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NOD2 plays an important role in the inflammatory responses of microglia and astrocytes to bacterial CNS pathogens

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Abstract

While glial cells are recognized for their roles in maintaining neuronal function, there is growing appreciation that resident central nervous system (CNS) cells initiate and/or augment inflammation following trauma or infection. We have recently demonstrated that microglia and astrocytes constitutively express nucleotide-binding oligomerization domain-2 (NOD2), a member of the novel nucleotide-binding domain leucine-rich repeat region containing family of proteins (NLR) that functions as an intracellular receptor for a minimal motif present in all bacterial peptidoglycans. In the present study, we have confirmed the functional nature of NOD2 expression in astrocytes and microglia and begun to determine the relative contribution that this NLR makes in inflammatory CNS responses to clinically relevant bacterial pathogens. We demonstrate the increased association of NOD2 with its downstream effector molecule, Rip2 kinase, in primary cultures of murine microglia and astrocytes following exposure to bacterial antigens. We show that this cytosolic receptor underlies the ability of muramyl dipeptide to augment the production of inflammatory cytokines by glia following exposure to specific ligands for disparate Toll-like receptor homologues. In addition, we demonstrate that NOD2 is an important component in the in vitro inflammatory responses of resident glia to *N. meningitidis* and *B. burgdorferi* antigens. Finally, we have established that NOD2 is required, at least in part, for the astrogliosis, demyelination, behavioral changes, and elevated inflammatory cytokine levels observed following in vivo infection with these pathogens. As such, we have identified NOD2 as an important component in the generation of damaging CNS inflammation following bacterial infection.

Keywords

Meningitis; pattern recognition receptors; NLR; infection; cytokines

INTRODUCTION

Microglia and astrocytes are major glial cell types in the central nervous system (CNS) and have been recognized as important contributors to inflammatory immune responses within the brain. Exposure of microglia and astrocytes to microbial challenge has been demonstrated to induce cytokine production that may recruit leukocytes to sites of challenge, and activate those cells upon their arrival (for review see (Chauhan and Marriott 2007)). We have previously demonstrated that microglia and astrocytes respond to bacterial CNS pathogens including *Borrelia burgdorferi* and *Neisseria meningitidis* (Rasley et al. 2004b; Rasley et al. 2006). However, the mechanisms by which these cells identify bacterial

pathogens are only now becoming apparent. These resident CNS cell types express members of the Toll like family of pattern recognition receptors (TLRs) that are instrumental in perceiving extracellular bacterial pathogens (Bowman et al. 2003; Bsibsi et al. 2002; Carpentier et al. 2008; Kielian 2006; Kielian et al. 2005; Konat et al. 2006; Livengood and Gilmore 2006; Olson and Miller 2004). However, *B. burgdorferi* (Brouqui et al. 1996; Embers et al. 2004; Livengood and Gilmore 2006) and *N. meningitidis* (Nikulin et al. 2006) can be internalized by eukaryotic cells and the mechanisms by which intracellular bacteria are perceived by microglia and astrocytes have not been established.

We have recently demonstrated that microglia and astrocytes constitutively express nucleotide-binding oligomerization domain-2 (NOD2; previously designated as CARD15) (Sterka and Marriott 2006; Sterka et al. 2006), a member of the novel nucleotide-binding domain leucine-rich repeat region containing family of proteins (NLR) that functions as an intracellular receptor for a minimal motif present in all bacterial peptidoglycans (Girardin et al. 2003b; Girardin et al. 2003c; Inohara et al. 2003). The functional nature of such expression was inferred from the ability of TLR ligands and *B. burgdorferi* or *N. meningitidis* antigens to upregulate NOD2 expression in these resident CNS cells (Sterka and Marriott 2006; Sterka et al. 2006) and from the inducible expression of Rip2 kinase, a critical downstream effector molecule in the NOD2 pathway. Furthermore, we demonstrated that muramyl dipeptide, MurNAc-L-Ala-D-isoGln (MDP), a ligand for NOD2 (Girardin et al. 2003), can significantly augment TLR-induced inflammatory cytokine production by both microglia and astrocytes (Sterka and Marriott 2006; Sterka et al. 2006). However, it should be noted that MDP has recently been suggested to be a ligand for another NLR, NLR family pyrin domain containing 3 (NLRP3) (previously known as CIAS1, cryopyrin, PYPAF1 or NALP3) (Martinon et al. 2004), and this has called the specificity of this ligand into question.

In the present study, we have confirmed the functional nature of NOD2 expression in astrocytes and microglia and begun to determine the relative contribution that this NLR makes in inflammatory CNS responses to clinically relevant bacterial pathogens. We demonstrate the increased association of NOD2 with its downstream effector molecule Rip2 kinase in primary cultures of murine microglia and astrocytes following exposure to bacterial antigens and show that this cytosolic receptor underlies the ability of MDP to augment glial immune responses. Furthermore, we demonstrate in vitro and in vivo that NOD2 is an important component in the inflammatory responses of resident glia to *N. meningitidis* and *B. burgdorferi*.

