Published in final edited form as:

J Neuroimmunol. 2007 September ; 189(1-2): 75–87.

Differential *ex vivo* nitric oxide production by acutely isolated neonatal and adult microglia

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Abstract

Microglia are the macrophage population residing in the parenchyma of the central nervous system (CNS), and are thought to play critical roles in CNS development, homeostasis and defense against pathogens. Microglia are capable of rapidly responding to microbial pathogens through engagement of their Toll-like receptors (TLRs). We first compared the efficiency of these responses in primary microglia acutely isolated from adult and neonatal mice. While the cytokine and chemokine responses of adult microglia were generally higher than those of neonatal cells stimulated *ex vivo* through TLRs, the nitric oxide response of neonatal microglia was markedly enhanced relative to the adult cells. We then went on to identify culture conditions such as exposure to M-SCF or GM-CSF that markedly enhanced the nitric oxide response of microglia, particularly those from the adult CNS. Finally, we demonstrate that the differential nitric oxide response of neonatal and adult microglia is not limited to the mouse, but also extends to rat microglia.

Keywords

Toll-like receptors; microglia; nitric oxide; inducible nitric oxide synthase; cytokine; chemokine; central nervous system

1. Introduction

Microglia are the macrophage population residing in the parenchyma of the central nervous system (CNS), and are thought to play critical roles in CNS development, homeostasis and defense against pathogens. First described by Del Rio-Hortega (Rezaie and Male, 2002), they are derived from myeloid precursors that infiltrate the central nervous system early in CNS development (Vilhardt, 2005). In the neonatal brain, resident microglia have an amoeboid morphology, proliferate, and exhibit motility while actively phagocytosing apoptotic dead and dying neurons. As the animal matures, the resident microglia obtain a ramified morphology and, aside from their numerous processes extruding through the surrounding CNS tissue, become immotile. These ramified microglia are said to be in a resting state. Ramified microglia are no longer actively phagocytosing, do not proliferate in the resting state, express lower levels of a number of cell surface markers as compared to peripheral macrophages (Brannan and Roberts, 2004) and are distinguished from blood-derived macrophages infiltrating the CNS by their lower level of CD45 expression (Sedgwick et al., 1991). While the rate of microglial cell

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ramification varies with anatomical location within the brain, this process is completed by approximately 14 days postpartum in the mouse (Hirasawa et al., 2005) and 18 days postpartum in the rat (Dalmau et al., 2003).

Mononuclear phagocytes such as macrophages are a crucial cellular component of the innate immune system because they are capable of rapidly responding to pathogens through engagement of their Toll-like receptors (TLRs), with subsequent induction of pro-inflammatory cytokines, chemokines, and essential immune modulatory small molecules such as nitric oxide. Early recognition of pathogens by the innate immune system is essential for their successful removal, and also leads to recruitment and coordination of cells of the adaptive immune system responsible for cellular and humoral responses and eventually development of immunologic memory to the specific pathogen.

TLRs recognize pathogenic organisms through the binding of pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). TLR engagement leads to signaling within the cell and to the induction and expression of inflammatory genes according to the specific TLR engaged. Each TLR has a signaling adaptor molecule associated with its intracellular Toll-IL1 receptor (TIR) domain and these adaptor molecules are responsible for the activation of either the NF- κ B or the interferon regulatory factor 3 (IRF-3) signaling pathways. While a variety of adaptor molecules capable of binding to the TIR domain have been identified, it has been demonstrated that MyD88 and TICAM-1 (also known as TRIF) are responsible for the activation of the afore mentioned signaling pathways (Pandey and Agrawal, 2006). TLRs signaling through MyD88 lead to NF- κ B activation and expression of NF- κ B dependent genes. Of the TLRs characterized thus far, all except TLR-3 are capable of signaling through MyD88. Likewise, those TLRs that can signal alternatively through TICAM-1, namely TLR-3 and -4, activate IRF-3 and thus induce expression of interferon (IFN)- β and, in the case of TLR-3 engagement, IFN- α as well. Expression and secretion of the type-1 IFNs leads to the activation of the STAT-1 α signaling pathway by autocrine and paracrine signaling via engagement of IFN receptors on the cell surface. MyD88-independent signaling also activates NF- κ B signaling, albeit with delayed kinetics (Yamamoto et al., 2002).

In addition to secretion of inflammatory cytokines and chemokines, macrophages produce the toxic defense molecule nitric oxide in response to pathogens. Nitric oxide is a free radical that causes oxidative stress and damage to cells that it contacts. While production of nitric oxide regulates many physiological processes at low concentrations, at immunologically relevant levels nitric oxide mediates the killing of microorganisms and inhibits T- and B-cell proliferation *in vitro* (Albina et al., 1991). The enzyme responsible for the rapid production of nitric oxide at immunologically relevant levels is type 2 or inducible nitric oxide synthase (iNOS). In brief, the iNOS enzyme converts O₂ and L-arginine to nitric oxide and L-citrulline, with subsequent release of nitric oxide into the environment as a gaseous molecule. Induction of iNOS expression is dependent on the activation of both the Stat-1 and NF- κ B signaling pathways (Ohmori and Hamilton, 2001). The level of IFN- β produced by some macrophage populations (e.g. monocyte- and bone marrow-derived) in response to TLR-4-mediated IRF-3 activation *in vitro* can be sufficient to sustain a robust nitric oxide response to LPS in the absence of exogenous IFN (Franchini et al., 2006; Keller et al., 1995).

