothelial interactions and blood-brain barrier permeability.** *J Anat* 2002, **200:**629-638.

47. Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A: **The evolution of vertebrate Toll-like receptors.** *Proc Natl Acad Sci USA* 2005, **102:**9577-9582.

48. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S: **A toll-like receptor that prevents infection by uropathogenic bacteria.** *Science* 2004, **303:**1522-1526.

49. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, Yamamoto M, Takeuchi O, Itagaki S, Kumar N, Horii T, Akira S: **Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin.** *J Exp Med* 2005, **201:**19-25.

50. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C: **Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.** *Science* 2004, **303:**1529-1531.

51. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: **Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.** *Science* 2004, **303:**1526-1529.

52. Wagner H: **The immunobiology of the TLR9 subfamily.** *Trends Immunol* 2004, **25:**381-386.

53. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A: **TLR11 activation of dendritic cells by a protozoan profilin-like protein.** *Science* 2005, **308:**1626-1629.

54. Alvarez JI, Rivera J, Teale JM: **Differential release and phagocytosis of tegument glycoconjugates in neurocysticercosis: implications for immune evasion strategies.** *PLoS Negl Trop Dis* 2008, **2:**e218.

55. Phulwani NK, Esen N, Syed MM, Kielian T: **TLR2 expression in astrocytes is induced by TNF-alpha- and NF-kappaB-dependent pathways.** *J Immunol* 2008, **181:**3841-3849.

56. Syed MM, Phulwani NK, Kielian T: **Tumor necrosis factor-alpha (TNF-alpha) regulates Toll-like receptor 2 (TLR2) expression in microglia.** *J Neurochem* 2007, **103:**1461-1471.

57. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y: **Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages.** *J Immunol* 2000, **165:**5767-5772.

58. Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ, Miller SD: **Differential activation of astrocytes by innate and adaptive immune stimuli.** *Glia* 2005, **49:**360-374.

59. Esen N, Tanga FY, DeLeo JA, Kielian T: **Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium Staphylococcus aureus.** *J Neurochem* 2004, **88:**746-758.

60. Lee S, Hong J, Choi SY, Oh SB, Park K, Kim JS, Karin M, Lee SJ: **CpG oligodeoxynucleotides induce expression of proinflammatory cytokines and chemokines in astrocytes: the role of c-Jun N-terminal kinase in CpG ODN-mediated NF-kappaB activation.** *J Neuroimmunol* 2004, **153:**50-63.

61. Olson JK, Ludovic Croxford J, Miller SD: **Innate and adaptive immune requirements for induction of autoimmune demy-**

elinating disease by molecular mimicry. *Mol Immunol* 2004, **40:**1103-1108.

62. Olson JK, Miller SD: **Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs.** *J Immunol* 2004, **173:**3916-3924.

63. Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW: **Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis.** *Proc Natl Acad Sci USA* 2004, **101:**1315-1320.

64. Cameron JS, Alexopoulou L, Sloane JA, DiBernardo AB, Ma Y, Kosaras B, Flavell R, Strittmatter SM, Volpe J, Sidman R, Vartanian T: **Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals.** *J Neurosci* 2007, **27:**13033-13041.

65. Ma Y, Li J, Chiu I, Wang Y, Sloane JA, Lu J, Kosaras B, Sidman RL, Volpe JJ, Vartanian T: **Toll-like receptor 8 functions as a negative regulator of neurite outgrowth and inducer of neuronal apoptosis.** *J Cell Biol* 2006, **175:**209-215.

66. Gosselin D, Rivest S: **MyD88 signaling in brain endothelial cells is essential for the neuronal activity and glucocorticoid release during systemic inflammation.** *Mol Psychiatry* 2008, **13:**480-497.

67. Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, Greenberg N, Cohen H, Kipnis J, Schwartz M: **Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood.** *Nat Neurosci* 2006, **9:**268-275.

68. Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M: **Toll-like receptors modulate adult hippocampal neurogenesis.** *Nat Cell Biol* 2007, **9:**1081-1088.