MATERIALS AND METHODS

Wild type and NOD2-deficient mice

B6.129S1-*Nod2*^{tm1Flv}/J mice that are NOD2-deficient (NOD2^{-/-}) and bred for > 10 generations onto a C57BL/6 background, as well as wild type C57BL/6j mice, were purchased from Jackson Laboratory, Bar Harbour, Maine. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes.

Isolation of primary murine microglia

Mouse neonatal brain microglia were isolated as described previously by our laboratory (Bowman et al. 2003; Rasley et al. 2002a; Rasley et al. 2004a; Rasley et al. 2002b; Rasley et al. 2004b; Rasley et al. 2006; Sterka and Marriott 2006) and cultured in RPMI 1640 with 10% FBS and 20% conditioned medium from LADMAC cells (ATCC number CRL-2420) as a source of CSF-1. Cells isolated in this manner are > 95% authentic microglia as

assessed by their characteristic morphology and expression of CD11b and F4/80 as determined by immunofluorescent microscopy.

Isolation of primary murine astrocytes

Mouse neonatal brain astrocytes were isolated as described previously by our laboratory (Bowman et al. 2003; Rasley et al. 2004a; Rasley et al. 2006; Sterka et al. 2006) and cultured in RPMI 1640 containing 10% FBS. Cells isolated in this manner are > 97% authentic astrocytes due to their characteristic morphology, expression of glial fibrillary acidic protein and the absence of CD11b as determined by immunofluorescent microscopy.

Culture of *Borrelia burgdorferi* and *Neisseria meningitidis* and preparation of antigen lysates

We have utilized *B. burgdorferi* strain N40 and *N. meningitidis* strain MC58 in these studies. While these organisms are not associated with CNS disease in mice, both represent clinically relevant Gram-negative bacteria responsible for Lyme neuroborreliosis and bacterial meningitis, respectively. We have previously demonstrated that antigens from these organisms elicit robust inflammatory responses by both murine microglia and astrocytes (Rasley et al, 2002a; Rasley et al, 2004a; 2004b; Sterka et al, 2006; Sterka and Marriott, 2006; Chauhan et al, 2008). Importantly, both of these organisms express peptidoglycan motifs that can be recognized by NOD2, but *B. burgdorferi* lacks the expression of the TLR4 ligand, LPS, and has been shown to initiate TLR2 and TLR5 mediated innate immune responses (Shin et al, 2008), while *N. meningitidis* expresses LPS and can elicit TLR2 and TLR4-mediated responses (Zughaier et al, 2007; Hellerud et al, 2008).

A clonal low passage of *B. burgdorferi* strain N40 was used throughout these studies and were grown in BSK II medium at 34 °C while *N. meningitidis* strain MC58 was grown overnight in 5 ml of GC medium plus hemoglobin with Isovitaex enrichment solution at 37°C as previously described (Rasley et al. 2006; Sterka and Marriott 2006). For cell lysates, bacteria were centrifuged at $10,000 \times g$, washed 3 times with PBS prior to pulsing 3 times with a cell sonicator. *B. burgdorferi* antigen isolates generated in this manner have previously been demonstrated to be free of detectable levels of LPS (Rasley et al. 2002a; Rasley et al. 2004a; Sterka and Marriott 2006; Sterka et al. 2006). For in vitro exposure of isolated glia to *N. meningitidis* or *B. burgdorferi*, bacteria were harvested by centrifugation and washed twice in PBS. Glia were exposed to 1–10 µg/ml *B. burgdorferi* or *N. meningitidis* cell lysates as indicated. These doses were selected based upon their documented ability to induce submaximal cytokine responses by glial cells (Rasley et al. 2004b; Rasley et al. 2006).

Co-immunoprecipitation of NOD2 with Rip2 kinase

Co-immunoprecipitation was performed essentially as described previously by Clark and colleagues (Clark et al. 2001). Briefly, cells (2×10^6) stimulated with bacterial lysates were washed with ice-cold PBS and lysed at 4°C for two hours in Tris buffered saline with EDTA (150 mM NaCl, 5 mM EDTA, 20 mM Tris pH 7.5) plus 1% Brij-97 (Sigma-Aldrich) and 10 units/ml aprotinin (Calbiochem, San Diego, CA), 1 mM PMSF and 1 µg/ml pepstatin A. The lysates were incubated with protein A agarose beads (Pierce Endogen) conjugated with antibodies directed against NOD2 or Rip2 kinase for 18 hours at 4°C. The immunoprecipitated material was subsequently subjected to immunoblot analysis for the presence of NOD2 or Rip2 kinase.