Despite increasing evidence that microglia represent a unique macrophage population suited to the specialized environment of the CNS (Butovsky et al., 2006; Walton et al., 2006; Ziv et al., 2006), the TLR responses of acutely isolated primary microglia, i.e. cells obtained directly from the CNS and not treated with growth factors or co-cultured with other cell types prior to analysis, remain relatively poorly characterized. Primarily for pragmatic reasons, investigations into microglial TLR responses have utilized transformed cell lines derived from immature fetal or neonatal mouse brain, or primary microglia from fetal or neonatal mouse

brain that have been cultured extensively *in vitro* with astrocytes (i.e. expanded in mixed glial cultures prior to purification) or with growth factors. Mixed glial cultures are most commonly employed experimentally as a source of microglia because this method promotes cell survival and proliferation *in vitro*. Prolonged culture of microglia with the macrophage differentiation factor M-CSF also promotes their survival and proliferation *in vitro* (Ponomarev et al., 2005). However, these and related culture techniques may also impact other aspects of microglial function. For example, prolonged exposure to M-CSF or GM-CSF *in vitro* drives primary neonatal microglial cells to express surface markers associated with macrophages or dendritic cells respectively (Santambrogio et al., 2001) and may therefore also impact microglial immune function. More importantly, the extent to which such cells functionally resemble microglia from the adult CNS remains to be determined. As discussed above, microglia undergo marked morphologic alterations during ontogeny, so that primary microglia from the fetal/neonatal and adult brain cannot be considered as phenotypically equivalent. Indeed, although microglial cell lines (Shen et al., 2005), microglia purified from mixed glial cultures (Roy et al., 2006) and acutely isolated neonatal microglia (Brannan and Roberts, 2004) are capable of robust nitric oxide responses when stimulated *in vitro*, we have previously reported that microglia from adult mouse brain are relatively inefficient producers of nitric oxide in response to a wide variety of stimuli *in vitro* (Brannan and Roberts, 2004).

In the present study, we compared, for the first time, the immunological responsiveness of acutely isolated adult and neonatal murine microglia to stimulation via TLR-2, -3 and -4, focusing on cytokine, chemokine and nitric oxide induction. In addition, we investigated the impact of expansion in mixed glial cultures, or exposure to M-CSF or GM-CSF, on the nitric oxide response of murine microglia. Finally, we extended our previous observations (Brannan and Roberts, 2004) to include a direct comparison of the TLR-4-dependent iNOS mRNA, protein and nitric oxide responses of neonatal and adult microglia isolated from two different rodent species, namely mouse and rat.

2. Materials and methods

2.1. Acute isolation of microglia from rodent CNS

C57Bl/6 NCr mice were obtained from NCI (Fredrick, MD) and Sprague-Dawley rats were obtained from Taconic (Hudson, NY). Adult (6-8 week-old) or neonatal (8 day-old unless specified otherwise) mice were euthanized with a lethal dose of sodium pentobarbital and then exsanguinated by perfusion with $1 \times$ Dulbecco's phosphate buffered saline (PBS) (Gibco/Invitrogen; Carlsbad, CA). For the rat studies, adult (3 month-old) or newborn rats were first anesthetized with halothane and then euthanized and perfused as described above. Harvested brains and spinal cords were removed and placed in Dulbecco's modification of Eagle's medium (DMEM) containing 4.5 g glucose and 25 mM HEPES (Mediatech; Herndon, VA) with 10% fetal bovine serum and mechanically dissociated through a wire 60-mesh screen (Sigma, St. Louis, MO). The resulting homogenate was centrifuged at $486 \times g$ for 10 minutes. Microglia were isolated from the pelleted homogenate by a Percoll (Amersham, Uppsala, Sweden) step gradient as described previously (Sedgwick et al., 1991). Briefly, the pellet was suspended in 70% isotonic Percoll and layered first with a 37% and then a 30% isotonic Percoll solution. The gradient was spun at $553 \times g$ for 25 minutes and afterward the microglia were collected from the 70-37% interface. The isolation method for murine microglia was similar to that previously described (Brannan and Roberts, 2004) with one important modification. Following 30-37-70% Percoll gradient purification, microglia isolated from either adult or neonatal CNS were subjected either to lympholyte-Mouse or lympholyte-Rat (depending on the species being investigated) gradient centrifugation in order to remove dead cells prior to *in vitro* culture. This step was particularly important, since we routinely observed between 50-60% cell death (as determined by trypan blue exclusion), directly following Percoll centrifugation. Removal of

these dead or dying cells increased the sensitivity of iNOS mRNA and protein detection significantly. The lymphocyte gradient was centrifuged at $1350\times g$ for 20 minutes and the viable microglia were removed from the interface and rinsed in media. FACS analysis routinely revealed that >95% of the cells were a homogeneous group of CD11b+/CD45low cells, confirming their identity as microglia, as previously published by our laboratory (Brannan and Roberts, 2004).

Cells were plated at a density of 5×10^5 cells/well in a 12-well dish in DMEM containing 10% FBS. Unless otherwise indicated, cells were left in culture overnight before stimulation with TLR agonists (as described below). In one series of experiments, microglia were isolated as described above, and then treated with 10 ng/mL M-CSF or GM-CSF for five days. Prior to stimulation, cells were scraped and replated at a density of 5×10^5 cells/mL in fresh media without M-CSF or GM-CSF.

2.2. Mixed glial cultures

Mice were euthanized as described above and decapitated. Cerebral cortices were dissected with specific attention paid to the removal of all meningeal layers. The cortices were minced and placed in a solution containing 0.25% trypsin in HBSS/DMEM (1:1). The tissue was trypsinized at 37°C for 15 minutes and dissociated by vigorous pipetting. The resulting homogenate was passed through a 60-mesh screen and then centrifuged at $486\times g$. The pellet was resuspended, cells counted by hemocytometer, and plated in media at a concentration of 1.2×10^5 cells/cm² in a 75 cm² flask. Mixed glial cultures were expanded for approximately 12 days until confluent. The flasks were then sealed and shaken overnight at 37°C to isolate microglia. Detached microglia were plated at a density of 5×10^5 cells/mL prior to stimulation. Thus in all experiments, microglia were purified from the mixed glial cultures prior to stimulation and analysis.