Immunoblot analysis of NOD2, Rip2 kinase, and hPepT1 expression

Immunoblot analyses were performed as described previously by our laboratory (Sterka and Marriott 2006; Sterka et al. 2006). The primary antibodies used were a mouse monoclonal antibody reactive against mouse NOD2 (Clone 2D9; Novus Biologicals, Littleton, CO), a mouse reactive affinity purified rabbit polyclonal antibody directed against Rip2 kinase (Cell Sciences Inc., Canton, MA), and a rabbit polyclonal directed against hPepT1 (Abcam Inc. Cambridge, MA).

Quantification of glial IL-6 and TNF- α secretion

Specific capture ELISAs were performed to quantify IL-6 and TNF- α and secretion by glial cells as described previously by our laboratory (Rasley et al. 2002a; Rasley et al. 2004b; Rasley et al. 2006). Cytokine levels in brain homogenates were normalized to total brain weight and reported as pg/g of brain tissue.

Intracerebral administration of bacteria

Viable *N. meningitidis* or *B. burgdorferi* (1×10^6 bacteria) were administered via intracerebral (i.c.) injection as previously described by our laboratory (Rasley et al. 2006) into NOD2^{-/-} or wild type mice. Anesthetized animals were secured in a stereotaxic platform and stand and received an i.c. injection containing pathogens resuspended in Ringer's solution (final volume of 1 μ l), or vehicle only, 1 mm lateral and 1 mm posterior to the bregma using a Hamilton positive displacement syringe (7001 series) with a 25 gauge needle and a tubing guard to ensure constant depth of administration (3–3.5 mm). Five animals per group were used and animals displaying signs such as seizures, abnormal gait/ataxia, failure to open eyes, or other physical disabilities were euthanized immediately. At 3 days post infection, animals were euthanized and all brain tissue removed and weighed. The brain tissue was mechanically disrupted in a glass homogenizer in 3 ml PBS and then centrifuged. Supernatants were analyzed for cytokine content by specific capture ELISA.

Histochemical and immunofluorescent analyses of murine brain sections

Infected and non-infected mice were euthanized 72 hours post infection and the brains were perfused with Prefer fixative (Anatech, Battel creek, MI) via intra-atrial injection. Samples were embedded in paraffin and sectioned serially (5–7 μ m).

Demyelination was measured in coronal sections by Luxol fast blue-Cresyl violet staining according to the directions provided by the manufacturer (American MasterTech Scientific Inc., Lodi, CA). Briefly, paraffin embedded sections were heated and rehydrated with decreasing concentrations of ethyl alcohol prior to rinsing in distilled water and Luxol fast blue staining at 60°C for 18 hrs. Grey and white matter was further differentiated using 0.05% lithium carbonate and washing with 70% alcohol. Finally, the slides were placed in Cresyl Echt violet stain for 10 min and differentiated with alcohol. After dehydration, slides were washed in xylene and coverslips were mounted using permanent mounting media and myelination of the radiations of the corpus callosum visually assessed in a low power (20 $\times$ objective) microscopy field in animals from each experimental group.

Astrogliosis was assessed in deparaffinized coronal sections blocked with goat serum (Zymed laboratories, San Francisco, CA) according to glial fibrillary acidic protein (GFAP) expression. GFAP was detected using a monoclonal mouse antibody directed against murine GFAP (Molecular Probes, Eugene, Oregon) and an AlexaFluor 488 conjugated chicken anti-mouse secondary antibody (Molecular Probes). Cell nuclei were visualized with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) and the cover slip was mounted using ProLong[®] Gold antifade reagent (Invitrogen, Eugene, Oregon). GFAP expression was assessed in multiple microscopy fields (40 $\times$ objective) of the same cortical

locations in animals from each experimental group using an Olympus IX70 Fluoroview confocal microscopy system.

Locomotor activity following i.c. bacterial challenge

Locomotor activity was assessed in mice according to a modified protocol of Levin and colleagues (2001) using a radial arm maze (Coulbourn Instruments, Allentown, PA) following i.c. administration of bacteria. Briefly, NOD2^{+/+} and NOD2^{-/-} mice (n = 6) were acclimated to the handler and the maze apparatus for several days prior to bacterial challenge (Mitani et al, 2004; Wenk, 2004). Mice were allowed to move freely within the apparatus for 8 minutes during which time the total number of arms visited was counted and latency was assessed as the number of arms visited divided by time as described previously (Kreider et al, 2005; Levin, 2001). Values were obtained for three consecutive days prior to bacterial administration and at various time points up to 17 days following challenge.

Acute isolation and cytometric analysis of CNS cells

Mixed CNS cells were acutely isolated from infected and uninfected animals using a protocol modified from Campanella et al. (Campanella et al. 2002) Briefly, brains were rapidly removed and mechanically disrupted in a glass homogenizer, washed, and resuspended in PBS/30% Percoll (Fluka, Sigma Aldrich, St Louis, MO) solution. This was overlaid on a gradient containing 37 and 70% Percoll solutions and centrifuged at $600 \times g$ for 20 min at room temperature. Cells were then collected from the 37/70% Percoll interface and washed with PBS. Cells were permeabilized with CytoFix/CytoPerm Plus according to the directions provided by the manufacturers (BD PharMingen) prior to staining with a AlexaFluor 488 conjugated monoclonal mouse antibody directed against murine GFAP (Molecular Probes, Eugene, Oregon). These cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) to determine the intensity of GFAP-associated fluorescence relative to the signal obtained with an antibody directed against an irrelevant antigen. In each experiment a minimum of 50,000 cells were counted and data is reported as the geometric mean of the fluorescence signal.

Densitometric analyses

Densitometric analyses of Western blots were performed using NIH Image (obtained from the NIH Web site: <http://rsb.info.nih.gov/ni-image>). Results are presented as mean values $\pm$ SEM of arbitrary densitometric units corrected for background intensity and normalized to the expression of β -actin.

Statistical Analyses

All results are presented as the mean $\pm$ SEM and were tested statistically by Student's t-test, one-way analysis of variance (ANOVA) with Tukey's post-hoc test, or repeated measures ANOVA with Tukey's post-hoc test, as appropriate using commercially available software (Sigma Stat, Systat Software, Inc., San Jose, CA). Results were considered to be statistically significant at a probability of < 0.05 .

RESULTS

Increased association of NOD2 with Rip2 kinase in murine microglia and astrocytes following bacterial challenge

To begin to establish the functional relevance of NOD2 expression in resident CNS cells, we investigated the ability of NOD2 to associate with its downstream effector molecule, Rip2 kinase, in isolated cultures of primary murine microglia and astrocytes using co-immunoprecipitation techniques. As shown in Figure 1, protein isolates from resting cultures

of microglia and astrocytes exhibit constitutive association between NOD2 with Rip2 kinase. Importantly, the level of association between NOD2 and Rip2 kinase is elevated in both microglia and astrocytes following exposure to lysates prepared from *N. meningitidis* or *B. burgdorferi*, two clinically relevant bacterial pathogens of the CNS that have been demonstrated to be internalized by glial cells (Livengood and Gilmore 2006). As such, the increased association of these molecules provides circumstantial evidence supporting a functional role for NOD2 in the immune responses of resident CNS cell types following bacterial challenge.

MDP augmentation of inflammatory cytokine production by stimulated microglia and astrocytes is mediated by NOD2

We have previously demonstrated that the NOD2 ligand, MDP, augments inflammatory cytokine production by microglia and astrocytes derived from BALB/c mice (Sterka and Marriott 2006). In the present study we have confirmed these findings in microglia and astrocytes derived from C57BL/6 mice. As shown in Figures 2A and 2C, MDP alone fails to elicit significant production of IL-6 or TNF- α . However, this NOD2 ligand significantly augments the production of both of these inflammatory cytokines by microglia following exposure to the TLR4 and TLR5 specific ligands, LPS and flagellin, respectively. Similarly, MDP augments LPS and flagellin induced IL-6 and TNF- α production by isolated astrocytes (Figures 3A and 3C). The dependence of the effects of MDP on TLR co-stimulation do not appear to be due to changes in the expression of the MDP transporter, hPepT1 (Ismair et al, 2006), as total protein levels of this molecule are unchanged following 12 hour exposure to LPS (5 ng/ml) or flagellin (5 ng/ml) as assessed by immunoblot analysis ($n = 3$; data not shown) of protein isolates from microglia (28 ± 2 , 33 ± 5 , and 29 ± 4 arbitrary densitometric units for untreated, LPS, and flagellin challenged microglia, respectively) or astrocytes (23 ± 3 , 17 ± 4 , and 16 ± 3 arbitrary densitometric units for untreated, LPS, and flagellin challenged astrocytes, respectively).

Importantly, we have confirmed that the effects of MDP are mediated by NOD2 by performing parallel experiments using microglia and astrocytes derived from animals genetically deficient in the expression of NOD2 (NOD2 $^{-/-}$). As shown in Figures 2B and 2D, MDP fails to augment inflammatory cytokine production by NOD2 $^{-/-}$ derived microglia challenged with LPS or flagellin. Similarly, MDP does not elevate TLR-induced IL-6 or TNF- α production by astrocytes derived from NOD2 $^{-/-}$ mice (Figure 3B and 3D). These data confirm that MDP acts via NOD2 and can augment TLR-induced inflammatory cytokine production by both microglia and astrocytes.