2.3. TLR agonist-mediated stimulation of microglia

Microglial cultures generated as described above were stimulated with the following agonists: lipopeptide PAM₃CSK₄ (PAM) (2 µg/mL, EMC Microcollections; Tuebingen, Germany), lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (1 µg/mL, Sigma; St. Louis, MO), polyinosine-polycytidylic acid (I:C) (25 µg/mL, Invivogen; San Diego, CA), and recombinant mouse or rat IFN-γ (100 U/mL, R & D Systems; Minneapolis, MN).

2.4. Isolation of primary macrophages

Resident macrophages were isolated from adult (6-8 week-old) C57Bl/6 NCr mice by peritoneal lavage and plated in complete DMEM. After overnight incubation, non-adherent cells were washed from the plate and the remaining macrophages were scraped and replated at a density of 5×10^5 cells/mL prior to stimulation.

2.5. RNA isolation and real-time PCR

RNA was isolated using the RNeasy Micro kit (Qiagen; Valencia, CA). RNA was primed with random hexamers and converted into cDNA by reverse transcription (RT) using Superscript II reverse transcriptase (Invitrogen; Carlsbad, CA) by following the manufacturer's recommended protocol. The resulting cDNA was then subjected to real-time PCR with Titanium Taq Polymerase (BD/Clontech; Palo Alto, CA) containing a final concentration of 0.1x SYBR Green (Molecular Probes;) and 0.2 µM of the primer set of interest in a 25 µL reaction. The PCR mixture was run in the Opticon DNA engine (MJ research; Waltham, MA). After an initial 3-minute 95°C denaturation step, the reaction was run through 40 cycles at 95°C for 40 seconds, 66°C for 20 seconds, and 72°C for 30 seconds. After a final extension step (72°C for 1 minute), melting curve analysis was executed to ensure the resulting products from

the reaction had equivalent and appropriate melting temperatures. Murine primer sets used are listed in Table 1. All primers for rat, as well as those for mouse IFN- β were obtained from SuperArray (Frederick, MD). The mRNA levels for rat (and mouse in the case of IFN- β induction) were normalized using HPRT primers from the same vendor. The manufacturers recommended volumes and conditions for PCR were followed.

2.6. Enzyme-linked immunosorbent assays (ELISA) and Griess assays

Supernatants were collected at 24 hours post-stimulation for ELISAs and 48 hours post-stimulation for Griess assays. For all ELISAs, DuoSet kits from R & D systems (Minneapolis, MN) were employed to manufacturer's specifications. Nitric oxide levels were determined by employing a standard Griess assay that detects nitrites as byproducts of nitric oxide generation in culture supernatants. Supernatants were diluted two-fold in Modified Griess Reagent (Sigma; St. Louis, MO) and compared to a standard curve of sodium nitrite (Sigma; St. Louis, MO) in serial two-fold dilutions with a high standard of 5 $\mu\text{g/mL}$ and a low standard of 312.5 ng/mL. Plates were read at 540 nm and data was analyzed with the program DeltaSoft 3 (Biometallics, Inc.; Princeton, NJ).

2.7. Western blotting

Cells were first frozen and then lysed by boiling the samples for 10 minutes under reducing conditions. Lysates were run through SDS-PAGE and then transferred onto a PVDF membrane. The membrane was blocked with a 6% milk/PBS-Tween 0.5% (PBST) solution. The blot was then placed in the milk-PBST solution containing anti-NOS2 (Transduction Laboratories; Mississauga, Canada), anti- β -actin (Santa Cruz Biotechnology Inc.; Santa Cruz, CA), or anti- α -tubulin (Sigma; St. Louis, MO) antibodies for two hours at room temperature. After washing the blot in PBST, the blot was placed in a solution of milk-PBST containing HRPconjugated secondary antibodies specific for anti-NOS2, -actin, or -tubulin isotypes. After one hour, the blot was washed in PBST, and treated with ECL Western blotting detection reagent (Amersham; Piscataway, NJ) following the manufacturer's specifications.

2.8. Statistical Analysis

Data are presented as the mean of three independent experiments (i.e. $N=3$), each conducted with microglia pooled from multiple brains. For example, for studies with adult mice, " $N=3$ " represents three independently conducted experiments that each required microglia pooled from at least 12 adult brains; for studies employing neonatal mice or neonatal rats, " $N=3$ " represents three independently conducted experiments that each required microglia pooled from at least 20 brains; for studies with adult rats, " $N=3$ " represents three independently conducted experiments that each required microglia pooled from 4 brains. In addition, the " N " data point for each individual experiment was the mean value determined from duplicate or triplicate samples.

Differences between cell-types were analyzed for statistical significance by using two-tailed student's T tests. Only those differences with P values of less than 0.05 were considered significant.

3. Results

3.1. Cytokine and chemokine production by TLR stimulated adult and neonatal murine microglia

Functional differences between macrophage populations residing in different peripheral tissues have been described (Punturieri et al., 2004). However, while functional differences have been noted between acutely isolated primary microglia and peripheral macrophages (Brannan and

Roberts, 2004), the innate immune responses of primary microglia isolated in this fashion remain very poorly characterized. Furthermore, a comparison of the innate immune responses of microglia from adult and neonatal brain has not been described to our knowledge, despite marked differences in cell proliferation and morphology *in vivo* between the two. We therefore compared the TLR-induced cytokine and chemokine responses of microglia isolated from the brain of neonatal and adult mice. Microglia were isolated from the CNS of 6-8 week-old or 8 day-old C57Bl/6 mice and stimulated with TLR-2, -3, or -4 agonists as described in the Materials and Methods.