NOD2 is required for maximal in vitro responses of microglia and astrocytes to *N. meningitidis* and *B. burgdorferi*

To begin to determine the relative importance of the NOD2 receptor in inflammatory glial responses to bacterial pathogens, we have compared the responses of *N. meningitidis* and *B. burgdorferi* challenged cells derived from wild type animals (NOD2 $^{+/+}$) to those of cells derived from NOD2 $^{-/-}$ animals. As shown in Figure 4, microglia produce IL-6 and TNF- α in response to exposure to *N. meningitidis* or *B. burgdorferi* antigens. Importantly, cells derived from NOD2 $^{-/-}$ mice exhibited significantly lower responses than their NOD2 $^{+/+}$ derived counterparts (Figure 4). Similarly, NOD2 $^{-/-}$ derived astrocytes produce significantly lower amounts of IL-6 and TNF- α than wild type derived cells following bacterial challenge (Figure 5). Taken together, these data indicate that NOD2 is an important contributor to the in vitro inflammatory immune responses of resident CNS cell types to two clinically relevant bacterial pathogens.

Inflammatory CNS damage following in vivo bacterial administration is reduced in the absence of NOD2

To establish the in vivo role of NOD2 in bacterially-induced inflammatory glial responses, we have investigated the morphological changes associated with in vivo administration of *N. meningitidis* in the presence or absence of NOD2. As shown in Figure 6, i.c. injection of known numbers of *N. meningitidis* (1×10^6 organisms) elicits significant demyelination in wild type animals as evidenced by reduced Luxol blue staining of the radiations of the corpus callosum ($n = 3$). In addition, infection was associated with elevated numbers of astrocytes and GFAP-associated fluorescence in the cerebral cortex (Figure 6B). Interestingly, the infection-associated demyelination (Figure 6A) and astrogliosis (Figure 6B) was less severe in NOD2^{-/-} animals than that seen in their wild type counterparts.

To further assess the role of NOD2 in bacterially induced neurodegeneration, we have used a radial arm maze apparatus to measure the locomotor activity of wild type and NOD2 deficient mice following i.c. administration of *N. meningitidis*. As shown in Figure 6C, bacterial challenge elicits a significant increase in locomotor activity in wild type mice as assessed by the number of arms visited during the observation period and reduced latency. This effect is consistent with previous demonstrations of hyperactivity in mice following CNS infection with some bacterial and viral pathogens (Hoffman et al, 2004; Solbrig and Koob, 2003), and may correlate with the long-term hyperactivity observed in children following bacterial meningitis (Berg et al, 2002). Importantly, NOD2^{-/-} mice fail to demonstrate these bacterially induced behavioral changes (Figure 6C).

To confirm our qualitative findings, parallel experiments were performed using flow cytometry to assess GFAP expression in acutely isolated glial cells. As shown in Figure 7A, the significant elevation in GFAP-associated fluorescence observed in wild type mice following *N. meningitidis* infection ($p < 0.05$; $N = 8$) was not present in NOD2^{-/-} animals. Similarly, the significant increase in CNS levels of the pro-inflammatory cytokine TNF- α seen following *N. meningitidis* administration ($p < 0.05$; $N = 5$) was not apparent in NOD2^{-/-} mice (Figure 7B). Finally, we have extended our in vivo studies to include the disparate CNS pathogen, *B. burgdorferi*. As shown in Figure 7, i.c. administration of *B. burgdorferi* (1×10^6 organisms) significantly elevates GFAP-associated fluorescence in glia isolated from wild type animals, consistent with bacterially-induced astrogliosis, and this effect is associated with a significant increase in TNF- α levels in whole brain homogenates ($p < 0.05$; $N = 5$). Importantly, *B. burgdorferi* administration failed to elicit significant elevations in either GFAP-associated fluorescence or TNF- α levels in NOD2^{-/-} animals (Figure 7A and 7B, respectively) indicating that NOD2 plays an important role in the generation of in vivo inflammatory responses to this bacterial pathogen.

DISCUSSION

While it is likely that infiltrating immune cells contribute to inflammatory responses within the CNS, a growing body of evidence suggests that resident glial cells play an important role in the initiation and progression of inflammation following infection or trauma. To accomplish this role, resident cells like microglia and astrocytes express multiple microbial pattern recognition receptors including TLRs (Bsibsi et al. 2002; Carpentier et al. 2008; Esen and Kielian 2005; Kielian et al. 2005; Olson and Miller 2004; Rasley et al. 2002a) to perceive bacterial pathogens and to initiate potentially damaging CNS inflammation. Microglia are of myeloid lineage and respond to traumatic injury by migrating to the site of challenge and assuming immune effector functions (Hickey and Kimura 1988). Our laboratory and others have demonstrated the ability of microglia to produce key inflammatory cytokines including TNF- α , and IL-6 (Kiefer et al. 1993; Kielian 2004; Kielian et al. 2002; Rasley et al. 2002a; Rasley et al. 2004b; Streit et al. 1998). Similarly,

astrocytes, the major glial cell population, also express multiple TLRs and have immune effector functions (Benveniste et al. 1990; Bowman et al. 2003; Bsibsi et al. 2002; Carpentier et al. 2005; Dong and Benveniste 2001; Esen et al. 2004; Gottschall et al. 1994; Kimberlin et al. 1995; Lieberman et al. 1989; Oh et al. 1999; Takeshita et al. 2001). Pathogen stimulated astrocytes express a variety of inflammatory cytokines and chemokines. As such these resident glial cell types are ideally situated to perceive and respond to bacterial CNS pathogens.

NLR proteins such as NOD2 have been identified as cytosolic pattern recognition receptors that play a role in the initiation of inflammatory host immune responses to bacterial challenge (Dziarski and Gupta 2005; Strober et al. 2006). NOD2 appears to function as an intracellular receptor for a minimal motif common to all bacterial peptidoglycans (Fournier and Philpott 2005; Girardin et al. 2003; Takada and Uehara 2006). Previously, we have characterized the expression of NOD2 in primary cultures of murine glial cells (Sterka and Marriott 2006; Sterka et al. 2006). We have demonstrated that microglia and astrocytes not only express NOD2 and its downstream effector molecule Rip2 kinase constitutively, but we have also shown that expression of these molecules is upregulated in glial cells following bacterial challenge (Sterka and Marriott 2006; Sterka et al. 2006). However, the importance of NLR proteins in the generation of inflammatory host responses to bacterial pathogens within the brain and the associated CNS damage has not been established.

In the present study, we demonstrate that exposure of microglia and astrocytes to bacterial lysates increases the association between NOD2 and Rip2 kinase. In addition, we have confirmed our previous findings using BALB/c-derived cells, by demonstrating that MDP augments the TLR-induced inflammatory responses of microglia and astrocytes derived from C57BL/6 mice. Importantly, we have confirmed that these effects are mediated by NOD2, rather than other putative receptors for MDP such as NLRP3 (Martinon et al. 2004) by demonstrating that the ability of MDP to exacerbate responses to TLR ligands is abolished in cells derived from NOD2^{-/-} animals. Perhaps more importantly, we have assessed the relative importance of NOD2 in the immune responses of isolated cultures of primary glia cells to bacterial antigens from two disparate and clinically relevant bacterial pathogens of the CNS, *N. meningitidis* and *B. burgdorferi*. We show that the inflammatory responses of both astrocytes and microglia are significantly reduced in the absence of NOD2 expression. Finally, we have established the in vivo relevancy of our in vitro studies by studying the CNS inflammation associated with direct intracerebral bacterial administration in wild type animals and mice genetically deficient in the expression of NOD2. Our data indicates that the astrogliosis, demyelination, and increases in inflammatory cytokine levels within the CNS associated with bacterial infection are reduced in the absence of NOD2 expression. Furthermore, we have found that the increase in locomotor activity associated with in vivo *N. meningitidis* challenge is absent in NOD2 deficient animals. Taken together, these studies indicate that NOD2 represents an important component in the generation of damaging CNS inflammation following bacterial infection.

The present findings are consistent with the known role for NOD2 in the initiation and/or progression of bacterially induced inflammation. NOD2 mutations have been associated with the development of chronic inflammatory bowel diseases and monocytes/macrophages derived from humans or mice with such mutations demonstrate altered NF- κ B activity and inflammatory cytokine production (Beynon et al. 2008; Hugot et al. 2001; Maeda et al. 2005; Ogura et al. 2001). With regard to resident CNS cells, we have shown that MDP alone fails to elicit inflammatory cytokine production despite constitutive expression of NOD2, its adaptor molecule Rip2 kinase, and the MDP transporter hPepT1 by both microglia (Sterka and Marriott, 2006) and astrocytes (Sterka et al, 2006) but can augment responses to TLR ligands. This finding is in agreement with studies in other cell types (Pauleau and Murray,

2003), and is consistent with the recent findings of Chen and co-workers (Chen et al. 2008) showing that NOD2 functions in conjunction with TLR2 to up-regulate the expression of formyl peptide receptor 2 (mFPR2), a G-protein coupled receptor that mediates the chemotactic activity of pathogen and host-derived peptides including amyloid beta 42, the key causative factor in Alzheimer's disease. Similarly, another recent study has demonstrated that NOD2 acts in concert with TLRs to induce nitric oxide and TNF- α production and to upregulate the expression of P2X4R, an ATP-regulated ion channel associated with CNS inflammation (Guo et al. 2006).