We first evaluated the relative mRNA levels of three pro-inflammatory cytokines, TNF α , IL-1 β , and IL-6, and also of the chemokine RANTES (CCL5), by real time RT-PCR following 4 hours of TLR stimulation (Fig. 1a-d). As shown in Fig. 1, both neonatal and adult microglia showed qualitatively similar mRNA expression patterns in response to stimulation with TLR-2, -3, or -4 agonists, with stimulation through TLR-4 inducing the most robust levels of cytokine mRNA. Adult microglia expressed significantly higher levels of TNF- α and IL-1 β mRNA than neonatal cells when stimulated through the three TLRs tested (Fig. 1a, b) and higher IL-6 mRNA levels only when stimulated through TLR-4 (Fig. 1c). RANTES mRNA was expressed to similar levels in both adult and neonatal cells when stimulated TLR-3 and TLR-4. TLR-2 stimulation was the least efficient mode of RANTES mRNA induction for both neonatal and adult microglia (Fig. 1d), consistent with the MyD88-independent regulation of this chemokine reported for other systems (Fitzgerald et al., 2003).

We subsequently compared the neonatal and adult microglial TLR-mediated expression of MIP-1 α (CCL3), MIP-1 β (CCL4), MCP-1 (CCL2) and KC (CXCL1) mRNA (Fig. 1e-h). Expression of these chemokines in brain parenchyma has been correlated with increased lymphocyte infiltration into the CNS during experimentally induced disease (Cardona et al., 2003; Kielian and Hickey, 2000). Again, we observed similar qualitative chemokine responses for neonatal and adult microglia although some qualitative differences were observed. Adult microglia expressed significantly more MIP-1 α and MIP-1 β mRNA compared to neonatal cells when stimulated via TLR2 or TLR3, (Fig. 1e and f), while neonatal microglia expressed significantly higher levels of MCP-1 mRNA when stimulated through these TLRs (Fig. 1g). Adult and neonatal microglia exhibited no statistically significant differences in the mRNA levels of these three chemokines when stimulated through TLR-4, or for KC expression regardless of the TLR agonist used (Fig. 1h).

To confirm our real-time PCR findings, we evaluated the levels of TNF- α , IL-1 β , IL-6, and RANTES protein produced by adult and neonatal primary microglia in response to TLR stimulation. Microglia were isolated from 6-8 week-old adult and 8 day-old neonatal brains as before, and stimulated with the appropriate TLR agonist for 24 hours. Supernatants were then collected and cytokine protein levels evaluated by ELISA (Fig. 2). Overall, the ELISA data for the pro-inflammatory cytokines were qualitatively similar to the RNA data above, with robust induction by TLR-4 and less efficient induction by TLR-3. However, we observed that neonatal microglia produced significantly higher levels of TNF- α protein relative to adult cells when stimulated via TLR-2 or TLR-4 ($P < 0.01$ and $P < 0.05$ respectively). This differs from the real-time PCR analysis, and did not appear to be due to delayed kinetics of TNF- α mRNA induction by neonatal as compared to adult microglia (data not shown). The RANTES protein data were consistent with RANTES mRNA expression levels in that stimulation through TLR-3 and -4 generated a more efficient response than that mediated by TLR2. Consistent with the real-time PCR data, adult microglia cultures produced significantly higher levels of IL-6 (when stimulated through TLR-4) and IL-1 β (in response to all three TLRs) compared to neonatal cultures ($P < 0.05$).

3.2. Inducible nitric oxide synthase mRNA, protein and nitric oxide production by microglia from neonatal and adult mouse

Previous reports have described efficient induction of iNOS mRNA, iNOS protein, and nitric oxide following TLR-2, -4, or -9 stimulation of microglia isolated from mixed glial cultures (Esen and Kielian, 2006; Olson and Miller, 2004) and following TLR-3 stimulation of astrocyte cultures (Scumpia et al., 2005). We therefore evaluated the relative efficiency of iNOS mRNA induction by acutely isolated primary microglia from adult or neonatal mouse brain. Optimal iNOS induction requires activation of both Stat-1 and NF- κ B signal transduction pathways. Since Stat1 is activated in response to type 1 or type 2 interferons, either IFN- α/β or IFN- γ serve to potentiate nitric oxide production by macrophages *in vitro* (Kamijo et al., 1994). In order to ensure an optimal nitric oxide response by primary microglia, we first evaluated TLR-mediated iNOS induction in the presence of exogenous interferon. In all studies involving exogenous interferon, IFN- γ was employed for pragmatic reasons related to the relative stability of the recombinant protein. Microglia isolated from both adult and neonatal mice expressed iNOS mRNA to similar extents by 24 hours post-stimulation (Fig. 3a). For both neonatal and adult cells, LPS was the most effective activator of iNOS mRNA expression, followed by the TLR-2 agonist PAM₃CSK₄. The TLR-3 agonist poly I:C was the least efficient activator of iNOS mRNA expression, likely due to the delayed kinetics of NF- κ B activation through this TLR (Yamamoto et al., 2002).

The above data demonstrate that both neonatal and adult microglia are capable of expressing iNOS mRNA to similar extents. However, we have previously reported that microglia isolated from the adult mouse CNS and subsequently cultured for 48 hours in the presence of LPS and IFN- γ (Brannan and Roberts, 2004), produce nearly undetectable levels of nitric oxide. In order to determine whether this phenomenon is dependent on the developmental stage of the cells, we isolated microglia from adult and neonatal mice (12 and/or 8 day-old) and evaluated the level of nitrite in microglial cultures exposed to LPS and IFN- γ for 24 (Fig. 3b) or 48 hours (Fig. 3c) *in vitro*. Microglia from 8 day-old mice produced significantly more nitrite than cells isolated from either 12 day-old or adult mice in the culture supernatants. As we have previously reported (Brannan and Roberts, 2004), only small amounts of nitrite were detected for microglia isolated from 6-8 week-old adult animals. We then went on to examine iNOS protein levels in microglia isolated from 8 or 12 day-old neonatal brains and compared them to microglia from 6-8 week-old adult mouse brains. These microglia were cultured overnight and subsequently stimulated with LPS and IFN- γ for 24 hours. Despite the barely detectable nitrite levels in 12 day-old and adult microglial cultures, only slightly reduced iNOS protein levels were observed in these cells (Fig. 3d).