There do not appear to be marked differences between the responses of microglia to *N. meningitidis* or *B. burgdorferi* antigens in the presence or absence of NOD2. However, astrocytes challenged with *B. burgdorferi* exhibit more NOD2/Rip2 kinase association and higher production of both IL-6 and TNF- α than cells exposed to *N. meningitidis*. In addition, in vivo administration of *B. burgdorferi* tended to elicit greater astrogliosis as assessed by GFAP-associated fluorescence than that seen in *N. meningitidis* infected mice. Since *B. burgdorferi* has been shown to initiate TLR2 and TLR5 mediated innate immune responses (Shin et al, 2008) while *N. meningitidis* can elicit TLR2 and TLR4-mediated responses (Zughaier et al, 2007; Hellerud et al, 2008), it is tempting to suggest that the TLR5/NOD2 interaction might elicit a more robust response in astrocytes than that associated with TLR4/NOD2 signaling. While verification of such a hypothesis will require further study, it is interesting to note that the TLR5 ligand, flagellin, elicits higher inflammatory cytokine production by astrocytes when co-administered with MDP than a combination of LPS and MDP (Figure 3).

To date, the mechanisms underlying TLR and NOD2 interaction/synergy have yet to be determined but it is probable that crosstalk exists between these disparate pattern recognition receptors and recent studies have focused on this issue (Kim et al, 2008). Indeed, we have previously demonstrated that ligands such as Pam3Cys (TLR2), LPS (TLR4), flagellin (TLR5), and a TLR9-activating CpG oligonucleotide, can all augment NOD2 and Rip2 kinase protein expression in both microglia (Sterka and Marriott, 2006) and astrocytes (Sterka et al, 2006) without altering hPepT1 expression. However, given the constitutive expression of these molecules in resting cells, and the rapid immune responses of glial cells to MDP and TLR ligand co-administration, a mechanism in which TLR stimulation renders glial cells sensitive to this NOD2 ligand would seem unlikely. Clearly, the interaction between these pattern recognition receptor families requires additional study and experiments to determine the mechanisms underlying TLR and NOD2 interaction/synergy in glial cells are ongoing in our laboratory.

While it is readily apparent that the MDP-associated exacerbation of glia responses to TLR ligands are absent in NOD2^{-/-} derived cells and the responses of these cells to bacterial lysates are lower than those seen in wild type derived cells, it is interesting to note that NOD2^{-/-} glial cells demonstrated greater cytokine responses to TLR ligands than wild type-derived cells. Similarly, we observed that uninfected NOD2^{-/-} mice exhibited less myelination, higher astrocyte-associated GFAP signals and higher basal cytokine levels than their wild type counterparts. At present we cannot explain these findings, but it is conceivable that an endogenous ligand for NOD2 might exist that serves to limit TLR-mediated inflammatory responses rather than exacerbate them. Further studies will be required to resolve this issue.

Taken together, the present studies indicate that NOD2 acts in a co-operative manner with disparate TLR homologues in the induction of inflammatory cytokine responses of primary microglia and astrocytes. In addition, we have shown that NOD2 is required for maximal in vitro inflammatory responses of resident CNS cells to bacterial lysates. Finally, we have

established that NOD2 is required for the astrogliosis, demyelination, and elevated inflammatory cytokine levels observed following infection with two clinically relevant bacterial pathogens. As such, we have identified NOD2 as an important component in the generation of damaging CNS inflammation following bacterial infection.

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FIGURE 1.

Association of NOD2 with Rip2 kinase in murine microglia and astrocytes increases following challenge with bacterial antigens. Primary murine microglia and astrocytes (2×10^6 per well) were untreated (0) or exposed to *N. meningitidis* (Nm: 1 and 5 ug/ml) or *B. burgdorferi* (Bb: 1, 5 and 10 ug/ml) antigens. At 2 hrs following bacterial challenge, protein isolates were immunoprecipitated with antibodies directed against Rip2 kinase or NOD2 and subsequently analyzed by denaturing immunoblot for the presence of NOD2 (upper panel) or Rip2 kinase (lower panel), respectively. As a positive control, NOD2/Rip2 kinase association was measured in thymus protein isolates (Thy). These experiments were performed three times with similar results.

FIGURE 2.

MDP augmentation of inflammatory cytokine production by stimulated microglia is mediated by NOD2. Cells (2×10^6) from wild type (NOD2^{+/+}) and NOD2 knockout (NOD2^{-/-}) animals were untreated (0) or exposed to bacterial flagellin (Flg) or LPS (1, 5 and 10 ng/ml) in the presence or absence of MDP (5 ug/ml). At 12 hrs following bacterial challenge, culture supernatants were assayed for the presence of IL-6 (Panels A and B) and TNF- α (Panels C and D) by specific capture ELISA. Data are presented as the mean of triplicate determinations of samples from three separate experiments $\pm$ SEM. Asterisks indicate statistically significant differences between cytokine levels in the absence or presence of MDP.

FIGURE 3.

MDP augmentation of inflammatory cytokine production by stimulated astrocytes is mediated by NOD2. Cells (2×10^6) from wild type (NOD2^{+/+}) and NOD2 knockout (NOD2^{-/-}) animals were untreated (0) or exposed to bacterial flagellin (Flg) or LPS (1, 5 and 10 ng/ml) in the presence or absence of MDP (5 ug/ml). At 12 hrs following bacterial challenge, culture supernatants were assayed for the presence of IL-6 (Panels A and B) and TNF- α (Panels C and D) by specific capture ELISA. Data are presented as the mean of triplicate determinations of samples from three separate experiments $\pm$ SEM. Asterisks indicate statistically significant differences between cytokine levels in the absence or presence of MDP.

FIGURE 4.

Inflammatory cytokine responses of microglia to *N. meningitidis* and *B. burgdorferi* are significantly lower in the absence of NOD2 expression. Cells (2×10^6) from wild type (NOD2+/+) and NOD2 knockout (NOD2-/-) animals were untreated or exposed to either *B. burgdorferi* antigens (Bb: 1, 5, and 10 ug/ml) or *N. meningitidis* antigens (Nm: 1, 5 and 10 ug/ml). At 12 hrs following bacterial challenge culture supernatants were isolated and assayed for the presence of IL-6 (Panels A and B) or TNF- α (Panels C and D) by specific capture ELISA. Data are presented as the mean of triplicate determinations of samples from three separate experiments $\pm$ SEM. Asterisks indicate statistically significant differences in cytokine production between cells derived from wild type and NOD2 deficient animals.

FIGURE 5.

Inflammatory cytokine responses of astrocytes to *N. meningitidis* and *B. burgdorferi* are significantly lower in the absence of NOD2 expression. Cells (2×10^6) from wild type (NOD2+/+) and NOD2 knockout (NOD2-/-) animals were untreated or exposed to either *B. burgdorferi* antigens (Bb: 1, 5, and 10 ug/ml) or *N. meningitidis* antigens (Nm: 1, 5 and 10 ug/ml). At 12 hrs following bacterial challenge culture supernatants were isolated and assayed for the presence of IL-6 (Panel A) or TNF- α (Panel B) by specific capture ELISA. Data are presented as the mean of triplicate determinations of samples from three separate experiments $\pm$ SEM. Asterisks indicate statistically significant differences in cytokine production between cells derived from wild type and NOD2 deficient animals.

FIGURE 6.

Bacterially induced in vivo CNS effects are reduced in the absence of NOD2. Vehicle or *N. meningitidis* (1×10^6 bacteria) was administered via intracerebral injection into C57BL/6 wild type (NOD2^{+/+}) and NOD2 deficient (NOD2^{-/-}) mice. Panels A and B: At 72 hours post infection, brain tissue was perfused in situ, isolated, and prepared for histochemical analysis by lightfield microscopy or immunofluorescent microscopy. Panel A shows representative Luxol blue staining of the radiations of the corpus callosum in coronal fields (20 $\times$ objective) while Panel B shows representative GFAP (green) and DAPI (blue) immunofluorescence (40 $\times$ objective) in coronal cortical fields from one of the three animals in each group. Panel C: Locomotor activity was assessed in infected NOD2^{+/+} and NOD2^{-/-} mice at 3, 7, 10, 14 and 17 days following i.c. bacterial administration using a radial arm maze apparatus. Data is shown as the mean number of arms visited during an 8 min observation period and latency was calculated as the number of arms visited per min (n = 6). Asterisks and dagger symbols indicate a statistically significant difference from baseline (Day 0) values and a statistically significant difference between wild type and NOD2 deficient animals, respectively.

FIGURE 7.

In vivo astrogliosis and inflammatory cytokine production are markedly reduced in the absence of NOD2. Wild type C57BL/6 mice were given vehicle (0) or were infected with *N. meningitidis* (Nm) or *B. burgdorferi* (Bb) (1×10^6) via intracerebral injection. Panel A: at 72 hours post infection, glial cells were isolated, permeabilized, and GFAP expression determined by flow cytometry. N = 8; asterisk indicates significant difference from uninfected animals. Panel B: at 72 hours post infection tissue homogenates were isolated for measurement of TNF- α protein expression by specific capture ELISA. N = 5; asterisk indicates significant difference from uninfected animals.