In a previous report, microglia purified from mixed glial cultures were capable of efficient upregulation of the type 1 interferons (IFN- α and IFN- β) in response to direct TLR-4 stimulation, and were therefore capable of efficient iNOS and nitric oxide production in response to LPS in the absence of exogenous interferons (Olson and Miller, 2004). We therefore examined the extent to which the iNOS response of acutely isolated murine microglia was dependent on exogenous interferon. We observed a significant decrease (4.5-fold, $P < 0.001$) in the amount of nitric oxide produced by neonatal microglia from 8 day-old brains when these cultures were stimulated with LPS in the absence of exogenous interferon (Fig. 4a). Further, we detected iNOS protein in adult and neonatal microglia stimulated with LPS in the presence, but not the absence of exogenous interferon (Fig. 4b).

3.3. Impact of culture conditions on nitric oxide production by microglia

The colony-stimulating factors M-CSF and GM-CSF are capable of differentiating monocytes to macrophages *in vitro* and are involved in terminal differentiation of resident macrophages *in vivo* (Akagawa, 2002; Shibata et al., 2001; Witmer-Pack et al., 1993). The extent to which

neonatal and adult microglia represent a fully differentiated mononuclear phagocyte remains an open question. M-CSF is constitutively produced by astrocytes throughout development, whereas GM-CSF is scarcely present in the adult CNS (Dame et al., 1999; Malipiero et al., 1990). M-CSF production by astrocytes has been implicated in the enhanced proliferation and survival of microglia in mixed glial cultures (Mizuno et al., 1994), and neonatal microglial cells treated with M-CSF assume some of the markers characteristic of macrophages (Santambrogio et al., 2001). We therefore first asked whether the robust nitric oxide response of neonatal microglia could be augmented further by exposure to M-CSF *in vitro* or expansion in mixed glial cultures. Microglia were acutely isolated from 8 day-old neonatal animals and cultured in M-CSF *in vitro* for 5 days, or isolated from mixed glial cultures also generated from 8 day-old mice (as described in Materials and methods). Microglia were plated at identical densities and stimulated with LPS and IFN- γ . After 48 hours, the supernatant was collected and analyzed for accumulated nitrite levels (Fig. 5). Microglia isolated from 8-day old brains and subsequently cultured in M-CSF prior to stimulation produced significantly more nitric oxide than acutely isolated cells from 8-day old brains (7.7-fold) or cells from mixed glial cultures (2.6-fold). In contrast to the acutely isolated cells, nitrite levels observed for neonatal microglia cultured in MCSF approached those observed for primary peritoneal macrophages.

In a second series of experiments, we went on to determine whether the impaired NO response of adult microglia could be overcome by exposure to either M-CSF or GM-CSF. We observed that adult microglia pre-treated with M-CSF or GM-CSF for 5 days could subsequently produce high levels of nitric oxide in response to IFN- γ and LPS stimulation (Fig. 5b). Indeed, the nitric oxide response of GM/M-CSF pre-treated adult microglia approached that of resident peritoneal macrophages examined in parallel. As before, nitrite levels for acutely isolated untreated cells were barely detectable. Pre-treatment with M-CSF for two days was insufficient time for development of the NO-competent phenotype (data not shown), suggesting that acquisition of this phenotype requires extended exposure.

3.4. Inducible nitric oxide synthase mRNA, protein and nitric oxide production by microglia from neonatal and adult rat

In order to determine if the differential nitric oxide response of acutely isolated neonatal and adult microglia extended to rodent species beside mouse, we examined the iNOS response of microglia isolated from rat brains. Specifically, microglia were isolated from 3 month-old adult or newborn Sprague-Dawley rats and stimulated with LPS in the presence or absence of exogenous IFN- γ . Adult rat microglia produced markedly less nitric oxide than those isolated from neonatal rat brains, both at 24 and 48 hours post-stimulation (7-fold and 5-fold for cultures stimulated with LPS and IFN- γ respectively) (Fig. 6a, b), a trend also observed for microglia isolated from the mouse. All differences in nitrite production seen between the neonatal and adult microglia were highly significant ($P < 0.001$). The kinetics of nitric oxide production appeared faster for microglia from neonatal rat as compared to neonatal mouse, with significant levels of nitrite detected by 24 hours (Fig. 6a). The accumulated nitrite levels in adult rat cultures were similar to those observed for neonatal mouse cultures. Most notably, unlike microglia isolated from the neonatal mouse CNS, neither adult nor neonatal rat microglia required exogenous IFN- γ for efficient nitric oxide production when stimulated through TLR-4. However, the addition of exogenous IFN- γ was required to sustain nitric oxide responses when the cells were stimulated with lower concentrations of LPS (1 ng - 5 pg range, data not shown).

Overall, induced iNOS mRNA levels were slightly lower for adult relative to neonatal rat microglia. However, this difference was not statistically significant (Fig. 6a, b and c). Western blot analysis of microglial lysates demonstrated reduced iNOS protein expression for adult versus neonatal rat microglia (Fig. 6d), correlating with a similar, albeit less marked, trend seen

in the mouse. In contrast to the mouse however (Fig. 4b), iNOS protein and nitric oxide levels were not significantly augmented by exogenous IFN- γ at the LPS concentration used (Fig. 6a and b). Taken at face value, these data are consistent with the higher fold induction of IFN- β mRNA by rat versus murine microglia, in response to LPS stimulation (Fig. 7c). Finally, in contrast to the less efficient nitric oxide response of adult versus neonatal rat microglia, adult cells exhibited a more robust cytokine mRNA response than those isolated from the neonatal brain (Fig. 7a, b and c).

4. Discussion

Macrophages are a critical cellular component of the immune system because they mount rapid innate responses to microbial pathogens that also serve as a bridge to adaptive immune responses. When TLRs on the surface of macrophages recognize and engage their corresponding PAMP, signaling events lead to cellular activation and induction of pro-inflammatory gene products. Depending on the TLR engaged, tissue macrophages can respond to pathogens through activation of the NF- κ B or IRF signaling pathways (Kaisho and Akira, 2006). Microglia are the resident macrophages of the CNS, and accumulating evidence suggests these cells exhibit specialized characteristics uniquely suited to this role.

All glial cell types in the healthy CNS have been shown to express TLRs 1-9 (Konat et al., 2006) and available evidence supports a role for TLRs expressed on microglia as key sensors of pathogens invading the CNS. For example, mice deficient in TLR-3 are resistant to lethal encephalitis induced by West Nile Virus infection as well as microgliosis in response to local poly I:C injection (Town et al., 2006; Wang et al., 2004), and studies involving intracranial injection of LPS have demonstrated that microglia respond rapidly to this TLR-4 ligand *in vivo* (Herber et al., 2006). While these studies focus on microglial function *in vivo*, the *in vitro* studies presented here allow consideration of TLR function in the absence of cell-cell contacts or secreted factors present in the CNS following TLR stimulation.

The majority of previously published *in vitro* studies investigating microglial function have involved microglia co-cultured with astrocytes for a period of 10-12 days prior to analysis. We hypothesized that exposure to growth factors secreted by astrocytes throughout the co-culture period, such as M-CSF (Mizuno et al., 1994), may result in functional differences when compared to acutely isolated cells. Mature ramified microglia residing in the adult CNS exhibit morphological and phenotypic differences from the immature amoeboid cells residing in the neonate. The possibility that microglia exhibit differential immune function during ontogeny or in response to *ex vivo* culture conditions prompted us to investigate whether adult microglia differ from neonatal microglia in their pro-inflammatory innate responses when stimulated through TLRs. In addition, we examined the impact of *ex vivo* culture conditions, including macrophage differentiation factors, on nitric oxide production by microglia. Finally, we asked whether the differential pro-inflammatory responses we observed for murine microglia isolated from adult versus adult brain could be extended to the rat.

Overall, we observed that neonatal and adult murine microglia exhibited a qualitatively similar pro-inflammatory cytokine and chemokine profile in response to stimulation by TLR-2, -3, or -4 (Figs. 1 and 2). With respect to quantitative differences, adult murine microglia upregulated significantly higher levels of TNF- α mRNA and IL-1 β mRNA and protein than neonatal cells, in response to each of the three TLRs tested. Adult murine microglia also produced significantly higher levels of IL-6 mRNA and protein in response to TLR4 stimulation. In contrast to the TNF- α mRNA data however, we found that murine neonatal cells produced significantly more TNF α protein than adult microglia, when stimulated with TLR-2 or TLR-4 agonists for 24 hours. This discrepancy between the mRNA and protein data does not appear to be due to differential TNF- α induction kinetics between neonatal and adult cells, since adult microglia

retain elevated TNF- α mRNA levels relative to neonatal cells when examined at 18 hours post-stimulation (data not shown). It is possible that adult microglia may be relatively efficient at utilizing TNF- α produced during cell culture. Indeed, resident microglia upregulate TNF receptors following activation (Lambertsen et al., 2007). Cytokine mRNA responses for rat microglia were similar to those for murine cells, with stimulated adult microglia expressing markedly higher levels of TNF- α mRNA and IL-6 mRNA compared to their neonatal counterparts (Fig. 7). Overall, these data suggest that the early pro-inflammatory response of microglia to pathogens may be more efficient in the mature as compared to the developing CNS. Consistent with this notion, several studies report differential sensitivity of the neonatal versus adult brain to infection. For example, the CNS of transgenic mice older than three weeks of age (and therefore bearing mature microglia) is resistant to infection by the measles virus, whereas that of the neonate is highly susceptible (Lawrence et al., 1999). Obviously cell types other than, or in addition to, microglia may be involved in age-dependent susceptibility to CNS infection, and future studies to compare the *in vivo* innate immune response of microglia residing in the adult versus immature brain will be informative.

We have previously reported that cultures of acutely isolated microglia from adult mouse brain exhibit barely detectable levels of nitrite, iNOS mRNA and protein relative to purified resident macrophages, following stimulation with LPS and IFN- γ (Brannan and Roberts, 2004). In the current report, we directly compared the iNOS mRNA and protein expression of acutely isolated neonatal and adult murine microglia (Fig. 3). Modification of our microglial isolation protocol increased the sensitivity of iNOS detection compared to that previously reported by us (Brannan and Roberts, 2004). We observed that both adult and neonatal cells upregulated iNOS mRNA to similar levels in response to stimulation. While we observed a slight increase in iNOS protein levels of microglia from 8 day-old mice, compared to cells from adult and 12 dayold mice, nitrite levels were markedly lower in the latter microglial cultures. Taken together, these data suggest that nitric oxide production is developmentally regulated at the post-translational or enzymatic level. *In vivo*, nitric oxide production may be permitted in the early stages, when cellular turnover in the developing CNS can repair damage to local tissue. As the animal ages and cells in the CNS become quiescent, nitric oxide production may then be more strictly regulated as the potential for repair becomes limited.

We next asked whether *ex vivo* culture conditions could augment the nitric oxide response of microglia to LPS and IFN- γ . Although prior *in vitro* expansion in mixed glial cultures had some impact on the nitric oxide capacity of neonatal microglia, M-CSF treatment had the most effective outcome (Fig. 5a). Prior exposure to either M-CSF or GM-CSF also boosted the normally weak nitric oxide response of adult microglia to that of peritoneal macrophages (Fig. 5b). M-CSF and GM-CSF are essential *in vivo* differentiation factors for several classes of resident macrophage, and support the *in vitro* differentiation of bone marrow stem cells to macrophages. It has been suggested that microglia represent a relatively undifferentiated mononuclear phagocyte population (Carson et al., 1998), and several studies have demonstrated that adult and neonatal microglia exposed to M-CSF or GM-CSF undergo a number of phenotypic alterations, including acquisition of surface markers characteristic of macrophages and dendritic cells respectively (Hao et al., 2002; Ponomarev et al., 2005; Santambrogio et al., 2001). It has been reported that bone marrow-derived macrophages differentiated in M-CSF exhibit an increased capacity for LPS-induced nitric oxide production with increasing maturation stage *in vitro* (Oliveira et al., 2003). M-CSF is constitutively produced, albeit at low levels, throughout CNS development, whereas GM-CSF is barely detectable in the adult CNS (Dame et al., 1999). Both M-CSF and GM-CSF are induced in the CNS in response to inflammation, and a growing body of evidence points to a role for these factors in the pathophysiology of brain disease. These CSFs are elevated in the cerebrospinal fluid of patients with Alzheimer's disease (Tarkowski et al., 2001), multiple sclerosis (Carrieri et al., 1998), HIV-associated dementia (Gallo et al., 1994; Perrella et al., 1992), and acute stroke

(Tarkowski et al., 1999), and can be produced by a variety of cells within the CNS, including infiltrating and/or perivascular macrophages.

In order to determine if the differential nitric oxide response of adult versus neonatal murine microglia extended to other rodent species, we examined microglia isolated from rats. Again, acutely isolated microglia from the adult rat brain produced significantly lower levels of nitric oxide in response to TLR-4 stimulation than those from the neonatal rat brain (Fig. 6). The differential nitric oxide response of adult and neonatal rat microglial cultures may be regulated via a post-transcriptional mechanism(s), since lower iNOS protein levels were observed for adult relative to neonatal cells without a significant reduction in iNOS mRNA. In contrast to the enhanced nitric oxide response of neonatal relative to adult rat microglia, mRNA levels of the cytokines examined were markedly higher in stimulated adult versus neonatal cells (Fig. 7). These data indicate that adult and neonatal microglia exhibit a highly selective differential nitric oxide response for both the mouse and the rat, since the pro-inflammatory cytokines response was intact in both systems. Interestingly, efficient induction of iNOS protein and nitric oxide appeared to be relatively independent of exogenous interferon for rat as compared to murine microglia (Fig. 4), suggesting that TLR-4-mediated stimulation of primary rat microglia can induce type I IFN at levels sufficient to activate the Stat-1 pathway *in vitro*. This apparent difference between the rat and mouse system is not absolute however, since exogenous interferon does augment the nitric oxide response of rat microglia when low doses of LPS are employed (data not shown). Our observation that rat microglia upregulate IFN- β transcript more efficiently than mouse cells, suggests that the type I IFN response of rat microglia to LPS may be more efficient than that of murine cells, consistent with the more efficient IFN- β mRNA response of the former.

In summary, for both the rat and mouse species, microglia from the adult brain exhibit a weak nitric oxide response relative to neonatal cells, despite possessing a similar or even greater capacity for pro-inflammatory cytokine/chemokine responses. Regulation of nitric oxide production by the iNOS gene has been reported to be exerted primarily at the transcriptional level in macrophages (Kleinert et al., 2004) and post-transcriptional regulation of iNOS expression has been reported (Mori and Gotoh, 2000; Ratovitski et al., 1999). The differential nitric oxide production of rodent microglia may be regulated at the level of protein translation/processing, since adult rat microglia express lower levels of iNOS protein than their neonatal counterparts. Potential mechanisms include a failure of iNOS to dimerize or differential transport of the substrate arginine from the extracellular environment into the cytoplasm (Closs et al., 2000; Ratovitski et al., 1999). While the enzyme arginase has been observed differential arginase expression between adult and neonatal murine microglia (data not shown). Although *in vivo* studies are required to establish the physiological relevance of the differential nitric oxide capacity of adult and neonatal microglia observed *in vitro*, a dampened nitric oxide response by microglia residing in the adult brain may be desirable. Nitric oxide can mediate neuronal apoptosis (Palluy and Rigaud, 1996) and the capacity of the adult CNS for neuronal regeneration and repair is less than that of the immature CNS (Eriksson, 2003). The toxicity of nitric oxide in the CNS is demonstrated clinically by the increased neurodegeneration found in diseases such as multiple sclerosis, Alzheimer's, stroke, influenza induced encephalitis, and HIV infection (Dawson and Dawson, 1998; Torreilles et al., 1999). The relative contribution of infiltrating macrophages and resident microglia to nitric oxide production in these and related CNS diseases remains to be determined.

Acknowledgements

This research was sponsored in part by the National Institute of Health; Grant number R01 NS-041213 (MRR). We are particularly grateful to Dr. Kevin Lee for all of his support for these studies.

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Figure 1. Cytokine and chemokine mRNA induction by microglia from adult or neonatal mouse CNS

Microglia were acutely isolated from adult (black bars) or 8 day-old neonatal (striped bars) mouse CNS, and after overnight culture, stimulated for 4 hours with TLR-2, -3, or -4 agonists. TNF- α (a.), IL-1 β (b.), IL-6 (c.), RANTES, (d.), MIP-1 α , (e.), MIP-1 β (f.), MCP-1 (g.), or KC (h.) mRNA expression was analyzed by real-time RT-PCR as detailed in *Materials and Methods*. The data is presented as RNA units relative to HPRT (n=3 for both adult and neonate).

Figure 2. Cytokine and chemokine protein production by microglia from adult or neonatal mouse CNS

Microglia were acutely isolated from adult or 8-day neonatal mouse CNS, and stimulated for 24 hours with TLR agonists. Supernatants were then harvested, and TNF- α (a.), IL-1 β (b.), IL-6 (c.), or RANTES (d.) levels determined by ELISA (n=3 for both adult and neonate).

Figure 3. Inducible nitric oxide synthase mRNA, protein and nitric oxide production by microglia from neonatal and adult mouse CNS

(a.) Microglia were isolated from adult or 8-day neonatal mouse CNS, and stimulated with TLR agonists in the presence of exogenous IFN- γ for 24 hours. Inducible NOS mRNA expression was analyzed by real-time RT-PCR as detailed in *Materials and Methods* and is presented as RNA units relative to HPRT ($n=3$ for both adult and neonate); (b.) Microglia were isolated from 8 day-old and adult mouse CNS, and stimulated by LPS and IFN- γ or left unstimulated, for 24 hours. Supernatants were then harvested and nitrite levels, as a measure of nitric oxide, determined by Griess assay ($n=3$ for both adult and neonate); (c.) Microglia were isolated from 8, 12 day-old, and adult mouse CNS, and stimulated by LPS and IFN- γ or left unstimulated, for 48 hours. Supernatants were then harvested and nitrite levels, as a measure of nitric oxide, determined by Griess assay ($n=3$ for both adult and neonate); (d.) Microglia were isolated from 8 day-old, 12 day-old, or adult mouse CNS, and stimulated for 24 hours with LPS and IFN- γ or left unstimulated for the same time period. Cellular lysates were then generated and iNOS and β -actin protein levels determined by Western analysis.

Figure 4. Role of exogenous interferon in inducible nitric oxide response of murine microglia
Microglia were isolated from adult or 8 day-old neonatal mouse CNS and cultured in media alone, or stimulated with LPS in the presence or absence of exogenous IFN- γ (a.) After 48 hours, supernatants were removed and nitrite levels determined by Griess assay; (b.) After 24 hours, microglial lysates were harvested, and iNOS and α -tubulin protein levels determined by Western analysis.

Figure 5. Impact of culture conditions on nitric oxide production by neonatal murine microglia
 (a.) Microglia that had been acutely isolated from 8-day neonatal mouse CNS and exposed to M-CSF for 5 days (striped bars), isolated from mixed glial cultures derived from 8-day cerebral cortices (white stippled bars), or acutely isolated from 8-day neonatal mouse CNS (black checkered bars), were stimulated with LPS and IFN- γ or left unstimulated for 48 hours. Supernatants were then harvested and analyzed for nitrite levels by the Griess assay. Resident macrophages isolated from adult murine peritoneal cavity were evaluated in parallel (black bars). (b.) Microglia were acutely isolated from adult mouse CNS and then cultured with M-CSF or GM-CSF for five days. After this period, cells were washed, resuspended in culture media in the absence of CSFs, and stimulated with LPS and IFN- γ or left unstimulated for 48 hours. Freshly isolated adult microglia and resident peritoneal macrophages were evaluated in parallel. Supernatants were harvested and analyzed for nitrite levels by the Greiss assay.

Figure 6. Inducible nitric oxide synthase mRNA, protein and nitric oxide production by microglia from neonatal and adult rat CNS

Microglia were isolated from adult and neonatal rat CNS, and stimulated with LPS alone, LPS and IFN- γ , or left unstimulated, for the appropriate time. Supernatants were analyzed for nitrite levels by the Griess assay at either 24 (a.) or 48 hours post-stimulation (b.) (n=3 for both adult and neonate for both time points); (c.) iNOS mRNA expression was analyzed by real-time RT-PCR as detailed in *Materials and Methods*. Data is represented as RNA units relative to HPRT (n=3 for both adult and neonate); (d.) Microglial lysates were analyzed for iNOS and α -tubulin protein levels by Western analysis.

Figure 7. Cytokine responses of microglia from neonatal and adult rat CNS, and relative IFN- β induction by microglia from mouse or rat CNS

Microglia from adult (solid bars) or neonatal (striped bars) rat CNS were cultured for 4 hours with or without LPS after overnight culture. TNF- α (a.) and IL-6 (b.) mRNA levels were analyzed by real-time RT-PCR as detailed in *Materials and Methods*. Data is presented as RNA units relative to HPRT ($n=3$ for both adult and neonate); (c.) Freshly isolated microglia from the adult or neonatal CNS of both rat and mouse were stimulated with or without LPS for 4 hours. IFN- β mRNA levels were analyzed by real-time RT-PCR, and data is presented as fold-induction relative to HPRT ($n=3$ for both adult and neonate for both species).

Table 1

Sequence name	Abbreviation	Accession #	Forward primer	Reverse primer	Product size
Hypoxanthine guanine phosphoribosyl transferase 1	HPRT-1	NM_013556	TGCCGAGGATTTGGAAAAAGTG	CACAGAGGGCCACAAATGTGATG	115 b.p.
Interleukin-6	IL-6	NM_031168	CAACGATGATGCACTTGCAGA	TGGTACTCCAGAAAGACCAGAGGAA	128 b.p.
Interleukin-1 beta	IL-1 β	NM_008361	CCGTGGACCTTCCAGGATGA	GGGAACGTACACACACAGCA	116 b.p.
Tumor necrosis factor alpha	TNF- α	NM_013693	CCACCACGCTCTTCTGTCTA	AGGGTCTGGGCCATAGAACT	102 b.p.
Chemokine (C-C) ligand 5 (CCL5)	RANTES	NM_013653	GCAGCTGCCCTCACCATCAT	GGCGGTTCTTCGAGTGACA	198 b.p.
Chemokine (C-C) ligand 3 (CCL3)	MIP-1 α	NM_011337	GGTCTCCACCACTGCCCTTG	CAACGATGAATTTGGCGTGGA	139 b.p.
Chemokine (C-C) ligand 4 (CCL4)	MIP-1 β	NM_013652	CTCCGGCAGCTTCACAGAA	GCCTCTCTGAAAGTGGCTCCTC	204 b.p.
Chemokine (C-C) ligand 2 (CCL2)	MCP-1	NM_011333	TGCAGGTCCCTGTCTATGCTT	AGCAGGTGAGTGGGGCGTTA	101 b.p.
Chemokine (C-X-C) ligand 1 (CXCL1)	KC	NM_008176	CCCAAAACCGAAAGTCATAGCC	TCAGAAAGCCAGCGTTACCCA	168 b.p.
Nitric oxide synthase 2 (NOS2)	iNOS	NM_010927	CGCTTGGGTCTTGTTCACTCC	AGGGGCAAGCCATGTCTGAC	104 b.p.